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MICROBIOLOGY AND MICROBIAL TOXICOLOGY:

QUALITY EVALUATION AND CONSUMER ACCEPTANCE:

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Effect of Freeze-Drying on Denaturation of Myosin from Rabbit Skeletal Muscle

SUMMARY

The effect of freeze-drying on the denaturation of myosin *A* and myosin *B* prepared from rabbit skeletal muscle and of heavy meromyosin derived from trypsin-treated myosin *A* was investigated by measurement of adenosine triphosphatase activities and by solubilities. The results show that the myosins undergo denaturation through the process of freeze-drying. Factors involved in the denaturation are the pH and ionic strength of the solution and the dehydration. The denaturation is inhibited by the addition of sucrose to the system.

Raw meat which has been subjected to freeze-drying is usually tougher and dried on reconstitution than the original meat and in addition shows a reduction in the water-holding capacity of the proteins (Hamm and Deatherage, 1960). These undesirable features have been considered to result from denaturation of muscle proteins (Cole and Smithies, 1960; Hunt and Matheson, 1959; Penny *et al.*, 1963; Sawant and Magar, 1961; Suden *et al.*, 1964). Fukazawa *et al.* (1961a,b) indicated that myosin *A* was an essential component of muscle in the development of the water-holding capacity of meat. Hashimoto *et al.* (1959) showed that the quality of meat products depends greatly on the state of myosin in the raw material. Physicochemical studies on the denaturation of myosin by different agents have been carried out in detail by several investigators (Yasui *et al.*, 1958, 1960; Takahashi *et al.*, 1962; Shikama, 1963; Hanafusa, 1962, 1964).

This study was undertaken to obtain an understanding of the denaturation of myosin during the process of freeze-drying. The effect of each stage of the freeze-drying process (preliminary operations, freezing, dehydration, and rehydration) on the denaturation of myosin was studied by the determination of solubilities and adenosine triphosphatase (ATPase) activities. Also studied was the protective effect of sucrose on the denaturation of myosin by freeze-drying.

Experimental

Preparation of myosins. Three preparations of myosin are known to possess ATPase activity: myosin *A*, myosin *B* (natural actomyosin), and heavy-meromyosin (HMM). The first two are extracted from fresh muscle, and the last one is derived from trypsin-treated myosin *A* (Szent-Györgyi, 1953). Since HMM maintains high ATPase activity, it is believed to be the subunit of myosin in which the enzymic-active site is localized. Used throughout this experiment were rabbit muscle myosin *B* of the Weber-Edsall type (Fukazawa, 1961a) purified three times by the usual dilution precipitation method and myosin *A* prepared by Perry's method (Perry, 1955) with slight modifications. Myosin A was extracted from rabbit muscle with Guba-Straub solution for 15 min at 5°C. Myosin *A* can be dissolved in 0.5*M* KCl solution and precipitated by dilution with water to 0.05*M* KCl solution. Purification was performed by repetitive precipitation and redissolution three times. For the elimination of myosin B as a contaminant in the solution, only the fraction soluble in 0.25*M* KCl was taken from the first precipitate, and the final preparation of the purified myosin *A* was restored in 0.5*M* KCl. HMM was prepared from fresh myosin *A* by a trypsin hydrolysis procedure similar to that of Szent-Györgyi (1953, 1960). Trypsin in 0.001*M* HCl was added to a myosin stock solution (10 mg per ml in 0.5*M* KCl-0.005*M* borate buffer, pH 7.0), in sufficient quantity to give a weight ratio of 1 part enzyme to 100 parts protein, and digestion was allowed to proceed at room temperature for some 15 min. The reaction was stopped by the addition of soybean trypsin inhibitor added in the weight ratio of two parts inhibitor to one part enzyme. Purified HMM was obtained by following the procedure of Szent-Györgyi (1953) in which ammonium sulfate fractionation is incorporated in the purification of HMM. Salt concentrations of stock solutions of the myosin preparations were as follows: myosin *B* in 0.6*M* KCl, myosin *A* in 0.5*M* KCl, and HMM in 50m*M* borate buffer. The pH values of the final preparations were approximately 6.5, and the protein concentration of each stock solution was adjusted to about 10 mg myosin/ml.

Freeze-drying. Ten ml of the stock solution of each myosin (approximately 10 mg myosin/ml) dialyzed for 48 hr at 3°C against various systems or buffered with tris-maleate at various pH values

was pipetted into a glass bottle and frozen within 5 min in a mixture of acetone–dry ice (−75°C). Then the bottle was connected to a vacuum pump, and the ice was removed at room temperature under a vacuum of 200 μ Hg for 2–4 hr. After this the bottle was sealed with a glass stopper and stored in a freezer at −15°C. To the freeze-dried sample of protein 10 ml of ice-cold deionized water was added and equilibrated overnight before use. Further dialysis was applied for some purposes (see text). When used, powdered sucrose was added before the solution was lyophilized in an amount equal to 0.5–3 times the weight of protein present.

Protein determination. Protein concentration was determined by a micro-Kjeldahl procedure, using a factor of 6.25 to convert nitrogen to protein. The protein recovery listed in Table 2 was the percentage of the original protein remaining in the supernatant fraction after centrifugation of the freeze-dried protein sample for 20 min at 10,000 × G and 0°C. Since the degree of denaturation of myosin *A* or myosin *B* upon freezing and thawing was found to be dependent on the protein concentration below 1.0 mg/ml (Hanafusa, 1962; Shikama, 1963), myosin solutions containing more than 2 mg/ml were used for denaturation studies throughout this experiment.

Enzymic activity. Basal media for ATPase activity measurements varied somewhat with the kind of myosin and the procedures employed as specified in each experiment in text. Standard components throughout the experiments, however, were 5m*M* $CaCl_2$, 20–50m*M* tris-maleate buffer, pH 7.0, 1m*M* ATP, and 0.1 mg of HMM or 0.2 mg of myosin *A* and myosin *B* per ml of reaction mixture. The assays were performed at 21°C. The amount of orthophosphate produced at measured intervals of time was determined by the method of Martin and Doty (1949).

Reagents. Adenosine triphosphate (ATP, sodium salt) was purchased from Sigma Chemical Co. All chemicals used were of the best reagent grade.

RESULTS

Tables 1 and 2 show the effect of freeze-drying on the ATPase activity of each myosin in the different systems listed in the tables. It is evident from the tables that the ATPase activity of the myosins is destroyed by the freeze-drying procedure regardless of the nature of the system or the myosin. The loss of solubility of myosin *A* in 0.5*M* KCl was measured concomitantly with ATPase activity and the results (Table 2) showed a fairly good correspondence, thus indicating that the myosins undergo denaturation during the process.

The addition of a small amount of sucrose greatly inhibits the denaturation. The enzymic activity, which is usually below 50% after freeze-drying, is retained at about 80% by this treatment (Tables 1, 2), and moreover, loss of solubility is also inhibited (Table 2).

Fig. 1 illustrates the effect of KCl concentration on the denaturation of the myosins by freezing and thawing. After frozen storage for 7 days at −15°C followed by overnight thawing at 3±1°C, the denaturation of the myosins increases proportionally to the square root of ionic strength at pH 6.0, but that at pH 7.0 appears to be independent of electrolyte concentration. Sucrose added in an amount of two times as much as the protein concentration has no effect on the denaturation. The results thus far obtained indicate that under the conditions described above, all the myosins undergo substantially the same denaturation and that pH may play an important role in the denaturation of the proteins.

Figs. 1, 2, and 3 show the effect of pH on the denaturation of a myosin *A* solution in 0.5*M* KCl at low temperatures. Results are similar whether the conditions in the solutions are obtained by standing overnight at 3±1°C after

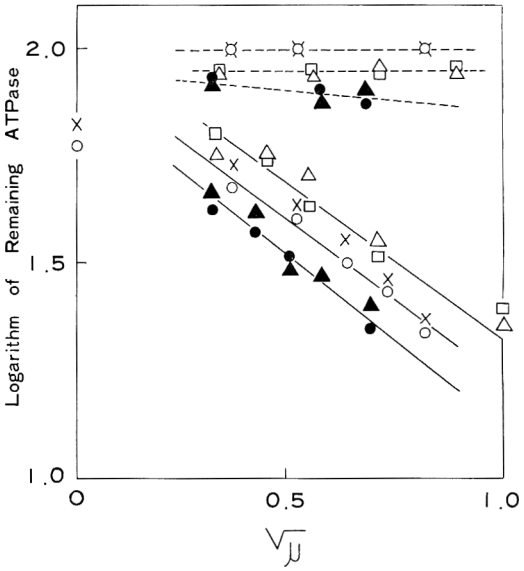

Fig. 1. Effect of ionic strength on inactivation of myosin *A*-ATPase by freezing and thawing. ○, HMM; ×, HMM plus sucrose; △, myosin *A*; □, myosin *A* plus sucrose; ●, myosin *B*; ▲, myosin *B* plus sucrose. Solid lines, initial pH 6.0; dashed lines, initial pH 7.0. pH values were adjusted with 50m*M* tris-maleate buffer. The stock protein solutions (2.0 mg protein/ml) at the various ionic strengths were stored in a freezer at −15°C for a week and thawed by standing overnight at 3±1°C. Weight ratio of sucrose to protein present was 2:1.

Table 1. Effect of freeze-drying on ATPase activity of H-meromyosin, myosin *A*, and myosin *B*.

Sample	System	Remaining ATPase activity (%) after dialysis
H-Meromyosin[a]	5m*M* borate buffer (pH 6.5)	17.3
H-Meromyosin[b]	0.1*M* KCl	12.0
	0.1*M* KCl + sucrose (2:1)	90.2
H-Meromyosin	H_2O	36.2
	0.1*M* KCl	29.4
	H_2O + sucrose (2:1)	63.3
	0.1*M* KCl + sucrose (2:1)	72.5
Myosin *A*	0.1*M* KCl	18.0
	0.1*M* KCl + sucrose (2:1)	64.4
Myosin *A*	0.1*M* KCl + sucrose (2:1)	73.9
Myosin *B*	0.6*M* KCl	40.1
	0.1*M* KCl	40.1
	0.6*M* KCl + sucrose (2:1)	86.1
	0.1*M* KCl + sucrose (2:1)	80.3

[a] HMM was freeze-dried immediately after preparation and was stored in the freezer at −15°C.
[b] Before freeze-drying HMM was dialyzed against 0.1*M* KCl solution for 24 hr at 3±1°C, and sucrose was added in an amount equal to two times the weight of protein present. When used, the sucrose added was removed by dialysis before ATPase assay.

adjusting to the various pH's with 50m*M* tris-maleate buffer, or by dialyzing at 3±1°C for 24 hr against 0.5*M* KCl solutions at different pH's; the protein was found to be unstable below pH 6.0 even at low temperatures. Denaturation of myosin *A* upon freezing and thawing is shown in Table 3 and Fig. 1. By quick-freezing at −75°C in 5 min followed by subsequent quick thawing at 20°C in 5 min, myosin *A* ATPase at pH 6.0 is inactivated by 25% of its activity, and almost no inactivation is observed at pH 6.5 or 7.0. The large difference in degree of inactivation observed between pH 6.0 and 7.0 at the higher freezing temperature (−15°C) in Fig. 1 may possibly be due to the mixed type of denaturation brought about by acid at low temperature (Figs. 2, 3) and by the slow freezing and thawing processes. When the myosin *A* which is partly denatured by freezing or unfavorable pH is dehydrated, the denaturation proceeds further until the sample loses more than 50% of its activity. This loss in activity is prevented by the presence of sucrose (Table 3, Fig. 2). Although the above results have shown that the presence of sucrose

Table 2. Retention of ATPase activity and protein recovery after freeze-drying of myosin *A*.

Sample	System	Remaining ATPase activity (%)	Protein recovery (%)
Myosin *A*[a]	0.1*M* KCl	45.5	61.8
	0.1*M* KCl + sucrose (0.5:1)[b]	68.1	76.5
	(2:1)[b]	72.7	74.2
Myosin *A*	0.5*M* KCl	47.1	53.7
	0.5*M* KCl + sucrose (0.5:1)[b]	74.3	61.0
	(1:1)[b]	85.7	63.9
	(2:1)[b]	85.7	68.0
Myosin *A*	0.5*M* KCl, pH 7.0	25.0	60.0
	0.5*M* KCl + sucrose (1:1)[b]	76.7	87.7
	(2:1)[b]	76.7	84.1
	ph 7.0 (3:1)[b]	76.7	83.6

[a] Myosin *A* was dialyzed against 0.1*M* KCl for 24 hr at 3±1°C before freeze-drying.
[b] Weight ratios of sucrose added to protein present are indicated in parentheses. Added sucrose was removed by dialysis before ATPase assay.

is effective in preventing the myosins from denaturation upon freeze-drying (Tables 1, 2, Fig. 2), the amount necessary for this protective effect is not necessarily the same in all cases (Figs. 1, 3).

Fig. 4 summarizes the effect of sucrose on the inhibition of the denaturation of myosin *A* by different conditions. The denaturation of myosin *A* caused by acid at low temperature (3±1°C), heat (35°C and pH 7.0), freeze-drying, and freezing and thawing was inhibited by the addition of a certain amount of sucrose. However, clear differences can be observed on the effective concentration of sucrose for the protective action in each case. This difference may possibly result from a concentration of sucrose upon freezing or freeze-drying. A reasonable explanation may thus be given to the discrepancy between results in Fig. 1, where no sucrose effect can be observed, and results in Tables 1 and 2, where sucrose does exert a protective effect on denaturation of the myosins.

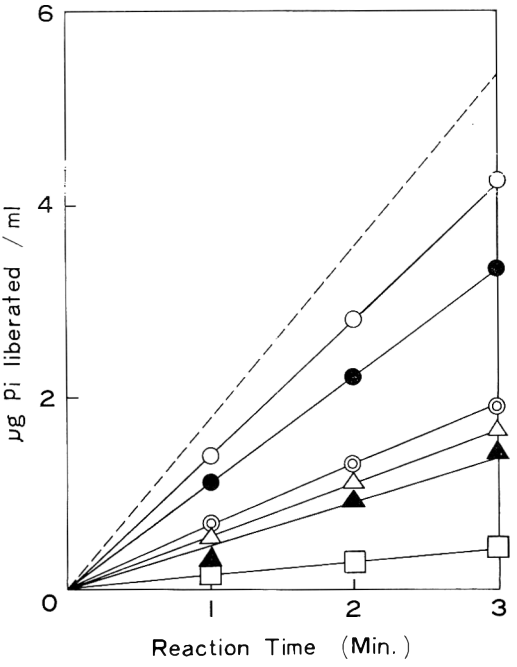

Fig. 2. Effect of pH values on myosin *A*-ATPase activity before and after freeze-drying in presence or absence of sucrose. —, control (immediately after preparation); ○, pH 6.5, adjusted by dialysis against 0.5*M* KCl with 5m*M* tris-maleate buffer; △, pH 5.8, adjusted by the same way as before. ●, (6.5) and ▲, (5.8) respectively represent the activities after lyophilization in presence of sucrose, and ⊙, (pH 6.5) and □, (pH 5.8) are those in the absence of sucrose. Weight ratio of sucrose added to protein in the system was 2:1.

DISCUSSION

The denaturation of myosin under our experimental conditions could occur in any of the following four stages of the freeze-drying process: 1) preliminary operations (dialysis or storage after preparation); 2) freezing; 3) dehydration; and 4) rehydration. In

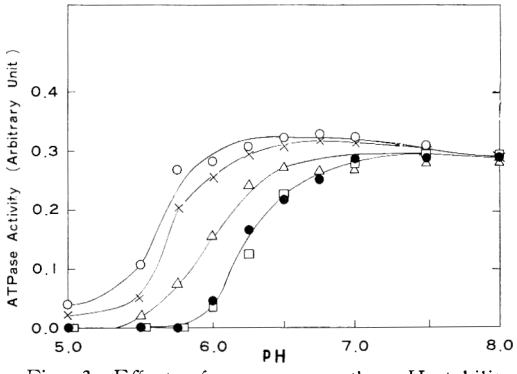

Fig. 3. Effect of sucrose on the pH-stability of myosin *A*-ATPase. ●, O*M* sucrose: □, 0.25*M* sucrose; △, 0.5*M* sucrose; ×, 1.0*M* sucrose; ○, 2.0*M* sucrose. Incubation: 0.5*M* KCl, 50m*M* tris-maleate buffer, 2.0 mg protein/ml for 18 hr at 3°C.

Table 3. Changes in ATPase activity of myosin *A* after freezing, thawing, and freeze-drying.

		ATPase activity (% of original)		
pH values[a] of system before treatment		6.0	6.5	7.0
Before freezing[b]	Control[d]	100.0	100.0	100.0
	0.1*M* sucrose[e]	102.0	101.3	100.0
After thawing[c]	Control[d]	75.7	100.0	92.3
	0.1*M* sucrose[e]	77.1	101.5	92.3
After freeze-drying	Control[d]	25.5	.....	47.7
	0.1*M* sucrose[e]	79.6	.....	86.0

[a] pH values were adjusted with 50m*M* tris-maleate buffer.
[b] Myosin *A* was frozen for 5 min at −75°C.
[c] Frozen samples were thawed at 20°C in 5 min.
[d] Five ml of myosin *A* (2.0 mg/ml) in 0.5*M* KCl.
[e] Five ml of myosin *A* (2.0 mg/ml) in 0.5*M* KCl and 0.1*M* sucrose.

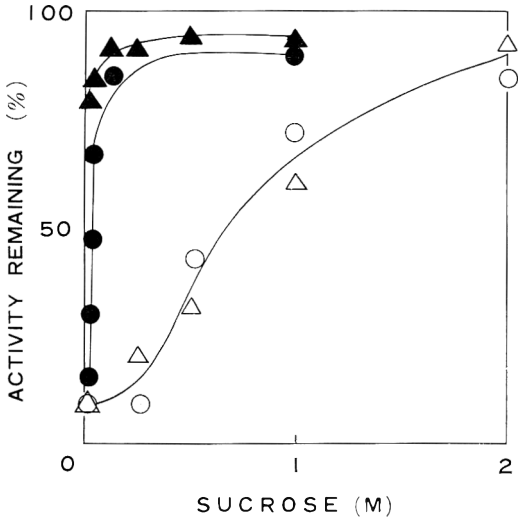

Fig. 4. Protective effect of sucrose on the inactivation of myosin ATPase. Freezing and thawing (▲): myosin *A* (2.0 mg/ml) in 0.5*M* KCl, 50m*M* tris-maleate buffer at pH 6.0 and 0 to 1.0*M* sucrose was stored in a freezer at −15°C for a week and thawed in a bath at 20°C within 5 min. Freeze-drying (●): myosin *A* (~ 10 mg/ml) in 0.5*M* KCl, 20m*M* tris-maleate buffer at pH 6.0 and 0 to 1.0*M* sucrose was freeze-dried and stored at −15°C. Incubation at 35°C and pH 7.0 for 90 min (△): incubation mixture contained 2 mg/ml myosin *A*, 50m*M* tris-maleate buffer, 0.5*M* KCl, and 0–2.0*M* sucrose. Incubation at pH 6.0 and 3°C for 18 hr (○): incubation mixture contained 2 mg/ml myosin *A*, 0.5*M* KCl, 50m*M* tris-maleate buffer, and 0–2.0*M* sucrose.

stages 1 and 4, myosin is in solution or suspension; and denaturation occurring here has been shown to depend on temperature and pH (Yasui *et al.*, 1958, 1960; Takahashi *et al.*, 1962), i.e., the denaturation of myosin usually consists of a pH-independent thermal denaturation and a temperature-independent acid or alkaline one. Since the denaturation study was carried out below 5°C, the rate of thermal denaturation should be very slow. There appeared to be no denaturation when the pH was in the neutral region (Figs. 1, 2). On the acid side, however, myosin ATPase was inactivated because of temperature-independent acid denaturation (Fig. 3).

As for the denaturation of stage 2, i.e., freezing, the physical state of ice is thought to play an important part in the denaturation of muscle proteins either in tissue cells (Dyer, 1951; Love, 1962) or in cell-free model systems (Hanafusa, 1962, 1964; Shikama, 1963). The former authors emphasize mechanical breakdown of cell membranes and the latter postulate some disturbance in or on the protein molecules. The nature of the destructive processes which take place when tissue cells are frozen and thawed was described by Lovelock (1954) and Love (1962). Briefly, damage on freezing is a consequence of the increase in concentration of electrolytes within and without the cell. The results in Fig. 1 and Table 3 indicate that the influence of freezing and thawing on the denaturation of myosin could be observed only in solution at pH 6.0, and that an increase in the electrolyte concentration exerted an additional effect on the acid denaturation at pH 6.0. Under certain conditions, both factors worked together, though the degree of denaturation was not very large. The freezing stage, therefore, plays only a minor role in this case.

In summing up the loss of ATPase activity by the factors so far described, one finds that it is far less than the total loss after freeze-drying. As shown in Fig. 2 and Table 3, about two-thirds of the total loss of ATPase activity is accounted for by the dehydration stage. Lyophilization has been widely used for preservation of proteins because most proteins resist denaturation during and after freeze-drying. However, some proteins and enzymes are denatured, and the results in Fig. 2 and Tables 1, 2, 3 clearly demonstrate that the myosins tested here are also susceptible to lyophilization. The total denaturation after freeze-drying appears to be a sum of the respective denaturation from the four different stages.

The protective ability of sugars and sugar alcohols against denaturation of some proteins varies among sugars, with glucose, fructose, and sucrose being the most efficient (Putnum, 1955; Haurowitz, 1963). It has also been shown that the addition of sugars and alcohols is effective in preventing deterioration of meat during frozen storage (Tamoto *et al.*, 1961; Love, 1962) or storage with salt (Nikkila and Linko, 1954a). The effect may be attributable to the dielectric properties of the added solute because it appears to be related to the concentration of the sugar (Fig. 3) or alcohol. The possibility of the formation of sugar-protein com-

plex has been discounted, and no satisfactory explanation of the effect has yet been offered. Whatever the mechanism is, the fact that sucrose is effective in preventing the myosins from denaturation by various conditions (Fig. 4) is significant.

The experimental results presented in this paper differ in some respects from those obtained with muscle tissues (Dyer, 1951; Love, 1962; Love and Elerian, 1964; Nikkila and Linko, 1954b; Penny *et al.*, 1963; Suden *et al.*, 1964). Nevertheless, it seems to indicate some fundamental suggestions for quality control in freeze-dried meats.

REFERENCES

Cole, L. J. N., and W. R. Smithies. 1960. Methods of evaluating freeze-dried beef. *Food Research* **25**, 363.

Dyer, W. J. 1951. Protein denaturation in frozen and stored fish. *Food Research* **16**, 522.

Fukazawa, T., Y. Hashimoto, and T. Yasui. 1961a. Effect of some proteins on the binding quality of an experimental sausage. *J. Food Sci.* **26**, 541.

Fukazawa, T., Y. Hashimoto, and T. Yasui. 1961b. The relationship between the components of myofibrillar protein and the effect of various phosphates that influence the binding quality of sausage. *J. Food Sci.* **26**, 550.

Hamm, R., and F. E. Deatherage. 1960. Changes in hydration on charges of muscle proteins during freeze-dehydration of meat. *Food Research* **25**, 565.

Hanafusa, N. 1962. Denaturation of myosin B due to freezing and thawing. *Low Temperature Science Ser. B.* **20**, 81.

Hanafusa, N. 1964. Denaturation of myosin A resulting from freeze-thawing. *Low Temperature Science* Ser. B. **22**, 119.

Hashimoto, Y., T. Fukazawa, R. Niki, and T. Yasui. 1959. Effect of storage conditions on some of the biochemical properties of meat and on the physical properties of an experimental sausage. *Food Research* **24**, 185.

Haurowitz, F. 1963. "The Chemistry and Function of Proteins." P. 165. Academic Press, New York.

Hunt, S. M. V., and N. A. Matheson. 1959. The relationship between the quality of dehydrated raw beef and the adenosine triphosphatase activity after storage at various moisture contents. *Food Research* **24**, 262.

Love, R. M. 1962. New factors involved in the denaturation of frozen cod muscle protein. *J. Food Sci.* **27**, 544.

Love, R. M., and M. K. Elerian. 1964. Protein denaturation in frozen fish. VIII. Temperature of maximum denaturation in cod. *J. Sci. Food Agr.* **15**, 805.

Lovelock, J. E. 1954. The protective action of neutral solutes against haemolyis by freezing and thawing. *Biochem. J.* **56**, 265.

Martin, J. R., and D. M. Doty. 1949. *Anal. Chem.* **21**, 565. Quoted by Lindberg, O., and L. Ernster. 1956. Determination of organic phosphorus by phosphate analysis. *In:* "Method of Biochemical Analysis." Vol. 3, p. 7. Interscience Publishers, New York.

Nikkila, O. E., and R. R. Linko. 1954a. Denaturation of myosin in fish muscle and in myosin sol by salts. *Suommen Kemistilehti.* B. **27**, 37.

Nikkila, O. E., and R. R. Linko. 1954b. Denaturation of myosin during defrosting of frozen fish. *Food Research* **19**, 200.

Penny, I. F., C. A. Voyle, and R. A. Lawrie. 1963. A comparison of freeze-dried beef muscles of high or low ultimate pH. *J. Sci. Food Agr.* **14**, 535.

Perry, S. V. 1955. Myosin adenosinetriphosphatase. *In:* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds). Vol. II, p. 582. Academic Press, New York.

Putnum, F. W. 1955. Protein denaturation. *In:* "The Proteins." (H. Neurath and K. Bailey, eds). Vol. I. B., p. 828. Academic Press, New York.

Sewant, P. L., and N. G. Mager. 1961. Studies on frozen fish. I. Denaturation of proteins. *J. Food Sci.* **26**, 253.

Shikama, K. 1963. Denaturation of catalase and myosin by freezing and thawing. *Sci. Rept. Tohoku univ. Ser. 4 (Biol).* **29**, 91.

Suden, J. R., A. M. Pearson, and L. R. Dugan, Jr. 1964. Rehydration of freeze-dried pork as related to pH and protein denaturation. *J. Food Sci.* **29**, 192.

Szent-Györgyi, A. G. 1953. Meromyosins, the subunits of myosin. *Arch. Biochem. Biophys.* **42**, 305.

Szent-Györgyi, A. G., C. Cohen, and D. E. Philpott. 1960. Light meromyosin fraction. I. A helical molecule from myosin. *J. Mol. Biol.* **2**, 133.

Takahashi, K., T. Yasui, Y. Hashimoto, and Y. Tonomura. 1962. Physicochemical studies on denaturation of myosin-adenosinetriphosphatase. II. Changes in chromatographic profile and optical rotation. *Arch. Biochem. Biophys.* **99**, 45.

Tamoto, K., S. Takeda, T. Fukumi, and K. Nishiya. 1961. Studies on freezing of "Surimi" (fish paste) and its application. IV. On the

effect of sugar upon the keeping quality of frozen Alaska pollack meat. *Bull. Hokkaido Regional Fisheries Research Lab.* **23**, 50.

Yasui, T., T. Fukazawa, Y. Hashimoto, S. Kitagawa, and A. T. Sasaki. 1958. Denaturation of myosin B. *J. Biochem.* **45**, 717.

Yasui, T., Y. Hashimoto, and Y. Tonomura. 1960. Physicochemical studies on denaturation of myosin-adenosinetriphosphatase. Arch. Biochem. Biophys. **87**, 55.

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Changes in the Concentration of Lactic Acid and Free Sugars in Post-Mortem Samples of Beef and Pork Muscle

SUMMARY

Investigations were carried out on changes of lactate concentration and pH values at various post-mortem times on different samples of beef and pork muscles (Expts. *A* and *B*). The differences in lactate concentrations between the method of Barker and Summerson and the enzymatic assay were considerable, fully confirming previous findings. Besides the lactate and pH values, glucose, fructose, maltose, and ribose were determined after feeding sucrose to some of the animals (Expt. *C*). Sucrose feeding decreased the pH values and increased the values for lactate in investigated muscles of exhausted animals which had been transported over 340 miles in approximately 24 hr. By this procedure some characteristics of the quality of the meat could be improved. Similar effects were not detected in animals transported only 3.1 miles, although glucose and fructose were present in significantly higher amounts in the sucrose-fed animals than in the control group.

INTRODUCTION

In post-mortem muscle, lactic acid and other carboxylic compounds are produced in considerable amounts during the degradation of glycogen. Since lactic acid is often considered to be the determining factor for the pH value of a meat, the lactate concentration at various times post-mortem has been investigated by several workers (Briskey, 1959; Wismer-Pedersen and Briskey, 1961a). In those experiments, the chemical method of Barker and Summerson (1941) was utilized (Briskey, 1959; Wismer-Pedersen and Briskey, 1961b; Briskey *et al.*, 1959). This method, however, is subject to certain disturbing influences, as was shown by Schweiger and Günther (1964) and Lundholm *et al.* (1963a,b), working independently. The determination of lactate with lactic dehydrogenase is more specific and accurate. After these results were examined, it seemed both advisable and interesting to repeat the investigation of the "time course" of lactate production in beef and pork muscle using these two methods in parallel experiments. As expected, the lactate concentration at various times found by the chemical and enzymatic method varied considerably. Thus the findings reported in the preceding paper (Schweiger and Günther, 1964) have been fully confirmed. In addition to lactic concentration, pH changes in tissue were determined. A comparison of the values should serve as an indicator of glycolysis. There is a direct relation between lactic acid and muscle carbohydrates via the glycolytic pathway. Therefore it is possible to exert some influence on the post-mortem production of lactic acid by changing certain conditions for the living animal. Heavy stress during transport can reduce the glycogen stock in muscle (Bate-Smith, 1948), but the feeding of sugar compensates for this loss. This paper describes the influence of sugar feeding on muscle lactic acid concentration and pH values, and the strong influence exerted upon the concentration of several simple sugars such as glucose and fructose. These sugars give additional information on the process of glycolysis in meat.

MATERIALS AND METHODS

Animals and samples of muscle. *Experiment A.* The sample of steer muscle (1 kg) was from the longissimus dorsi. It was excised immediately after slaughter and stored at 0°C. Small pieces of the sample were checked at 20 min, and 2, 4, 6, 8, 24, 32, 48, 72, 98, and 169 hr post-mortem. Samples of beef muscles were taken 2, 5.5 and 27.5 hr post-mortem from the longissimus dorsi.

Experiment B. Six pigs (German Landrace) were selected from 42 that had been transported by the Federal Railway over a distance of 550 km (340 miles) involving about 24 hr of travel time. Upon arrival, animals 1–3 (average weight 108 kg) were fed a mixture of 1 kg sucrose and ½ kg cereal with protein-concentrate. Control animals 4–6 (average weight 112 kg) were only allowed to drink. The schedule and conditions before and after slaughter were the same for all 6

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animals. Samples were taken from the suspended carcasses from the adductor muscle at 30, 50, 80, 150, and 210 min and 19 hr post-mortem. Samples from animals 4–6 were taken at 50, 60, 90, 115 min and 19 hr. After taking the first sample, the carcasses were transferred to a cold room and stored at 0–2°C. (Different times post-mortem for technical reasons.)

Experiment C. In this experiment, 12 pigs (German Landrace) were used. They had been transported over 5 km (3.1 miles), which took 30 min. Six animals (7–12) were fed 1 kg sucrose and ½ kg cereal with protein-concentrate. Control animals (1–6) were fed the usual mixture of cereal and protein-concentrate. All animals were the same age; there were only minor differences of weight (7–12 with a $\bar{x}$ = 105 kg; 1–6 with a $\bar{x}$ = 113 kg). Conditions during and after slaughter were the same in both groups. Samples were taken at 1, 2, 3, 5, and 27 hr post-mortem. The carcasses were transferred to a cold room after the first sample had been obtained.

Chemicals. All chemicals were reagent-grade purchased from Merck, Darmstadt; ribose and the test-combination TCB 15 972 for the enzymatic determination of lactate were from Boehringer and Soehne, Co., Mannheim, Germany. Cellulose powders MN 300 and MN 300 HR were the product of Macherey and Nagel, Düren, Germany. Used for paper chromatography was paper 2043b of Schleicher and Schüll, Dassel, Germany. Glucose was identified on paper and thin layers with the "Glucostat" reagent of Worthington Biochem. Corporation, Freehold, N. J.

Extractions and quantitative determinations. The pH values were measured with a pH-meter type PHM 22 of Radiometer, København. All given values represent the average of double estimations. Perchloric acid extraction and determination of lactate using lactic dehydrogenase were described in detail by Schweiger and Günther (1964). The values obtained were multiplied by 33.8 in order to calculate the amount of lactate in the cuvette. The chemical determination was performed according to the method of Barker and Summerson (1941). In experiment *C*, samples were obtained and stored (0–2°C) every few hr over a 72-hr period. This was necessary for proper timing of the experiment. Other portions of the samples were stored in perchloric acid before extraction. There were, however, no differences in the final lactate values due to the different methods of extraction, as the comparison in Table 1 shows.

Sugars were determined according to Günther (1962) and Grau *et al.* (1960). The method is described briefly as follows:

The perchloric extract of a 1.5- or 2.0-g sample of the muscle was neutralized with KOH and the supernatant was purified on a cation exchanger Merck I in the H^+-form (15 × 1-cm column). The eluate was concentrated in a rotary evaporator. Depending on the sugar concentration, 1–20 µl of the solution were spotted for paper chromatography. The following solvents were used: system I, a mixture of butanol-ethanol-water-isopropanol (2:4:0.5:1.5); or system II, a mixture of ethyl acetate-pyridin-water (2:1:2). After the chromatograms were dried, the sugars were stained with an alcoholic solution of p-anisidine-phthalate, a modification of Pridham's method (1956). The colors obtained were: glucose and fructose = brown-green; maltose = pink-brown; ribose and other pentoses = red-violet. To ensure an even staining procedure, the paper was dipped into the staining solution and placed in an oven for 15 min. The paper became transparent by placing it into paraffin liquid for 1–2 hr; surplus paraffin was removed under a pressure of 1 kg. For a quantitative determination of the spots, the chromatogram attachment CA_2 was used in the Spectrophotometer PMQ II (Zeiss-Oberkochen-Germany) (Fig. 1). The slit opening for glucose, fructose, and maltose was 0.1 mm at wavelength of 510 nm, and for ribose 0.04 mm at wavelength of 400 nm. The values were put in the form of a curve on paper for quantitative calculation (Fig. 2). A comparison with standard sugars that were handled in the same way as the samples extracted from muscle, gave the exact amount of sugars present in the muscle. The standard deviation is ±5% at the 2 s confidence limit. Recovery of added glucose was 93%. For additional identification of the sugars by paper chromatography, a butanol-pyridin-water mixture (6:4:3 = system III) and a butanol-acetic-water mixture (4:1:1 = system IV) were used.

Identifications of the substances. L(+) lactic acid was identified as its p-phenyl-phenacyl-ester by means of the infrared spectrum in the Perkin-Elmer Infracord 137 E. For this purpose the lactic acid from muscle was purified on a cellulose layer. The sugars were also chromatographed on 0.5-mm cellulose layers (MN 300 HR = high purity) in system II. Glucose and fructose (1–2 mg) were eluted from the layers and mixed with

Table 1. Lactate values (g/100 g muscle).

Animal	Immediately extracted in $HClO_4$	Stored in $HClO_4$	Stored in 96% ethanol
30	1.09	1.01	0.96 Barker a. S.
	1.01	0.96	0.90 enzymatic
22	1.21	1.06	1.17 Barker a. S.
	0.83	0.77	0.83 enzymatic

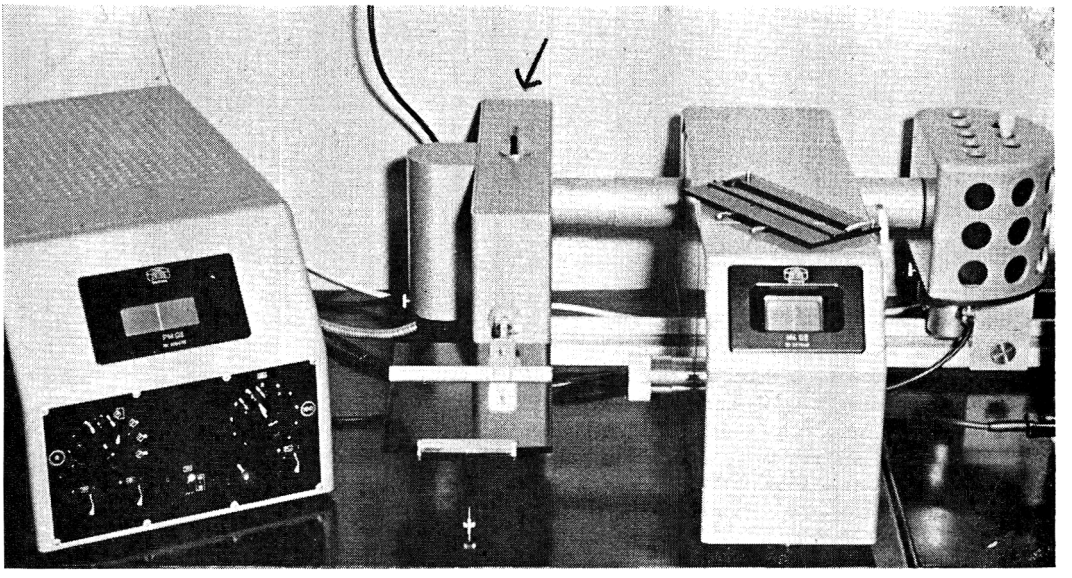

Fig. 1. Chromatogram attachment CA_2 for the spectrophotometer PMQ II (Zeiss, Oberkochen).

200 mg KBr in 0.5 ml solution. After desiccation, the material was pulverized and formed into discs for infrared spectroscopy. Glucose and maltose

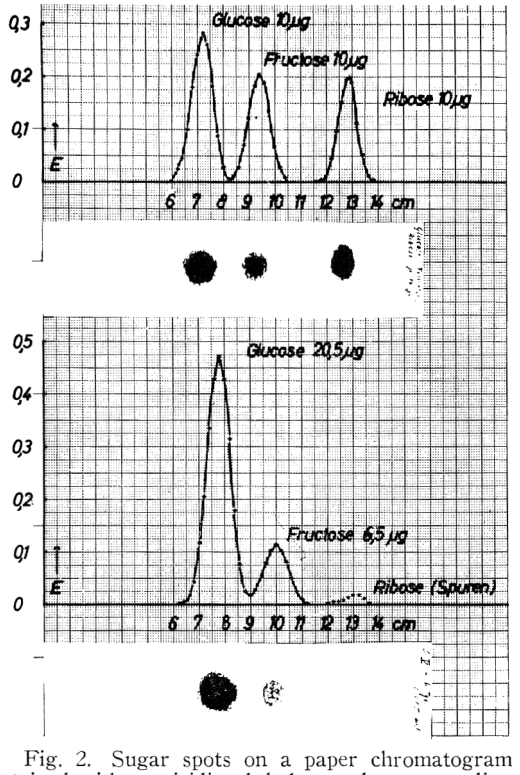

Fig. 2. Sugar spots on a paper chromatogram stained with p-anisidinephthalate and corresponding diagram of extinctions. Top: standards; bottom: sugars isolated from pig muscle.

(after 5 hr of hydrolysis in $N/10$ HCl) were identified on the paper and layers by the reaction with glucose oxidase (Salton, 1960). Ribose was refluxed for 30 min in 1 ml of water with 0.1 g sodium acetate and 0.1 g phenylhydrazine according to the method of Haynes (1961). Ribosyl-phenylosazone was precipitated in the cold, washed, recrystallized from ethanol, and formed into a disc with 200 mg KBr for infrared spectroscopy.

RESULTS

The determination of lactate in post-mortem beef and pig muscle by chemical and enzymatic methods confirms that there are concentration curves showing qualitative and quantitative differences in many cases. It is not always possible to detect any correlation between the lactate values and the muscle pH determined at the same time. Fig. 3 and Table 2 compare lactate values for experiment *A*. The slope of both curves, from enzymatic and chemical determinations, is about the same. However, the Barker and Summerson values are 20–25% lower (Table 2 = 10–60%). Differences in lactate concentration with the pig muscle experiments were much more obvious (Figs. 4, 5, 6). These figures show that there may be extreme differences, by a factor of 10–20, especially during the rigor mortis period. There were also considerable deviations in the qualitative pattern (Fig. 4). However, in some cases these deviations were negligible and the curves almost identical (Fig. 7). With all animals there was one common feature, at least when the enzymatic lactate determination was used, that being a significantly short period

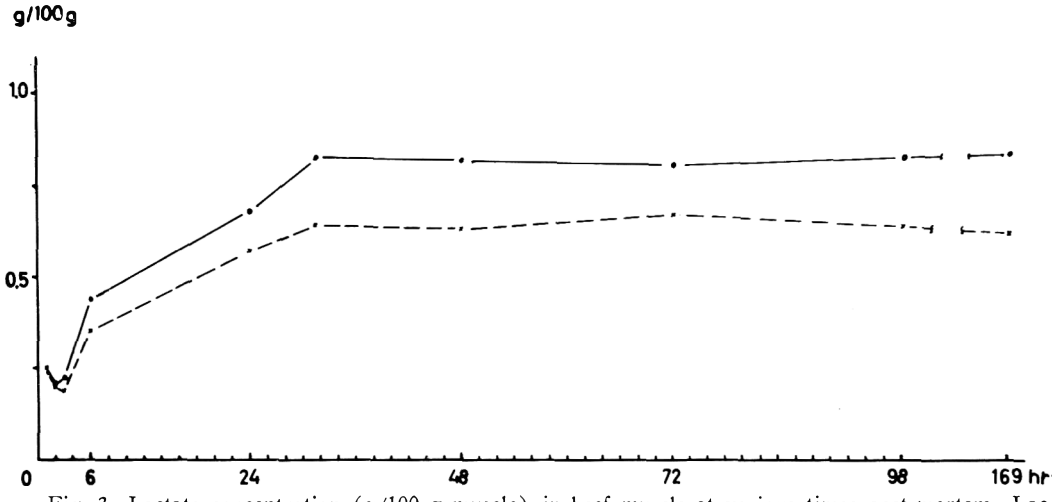

Fig. 3. Lactate concentration (g/100 g muscle) in beef muscle at various times post-mortem. Lactate according to the Barker and Summerson method, ×– – –×. Lactate with the LDH method •——•.

of decreasing lactate concentration at about 2–4 hr post-mortem.

It was shown in the feeding experiment that sucrose feeding influenced lactate production in animals subjected to long-distance transport. Fig. 8 gives the increase of lactate concentration in the muscle. It is evident that sucrose feeding causes a lactate increase of almost 100% during the first 5 hr post-mortem (Fig. 8a), whereas there is only a relatively small increase in the control animals (Fig. 8b). pH value curves correspond to lactate in this experiment. There was a large increase of 0.8 pH unit in the sucrose animals, compared to minor changes of pH in the control animals. On the other hand, the sucrose feeding had very little influence upon lactate and pH patterns of animals subjected to just short-distance transport (Figs. 9, 10).

In addition, the concentration of some free sugars was determined post-mortem in muscles of animals subjected to short-distance transport. Fig. 9 shows that glucose, with 46 mg, and fructose, with 21 mg/100 g muscle, were significantly increased in sucrose animals, compared with 22 and 4 mg, respectively, in the control animals. (Glucose concentration maximum at 3–5 hr post-mortem.) The concentrations of maltose and ribose were always below 10 mg in 100 g of muscle. Other sugars had lower concentration than 1 mg in 100 g.

The infrared spectra of glucose, fructose, and ribosylphenylosazone were in accordance with those of standards and spectra described in the literature (Tipson and Isbell, 1962; Otting, 1961). The main bands of D-glucose (Fig. 11) at 13.0, 11.7, and 10.9 μ, and of fructose (Fig. 12) at 10.75, 12.25, and 12.8 μ can be seen clearly. Ribosylphenylosazone has a spectrum with a band at 10.85 μ and a missing adsorption at 9.0 μ (Fig. 13). Thus, it is easy to distinguish ribose from xylose, which has a similar R_f value on chromatograms, by the phenylosazone of the former.

DISCUSSION

Lactate values. The experiments show that there are minor quantitative deviations of lactate values determined by either the chemical or enzymatic method in aliquots of a beef muscle extract, whereas these deviations are considerable in pork muscle. The

Table 2. Concentrations of lactic acid estimated by Barker and Summerson (** = ±8.2%) and by the enzymatic method (** = ±4.1%) in some samples of beef muscle at various times after slaughter.

	Time post-mortem in hr					
	2		5.5		27.5	
Animal	Enzym.	Chem.	Enzym.	Chem.	Enzym.	Chem.
1	0.355	0.395	0.240	0.355	0.389	0.450
2	0.159	0.275	0.203	0.340	0.254	0.350
3	0.081	0.145	0.064	0.170	0.610	0.940
4	0.425	0.650	0.230	0.330	0.575	0.905

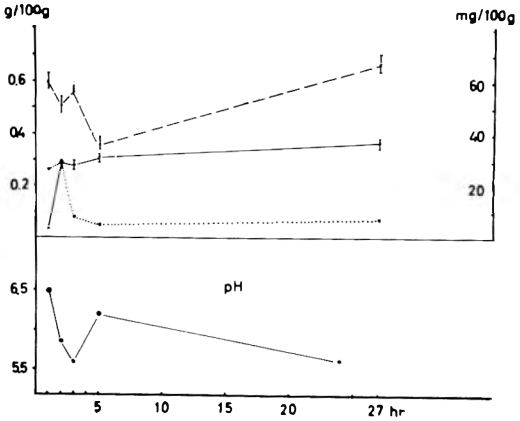

Fig. 4. Lactate concentration (g/100 g) and pH curve in pig muscle at various times post-mortem. Animal 3 (Control). Lactate according to Baker and Summerson, ×– – –×. Lactate with LDH method, •——•. Glucose, ○ . . . . ○. Upper curve: ordinate at left: g lactate/100 g muscle; ordinate at right: mg glucose/100 g muscle. Brackets indicate standard deviation of the method.

Barker and Summerson method appears to be disturbed by substances present over a period lasting several hours post-mortem. These substances may be identical with substances which were apparently separated from lactate by chromatography in our earlier experiments (Schweiger and Günther, 1964). It is interesting that there are cases that suggest quite different patterns of lactate production in different samples when chemical determination is used, whereas these patterns are identical at least in their main feature when enzymatic determination is used. In only a few cases was there a direct correlation between the production of lactate

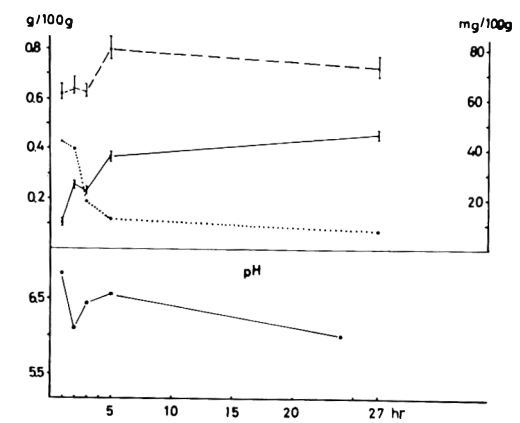

Fig. 5. Same explanation as in Fig. 4, animal 9. Brackets indicate standard deviation of the method.

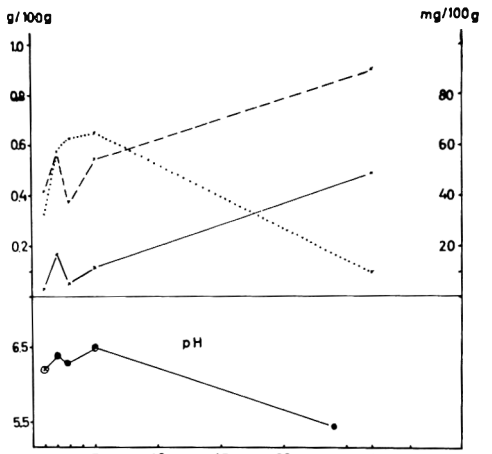

Fig. 6. Same explanation as in Fig. 4, animal 1 (Control).

and changes in pH. It was possible to alter the post-mortem pH pattern by subjecting the animals to different conditions before slaughter, especially by feeding them sucrose. This shows that the system of essentially 4 types of reactions for pigs, suggested by Briskey and Wismer-Pedersen (1960, 1961b), is certainly dependent on several factors and should not be regarded as a fixed scheme.

It is not completely known whether lactic acid, produced post-mortem, is the only compound that is responsible for the decreasing pH values in muscle. Grau and Günther (1962a) suggested the contribution of other strongly acidic carboxylic compounds (e.g.

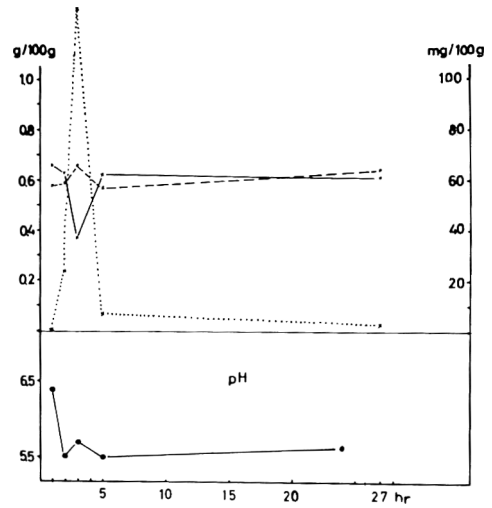

Fig. 7. Same explanation as in Fig. 4, animal 11

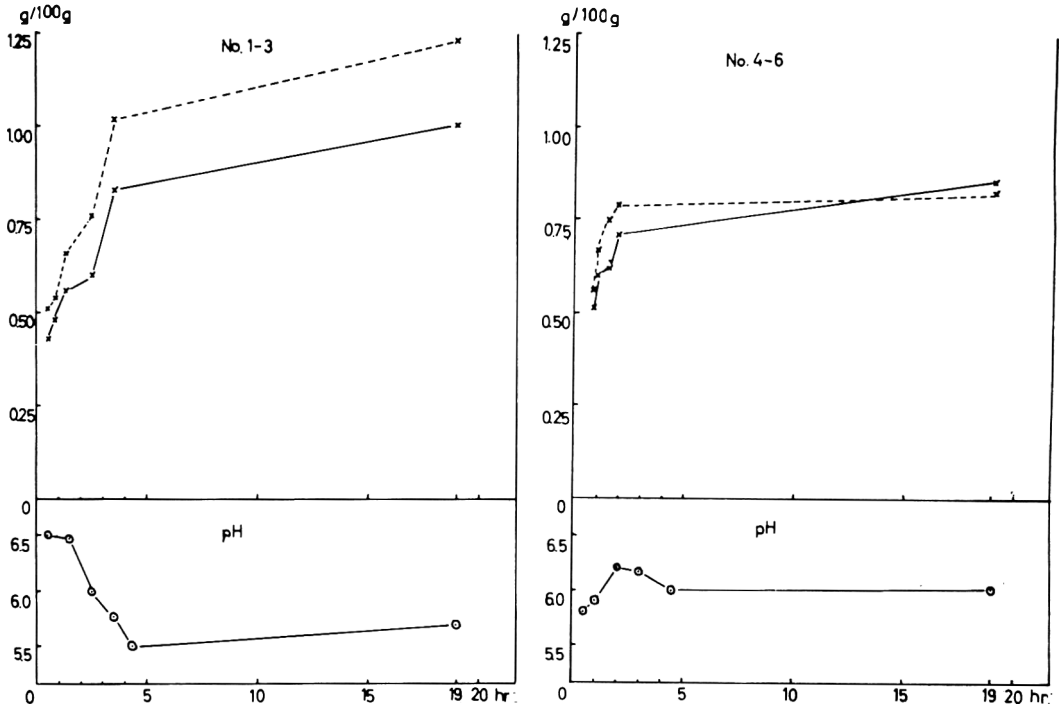

Fig. 8. Lactate concentration (g/100 g) and pH curve in pig muscle (averages from 3 animals) at various times post mortem; transport of 550 km, Expt. *B* (340 miles). Lactate according to Barker and Summerson, ×---×. Lactate with LDH method, ●——●. 8a) Animals fed with sugar—left. 8b) Controls—right.

citric acid) to the decreasing pH values. Most of the results given here also indicate that there is no simple correlation between lactate concentration and pH values. Only the sucrose-fed animals in experiments *B* and *C* showed, to a certain extent, such a correlation. In some cases, there was an increase of both lactic acid concentration and pH values at 3–5 hr post-mortem. It may be that the increasing pH was due to the influ-

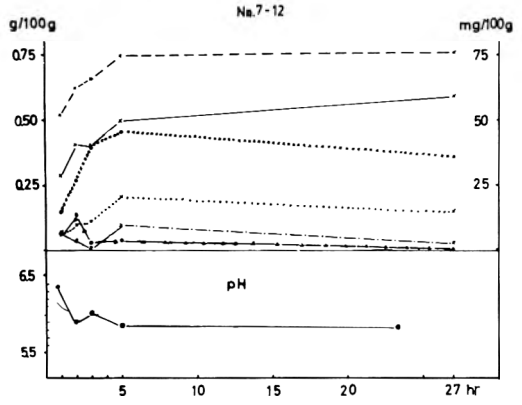

Fig. 9. Lactate concentration (g/100 g) and pH curve in pig muscle (averages from 6 animals) at various times post-mortem; transport of 5 km (3.1 miles). Expt. *C*. Lactate according to Barker and Summerson ×---×. Lactate with LDH method, ●——●. Glucose, ××××. Fructose, ×...×. Ribose, ×—•—×. Maltose, ▲——▲. Top: ordinate at left: g lactate/100 g muscle; ordinate at right: mg sugar/100 g muscle. Animals fed with sugar.

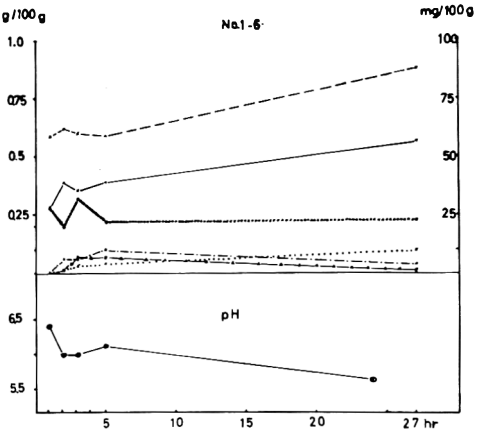

Fig. 10. Controls.

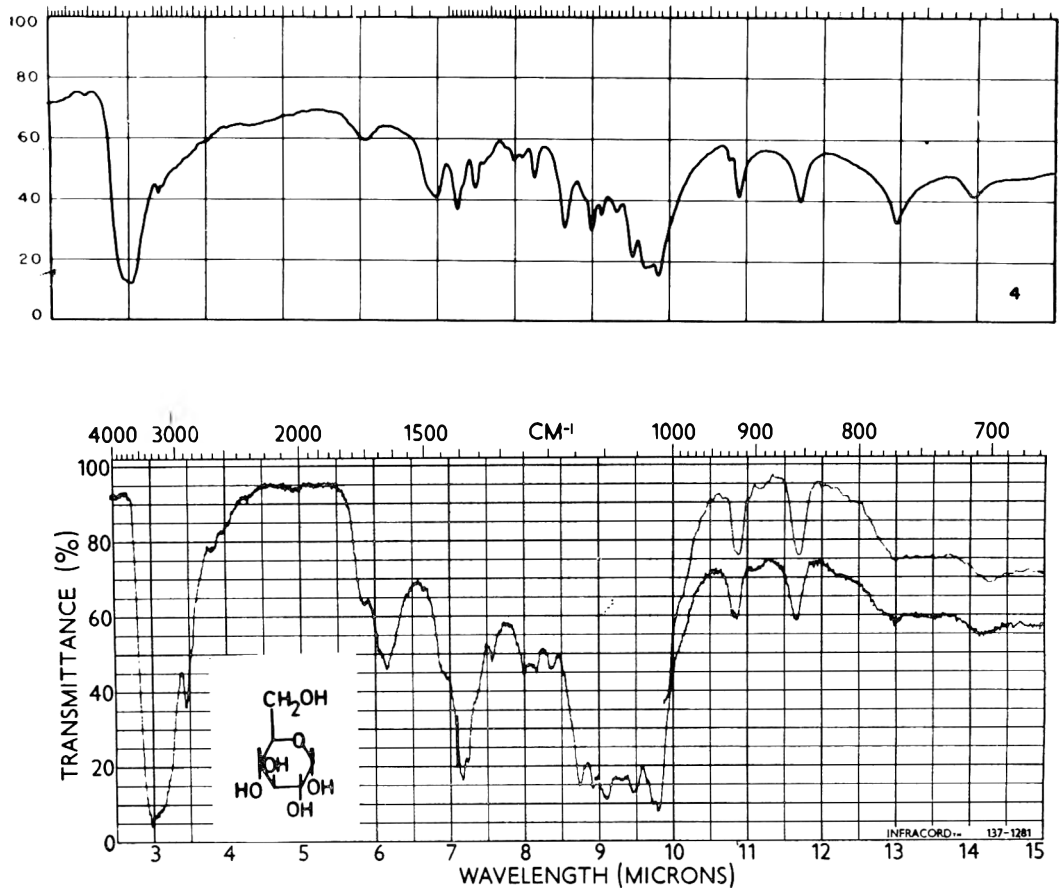

Fig. 11. Infrared spectra of D-glucose × H_2O. Top: after Tipson and Isbell (1962). Bottom: glucose isolated from muscle and purified on a cellulose layer.

ence of enzymatic deamination of adenine nucleotides described by Bate-Smith and Bendall (1956) and Bendall and Davey (1957).

The rigor mortis period was especially interesting. Among others, Amano *et al.* (1953) and Fujimaki and Kojo (1953) determined some intermediates in fish muscle at various post-mortem times. They found that there is a decrease in lactic acid concentration after 1–2 hr combined with an increase of glycogen concentration in the presence of ATP. A resynthesis of ATP from creatinphosphate and ATP during the early period of post-mortem glycolysis is described by Bendall (1960). Some of these results were confirmed during our experiments. In steer muscle (Expt. *A*, Fig. 4), the lactic acid value was at a minimum at 2–3 hr, followed by an increase. A very similar type of curve was obtained for most pig muscles (Expt. *B*, *C*,) at 2–3 hr post-mortem.

Effect of sugar feeding. The feeding of sucrose-exhausted animals caused a significant increase of lactate along with lower pH values in post-mortem muscle (Expt. *B*). This means that sucrose can restore a higher level of sugar in muscle. The production of lactic acid was less in the control animals and there were almost no pH changes. However, it is impossible to arrive at a definite conclusion without further experiments with larger numbers of animals. In contrast to the animals which were transported over long distances, the animals in experiment *C* were not influenced by the sucrose feeding. There were no differences in lactate and pH in the control group.

The sugar determinations demonstrated that the concentrations of glucose and fruc-

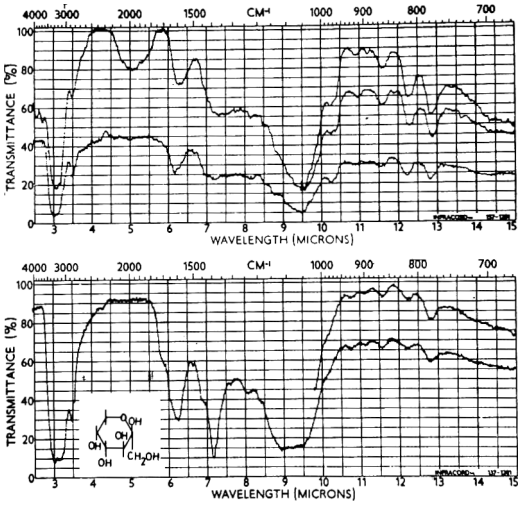

Fig. 12. Infrared spectra of D-fructose. Top: standard. Bottom: fructose isolated from muscle and purified.

tose are considerably higher in the muscle of all sucrose animals. These sugars are apparently derived from sucrose by enzymatic cleavage *in vivo*. The possibility that the sucrose molecule reaches the muscle before slaughter and is split during the perchloric extraction was ruled out in an experiment where pure sucrose was treated in the same way as the sample. With sucrose there are degradation products which during chromatography tend to run (paper and thin layer) close to the solvent front; these substances did not appear with the sample extracts.

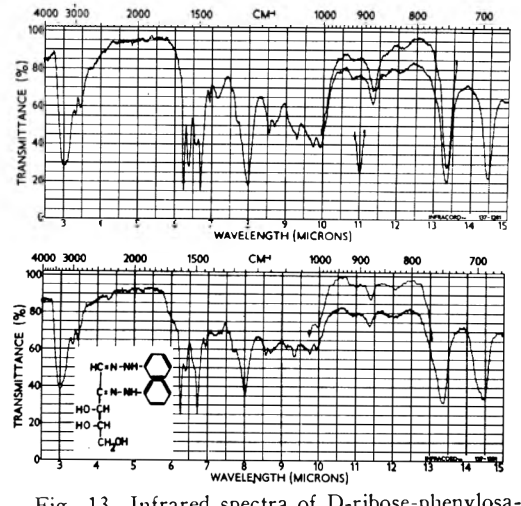

Fig. 13. Infrared spectra of D-ribose-phenylosazone. Top: standard. Bottom: ribose-phenylosazone from ribose isolated from muscle.

There is a minor pathway of degradation of glycogen to maltose under the influence of α-amylase. Maltose and oligosaccharides were found on paper chromatograms of veal extract (Grau *et al.*, 1960) and pig muscle extract (Grau and Günther, 1962b) in earlier experiments. During the present investigation (Expt. *C*) there was a maximum of maltose concentration 2 hr post-mortem in sucrose-fed animals. The work of Batzer *et al.* (1962) shows that glucose is necessary in order to produce the typical meat flavor. Scheper (1964), of the Bundesanstalt für Fleischforschung, determined certain quality characteristics in experiments *B* and *C* with pig muscle. He found that the long transport (Expt. *B*), especially below freezing temperatures, promotes an accelerated glycolysis and its undesirable consequences, such as low water-holding capacity, pale color, and soft consistency. However, a transport distance of 5 km (3.1 miles) (Expt. *B*) had little or no influence on the quality characteristics. The feeding of sucrose followed by a rest of several hours restores exhausted animals not only with normal glycolysis, as we have shown, but also with good water-holding capacity and consistency. There was evidence to indicate some breeds are not as susceptible to long-distance transport effects on quality of the meat.

REFERENCES

Amano, K., M. Bito, and T. Kawabata. 1953. Handling effect upon biochemical changes in the fish muscle immediately after catch. I. Difference of glycolysis in the frigate mackerel killed by various methods. *Bull. Japan. Soc. Sci. Fisheries* **19**, 49.

Barker, S. B., and W. H. Summerson. 1941. The colorimetric determination of lactic acid in biological material. *J. Biol. Chem.* **138**, 535.

Bate-Smith, E. C. 1948. The effect of fatigue on post-mortem changes in muscle. A. Report of Food Invest. Vol. 21. Cited from *Advances in Food Research* **1**, 11.

Bate-Smith, E. C., and J. R. Bendall. 1956. Changes in muscle after death. *Brit. Med. Bull.* **12**, 230.

Batzer, O. F., A. Santoro, and W. Landman. 1962. Identification of some beef flavor precursors. *Agr. Food Chem.* **10**, 94.

Bendall, J. R. 1960. Post-mortem changes in muscle. *In*: The Structure and Function of Muscle.

VIII. G. H. Bourne. Academic Press, New York and London.

Bendall, J. R., and C. L. Davey. 1957. Ammonia liberation during rigor mortis and its relation to changes in the adenine and inosine nucleotides in rabbit muscle. *Biochim. et Biophys. Acta* **26**, 93.

Bendall, J. R., O. Hall, and J. Wismer-Pedersen. 1963. Verfärbung und Wässrigkeit in der Muskulatur von Landrasse Schweinen nach der Schlachtung. *Fleischwirtschaft* **15**, 1115.

Briskey, E. J. 1959. Changes occurring during rigor mortis and subsequent ripening of muscle tissue. *Proc. 11th Recip. Meat Conf.*

Briskey, E. J., and J. Wismer-Pedersen. 1960. Biochemistry of pork muscle structure. I. Rate of anaerobic glycolysis and temperature change versus the apparent structure of muscle tissue. *J. Food Sci.* **26**, 297.

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.

Fujimaki, M., and K. Kojo. 1953. Handling effect upon biochemical changes in the fish muscle immediately after catch. II. Changes of acid-soluble phosphorus compounds of frigate mackerel muscle. *Bull. Japan. Soc. Sci. Fisheries* **19**, 49.

Grau, R., and H. Günther. 1962a. Über die beim Reifen von Fleisch und Fleischerzeugnissen vorkommenden organischen Säuren und zur Methode der Isolierung und Trennung. *Fleischwirtschaft* **14**, 184.

Grau, R., and H. Günther. 1962b. Wirkung von Chlortetracyclin als Futterzusatz auf die Glycolyse im Schweinemuskel. *Naturwissenschaften* **14**, 329.

Grau, R., H. Günther, and J. Scheper. 1960. Das biochemische Verhalten der natürlich im Fleisch vorkommenden Zucker beim Kalb unter Berücksichtigung der Schlachtqualität. *Fleischwirtschaft* **12**, 728.

Günther, H. 1962. Papierchromatographische Bestimmung von natürlich im Fleisch vorkommenden Zuckern. *Fleischwirtschaft* **14**, 502.

Haynes, B. 1961. Qualitative Organic Analysis. Cleaver-Hume Press, London. p. 110.

Lundholm, L., E. Mohme-Lundholm, and N. Svedmyr. 1963a. Comparative investigation of methods for determination of lactic acid in blood and in tissue extracts. *Scand. J. Clin. & Lab. Invest.* **15**, 311.

Lundholm, L., E. Mohme-Lundholm, and N. Vamos. 1963b. Lactic acid assay with L(+) lactic acid dehydrogenase from rabbit muscle. *Acta Physiol. Scand.* **58**, 243.

Otting, W. 1961. IR-Spektroskopische Untersuchung von Phenylosazonen. *Liebigs Ann. Chem.* **640**, 44.

Pridham, J. B. 1956. Determination of sugars on paper chromatograms with p-anisidinehydrochloride. *Anal. Chem.* **28**, 167.

Salton, M. 1960. Specific determination of glucose on paper chromatograms. *Nature* **186**, 966.

Scheper, J. 1964. Zum Problem des Transportes von Schlachtschweinen. *Schlacht- und Viehhof-Zeitung* **64**, 399.

Schweiger, A., and H. Günther. 1964. A comparison of two methods for the determination of lactic acid in muscle *J. Food Sci.* **29**, 808.

Tipson, R., and H. Isbell. 1962. Infrared absorption spectra in the study of mutarotational equilibria of monosaccharides. *J. Research Natl. Bureau of Standards. A. Physics and Chemistry Vol.* **66** A, 1. Jan.-Febr.

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

Wismer-Pedersen, J., and E. J. Briskey. 1961b. Relationship of post-mortem acidity and temperature. *Food Technol.* **15**, 232.

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Effects of Environmental Temperature and Humidity During Growth on Muscle Properties of Two Porcine Breeds

SUMMARY

Studies were conducted on the muscle properties of pigs reared in one of several combinations of environmental temperature and humidity. Longissimus dorsi muscle of pigs reared in alternating temperatures (29 and 18°C) was inferior in structure to that of pigs reared in 29 or 18°C constant temperatures if the relative humidity was low (30%). High relative humidity (85%) during growth tended to improve muscle structure and tenderness (68 and 72°C cooking temperature), regardless of environmental temperature. Breed-treatment interactions for muscle structure score indicated that Poland China and Hampshire pigs differed in response to environment. Breed comparisons over all treatments revealed that muscle from Poland China pigs was less tender than muscle from Hampshire pigs when it was heated to 60°C. The data on tenderness suggest that the major environmental effects were exerted on the muscle constituents which are susceptible to high cooking temperatures (protein hardening range) whereas the breed differences were evident at low cooking temperatures (collagen shrinkage range).

INTRODUCTION

The incidence of pale, soft, exudative (PSE) porcine muscle increases during the seasons of the year that temperatures are high or fluctuating (Ludvigsen, 1954; Judge *et al.*, 1959; Forrest *et al.*, 1963). Heat stress on pigs of the Poland China and Hampshire breeds causes rapid anaerobic glycolysis, elevated post-mortem temperature, and ultimate development of the PSE condition in the muscles (Sayre *et al.*, 1963; Kastenschmidt *et al.*, 1964, 1965). However, no research has been reported on the influences that specific conditions of the rearing environment may exert on the post-mortem temperature and ultimate properties of chilled porcine muscle.

Studies on the relationship of porcine muscle color or structure to tenderness have had conflicting results. Judge *et al.* (1960) reported that the tenderness of broiled chops was negatively correlated with the degree of muscle firmness prior to cooking, whereas Briskey (1963) reported that the PSE muscle of heat-stressed animals was less tender than normal muscle after moist-heat cookery. Although work of Sayre *et al.* (1964) indicated that resistance to shear was high in PSE muscle heated to 85°C, the effect of end-point cooking temperature on PSE muscle tenderness has not been investigated.

This experiment was designed to: 1) determine the effects of several combinations of environmental temperature and humidity during growth on the post-mortem temperature and ultimate color, structure, and tenderness of muscle from breeds of pigs known to have a high incidence of PSE muscle; and 2) determine the effect of end-point cooking temperature on the tenderness of muscles whose properties may have been influenced by rearing environment or breed.

EXPERIMENTAL

This experiment utilized muscle samples from 36 Poland China and 36 Hampshire barrows reared for 35 days in psychrometric chambers in which temperature and humidity were controlled. At the beginning and end of the experiment the respective mean weights of the animals were 68 and 95 kg. The animals were allotted into six treatment groups by breed (Table 1). They were kept in pens which provided 0.93 sq m of floor space per animal.

At the end of the growth period the animals were transported approximately 5 miles in an enclosed vehicle. Although precise humidity control was impossible during transport, the temperature

Table 1. Experimental design.

Group	Ambient temperature (°C)	Relative humidity (%)	Number of animals	
			Poland China	Hampshire
H[a]L[b]	29	30	6	6
LL	18	30	6	6
AL	29, 18[c]	30	6	6
HH	29	85	6	6
LH	18	85	6	6
AH	29, 18[c]	85	6	6

[a] Designates high, low, or alternating temperature.
[b] Designates high or low percent relative humidity.
[c] Three-day periods at each temperature.

in the vehicle was maintained at the environmental temperature at which the animals were reared. (Animals in the alternating temperature group were removed from treatment while in the 29°C temperature.) Excitement of the animals was minimized prior to exsanguination.

Carcass temperatures were observed at 45 min post-mortem by inserting a testing thermometer into the center of the ham. Color and structure scores for the chilled longissimus dorsi muscle were determined at 24 hr post-mortem. Color and structure were scored separately; muscles were classified as soft, intermediate, or firm, and as pale, grayish-pink, or dark, with each attribute receiving a score of 1, 2, or 3, respectively.

At 24 hr post-mortem the two longissimus dorsi muscles were removed from each carcass, wrapped in heavy freezer wrap, and stored at −18°C for later determinations of resistance to shear. After thawing, portions of the center sections of the muscles (excluding 10-cm portions at each end) were placed in plastic bags. Copper-constantan thermocouples were inserted into the center of the samples, air was evacuated from the bags, and the bags were sealed. The samples were heated in a water bath at 60, 64, 68, or 72°C. When temperature recordings indicated that the samples had reached the temperature of the water bath, heating was terminated and the samples were cooled to 4°C.

Resistance to shear was determined on the cooked samples and on raw samples with an Allo-Kramer shear press. Samples were sliced for shearing by the technique of Wise and Stadelman (1959) with the cutting device designed by Dodge and Stadelman (1960). All slices were weighed to the nearest 0.1 g, and resistance to shear was calculated in lb per g of tissue. A minimum of 10 slices per sample were sheared.

Data were analyzed by analysis of variance as described by Ostle (1956). Treatment means were tested with the Duncan (1955) multiple-range test.

RESULTS AND DISCUSSION

Differences in carcass temperature at 45 min post-mortem approached statistical significance at the .05 testing level (Table 2). The data indicated that high (29°C) and alternating (29 and 18°C) environmental temperatures during growth tended to elevate carcass temperature, a condition that may be detrimental to ultimate muscle quality (Wismer-Pedersen and Briskey, 1961).

The environmental conditions in this experiment had little influence on the color of the chilled muscle, but its structure was affected (Table 2). The combination of alternating temperature and low relative humidity (30%) resulted in muscle structure scores that were lower ($P < .05$) than those of the other environmental treatments. High relative humidity (85%) tended to improve structure score regardless of the environmental temperature. A significant ($P < .05$) breed-treatment interaction for muscle structure score indicated that the superior structure in the high-humidity groups resulted from the responses of the Poland China pigs (Fig. 1). High and low environmental humidity appeared to produce similar muscle structure in Hampshire pigs.

Allo-Kramer shear values indicated that the effects of rearing environment on tenderness were more evident when relatively high cooking temperatures (68 and 72°C) were used (Table 3). In addition, the magnitude

Table 2. Effects of rearing environment on post-mortem properties of porcine muscle

	Significant mean differences[a]						F
	LH[b]	LL	HL	AH	AL	HH	
Temperature (°C)[c]	40.7	40.8	41.0	41.2	41.5	41.6	2.1
	AL	AH	HH	HL	LL	LH	
Color score[de]	1.67	1.67	1.83	1.90	1.92	1.92	0.7
	AL	HL	LL	AH	HH	LH	
Structure score[df]	1.48	1.63	1.67	1.75	2.00	2.08	2.6*

[a] Any group means not underlined by the same line are significantly ($P < .05$) different.
[b] See Table 1 for description of treatments.
[c] Internal temperature at the center of the ham 45 min post-mortem.
[d] Longissimus dorsi, 24 hr post-mortem.
[e] 1 = pale, 3 = dark.
[f] 1 = soft, 3 = firm.
* $P < .05$.

Table 3. Effects of rearing environment on shear values [a] in porcine longissimus dorsi muscle.

Cooking temperature	Significant mean of differences [b]						F
	LL [c]	HH	AH	HL	LH	AL	
Raw	19.5	19.9	20.5	20.8	21.1	21.4	0.2
	LH	AL	HH	HL	LL	AH	
60°C	26.8	27.8	28.4	29.5	29.7	30.7	1.7
	AL	HL	LL	LH	HH	AH	
64°C	20.9	22.4	23.0	24.2	24.9	25.2	1.1
	LH	AL	AH	HH	HL	LL	
68°C	25.1	28.2	28.7	29.3	32.3	32.5	3.6**
	LH	HH	AH	HL	AL	LL	
72°C	24.8	26.9	28.0	30.5	30.8	32.2	3.8**

[a] Mean lb shear/g tissue.
[b] Any group means not underlined by the same line are significantly ($P < .05$) different.
[c] See Table 1 for description of treatments.
** $P < .01$.

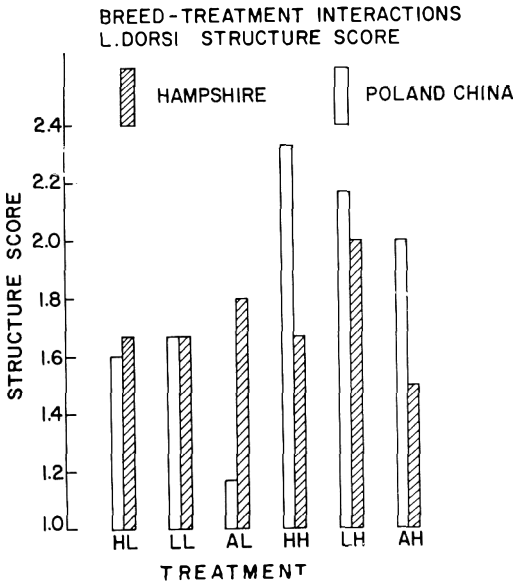

Fig. 1. Interaction of breed and environmental treatment for structure score of longissimus dorsi muscle. See Table 1 for description of treatments.

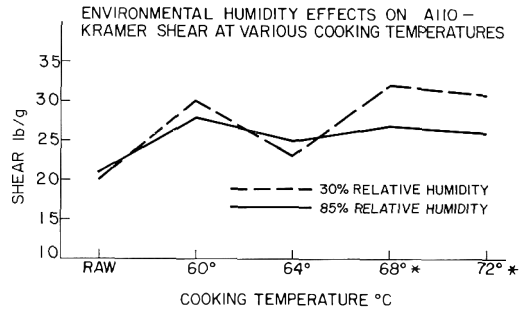

Fig. 2. Effect of environmental humidity during growth on Allo-Kramer shear values for longissimus dorsi muscle at various cooking temperatures. * $P < .05$.

of the shear values indicated that pigs reared in low humidity tended to have less tender muscle than pigs reared in high humidity. These observations were confirmed when the data were combined to compare the effects of relative humidity over all environmental temperatures (Fig. 2). The graphic representation of the data showed that muscle with inferior structure (from animals reared at low humidity) toughened when subjected to high cooking temperatures. These results are in agreement with research of Sayre *et al.* (1964), who found high shear values in PSE muscle which had been heated to 85°C. It is possible that the fibrillar proteins harden in muscle with inferior structure when cooking temperatures reach 68 or 72°C. This suggestion is supported by work of Machlik and Draudt (1963), who observed that collagen shrinkage was complete in beef muscle heated for 15 min or less in the temperature range of 60–65°C, whereas little protein hardening occurred after prolonged heating at those temperatures. In the present

Table 4. Breed differences in shear values[a] in longissimus dorsi muscle.

Cooking temperature	Breed		F
	Poland China	Hampshire	
Raw	21.2	19.8	1.3
60°C	30.0	27.7	6.5*
64°C	24.3	22.6	1.9
68°C	30.0	28.7	1.1
72°C	29.7	27.9	2.3

[a] Mean lb shear/g tissue.
* $P < .05$.

work, collagen shrinkage was probably complete at cooking temperatures below those at which treatment effects were evident.

Data on resistance of muscles to shear were subjected to analysis for breed differences. Muscles of Poland China pigs were less tender ($P < .05$) than muscles of Hampshire pigs when cooked to 60°C (Table 4). These breed differences in tenderness were independent of treatment effects (shown by analysis of variance), and they occurred at a cooking temperature that causes collagen shrinkage but probably does not cause hardening of the fibrillar proteins (Machlik and Draudt, 1963). Consequently, the muscle constituents responsible for breed contrasts in tenderness may differ from those responsible for treatment effects.

REFERENCES

Briskey, E. J. 1963. Influence of ante- and postmortem handling practices on properties of muscle which are related to tenderness. Proc. Meat Tenderness Symposium, Campbell Soup Co. 195.

Dodge, J. W., and W. J. Stadelman. 1960. Studies on tenderness evaluation. *Poultry Sci.* **29**, 184.

Duncan, D. B. 1955. Multiple range and multiple F tests. *Biometrics* **11**, 1.

Forrest, J. C., R. F. Gundlach, and E. J. Briskey. 1963. A preliminary survey of the variations in certain pork ham muscle characteristics. *Proc. 15th Research Conf. Circ.* **74**, 81. Am. Meat Inst. Foundation, Chicago, Ill.

Judge, M. D., V. R. Cahill, L. E. Kunkle, and W. H. Bruner. 1959. Pork quality. I. Influences of some factors on pork muscle characteristics. *J. Animal Sci.* **18**, 448.

Judge, M. D., V. R. Cahill, L. E. Kunkle, and F. E. Deatherage. 1960. Pork quality. II. Physical, chemical and organoleptic relationships in fresh pork. *J. Animal Sci.* **19**, 145.

Kastenschmidt, L. L., E. J. Briskey, and W. G. Hoekstra. 1964. Prevention of pale, soft, exudative porcine muscle through regulation of ante-mortem environmental temperature. *J. Food Sci.* **29**, 210.

Kastenschmidt, L. L., G. R. Beecher, J. C. Forrest, W. G. Hoekstra, and E. J. Briskey. 1965. Porcine muscle properties. A. Alteration of glycolysis by artificially induced changes in ambient temperature. *J. Food Sci.* **30**, 565.

Ludvigsen, J. 1954. Undersogelser Over Den Sakaldte "Muskeldegeneration" Hos Svin. *Beretn. fra forsogslaboratoriet Udgivet of Statens Husdrybrugsudvalg* **1**, 272.

Machlik, S. M., and H. N. Draudt. 1963. The effect of heating time and temperature on the shear of beef semitendinosus muscle. *J. Food Sci.* **28**, 711.

Ostle, B. 1956. Statistics in Research. 2nd ed. Iowa State College Press, Ames, Iowa.

Sayre, R. N., E. J. Briskey, and W. G. Hoekstra. 1963. Alteration of post-mortem changes in porcine muscle by preslaughter heat treatment and diet modification. *J. Food Sci.* **28**, 292.

Sayre, R. N., B. Kiernat, and E. J. Briskey. 1964. Processing characteristics of porcine muscle related to pH and temperature during rigor mortis development and to gross morphology 24 hr post-mortem. *J. Food Sci.* **29**, 175.

Wise, R. G., and W. J. Stadelman. 1959. Tenderness at various muscle depths associated with poultry processing. *Food Technol.* **13**, 689.

Wismer-Pedersen, J., and E. J. Briskey. 1961. Relationship of post-mortem acidity and temperature. *Food Technol.* **15**, 232.

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Changes in Aldolase Activity in Cod and Haddock During Frozen Storage

SUMMARY

The aldolase activity of cod and haddock flesh is significantly reduced by freezing and thawing, and falls progressively during storage at −14°. During both rigor mortis and frozen storage at −14°, much of the aldolase activity becomes inextractable in water.

INTRODUCTION

In contrast to the behavior of the myofibrillar groups of proteins, the extractability of the sarcoplasmic proteins of cod and several other species of fish remains high during frozen storage at −14° (Connell, 1964). In addition, the moving boundary electrophoresis patterns of the sarcoplasmic proteins extracted from fish frozen-stored under adverse conditions did not differ from similar patterns obtained from extracts of unstored fish (Connell, 1964). Recent work has shown that the zone electrophoretic patterns of the sarcoplasmic proteins from cod stored under severe conditions differ slightly from those from fresh cod (Mackie, 1965). However, the overall implication of these combined results is that the gross properties of the sarcoplasmic proteins are not much affected by frozen storage.

Much less information is available on changes in individual sarcoplasmic proteins during frozen-storage of fish. For example, data are limited on changes in the specific activity of the many enzymes making up this group (Gould, 1965a). However, it has been reported that the specific activities of "malic enzyme" and α-glycerophosphate dehydrogenase present in the supernatant solution of centrifuged fish muscle change in a regular manner on frozen storage of the muscle (Gould, 1965b). In particular, the specific activity of "malic enzyme" decreases in cod, haddock, and dab in storage at −7°. Earlier unpublished work had suggested that the aldolase activity of cod was stable during frozen storage (Rambeck and Connell, 1955); the present work reports a fuller investigation of the behavior of this enzyme.

EXPERIMENTAL

Materials. Cod (*Gadus callarias* L.) and haddock (*Gadus aeglefinus* L.) were caught in the North Sea by trawl net and either transferred while still live to a shore tank or killed, gutted, and kept in melting ice for 1–2 days before use. The iced fish were filleted and skinned, and the fillets then frozen in an air-blast, closely wrapped in aluminum foil to prevent desiccation, and stored at −14°. They were thawed by leaving them overnight at 1–2°.

Methods. Aldolase activity of homogenates of whole muscle was measured as follows: 30 g of muscle from the central part of the fillet was cut up into small pieces and homogenized with 300 ml distilled water at 0° in a chilled Atomix blender run at full speed for 75 sec. The homogenate was diluted with 15 volumes of water at 0° before use, and the diluted suspension held for no longer than a few hours at 0° before the assay. Occasionally the activities of the water-soluble portion of the homogenates were measured; used for this were filtrates of the diluted homogenates.

The method of enzyme assay was that of Bruns and Bergmeyer (1963) suitably modified for tissue homogenates: the reagents used were those described. One ml of homogenate was mixed with 1.75 ml collidine/hydrazine buffer, pH 7.4, and 0.25 ml fructose-1:6-diphosphate solution. After 20 min of incubation at 30°, the reaction was stopped by addition of 3 ml 10% aqueous trichloracetic acid. and the mixture was filtered. To 1 ml of the filtrate was added 1 ml 0.75*N* sodium hydroxide, and the mixture was allowed to stand for 10 min at room temperature in order to split the alkali-labile phosphates. One ml 2:4-dinitrophenylhydrazine solution was then added, the mixture incubated at 30° for 10 min, 7 ml 0.75*N* NaOH added, allowed to stand for a further 10 min at room temperature and the color read at 540 mμ. The blank was prepared by adding substrate to the mixture after the enzyme had been inactivated by trichloracetic acid. The enzyme assay was calibrated in terms of alkali-labile phosphate produced in the enzyme incubation. This calibration was performed as suggested by Sibley and Lehninger (1949), that is, by determining the amount of inorganic phosphate in the solution after addition of alkali by means of Gomori's (1941–42) method; the amount of inorganic phosphate was then compared with the color produced at 540 mμ in the same solution.

An enzyme incubation temperature of 30° was

chosen because this was found to be the temperature of maximum enzyme activity under these conditions. The enzyme activity was linear with time up to 20 min, so this period of enzyme incubation was chosen for all the assays.

The homogenates were not perfectly uniform, so three 1 ml portions from each homogenate were taken for separate enzyme assay, and the results averaged.

RESULTS AND DISCUSSION

Effect of freezing and immediate thawing. The effect on the aldolase activity of freezing and thawing without intervening storage was examined by means of the paired-fillet technique. In this, one fillet from each fish was frozen and then thawed whereas the other fillet from the same fish was stored at 0° unfrozen. Enzyme assays were then performed on the muscle from both fillets at the same time. Two cod and four haddock were examined in this way, and the results (Table 1) show that freezing reduced the aldolase activity in these species by 10–20%.

Effect of storage at −14°. The changes in aldolase activity of cod and haddock fillets stored at −14° are respectively shown in Figs. 1 and 2. Assuming that the activity changes linearly with time, the following regression lines were calculated:

$$\text{Cod:} \quad A = 0.674\ (\pm 0.001) - 0.0068\ (\pm 0.0006)\,t$$

$$\text{Haddock:} \quad A = 0.810\ (\pm 0.003) - 0.0103\ (\pm 0.0013)\,t$$

where A is the aldolase activity in arbitrary units of optical density at time t weeks. The values in parentheses are the standard errors.

Table 1. Effect of freezing on the aldolase activity of cod and haddock.

	Aldolase activity[a] (optical density at 540 mμ)	
	Unfrozen	Frozen
Cod A[b]	0.806 (0.023)[c]	0.660 (0.019)
Cod B	0.825 (0.013)	0.683 (0.011)
Haddock A	0.858 (0.002)	0.806 (0.015)
Haddock B	0.936 (0.012)	0.748 (0.018)
Haddock C	0.844 (0.005)	0.823 (0.023)
Haddock D	0.843 (0.017)	0.788 (0.022)

[a] The optical density values at 540 mμ, though arbitrary, are linearly related to the enzyme activity. They are the mean values of three determinations.

[b] The letters denote different fish.

[c] The values in parentheses are the standard errors of the means.

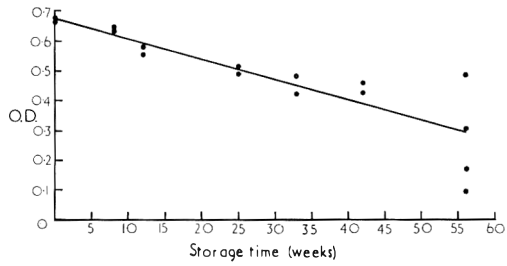

Fig. 1. Changes in the aldolase activity of cod flesh during storage at −14°. The enzyme activity is given in arbitrary units of optical density.

It is seen that there is a definite and progressive decline in enzyme activity during storage at −14°, and that the rate at which the activity declines is approximately the same in cod and haddock. Judged from the temperature-dependence of other protein changes during frozen storage, it can be confidently predicted that storage at below −14° would result in a lower rate of decline in aldolase activity. However, the report of the unpublished experiments of Rambeck and Connell (1955) is definitely incorrect in indicating that the aldolase activity is stable under all conditions of frozen storage.

General observations. The specific enzyme activities of 8 fresh unfrozen samples of cod and of 12 similar samples of haddock muscle were found on average to be, respectively, 43,000 and 46,000 μl fructose-1:6-diphosphate split/g wet tissue/hr at 30°. The standard error of these averages was 700 in each case. These values are to be compared with the average figure of 75,000 μl fructose-1:6-diphosphate split/g wet tissue/hr at 38° for rat skeletal muscle quoted by

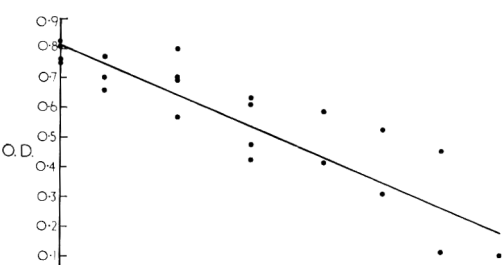

Fig. 2. Changes in the aldolase activity of haddock flesh during storage at −14°. The enzyme activity is given in arbitrary units of optical density.

Sibley and Lehninger (1949). The difference between the specific activities of cod and haddock muscle is significant, and it appears that cod muscle is more affected by freezing and immediate thawing than is haddock muscle.

There are two possible causes for the decline in aldolase activity during storage at −14°: denaturation of the enzyme; and progressive lack of availability of the enzyme in the homogenates. The first of these causes is the most obvious, for it has been widely invoked to explain the behavior of other proteins during the frozen storage of fish. For example, the myosin adenosine triphosphatase (ATP-ase) activity of cod declines during storage at −14° (Connell, 1960) and this behavior can be interpreted as a denaturation of the enzyme. It is interesting to note that ATP-ase activity declines more rapidly than aldolase activity at the same temperature of storage. Progressive inactivation of aldolase by oxidation of sulphydryl groups is also possible, though it should be noted that no decline in the numbers of either total or easily-reactible sulphydryl groups of the whole muscle proteins was observed during storage of cod at −14° (Connell, 1960).

However, it is important that during storage under these adverse conditions, the fish muscle becomes considerably firmer and increasingly resists efforts to reduce it to a fine state of subdivision by means of blending. If there is any tendency for the enzyme to adsorb on or otherwise become fixed to the insoluble part of the muscle, then it could be imagined that storage of the muscle might lead to an increasingly large degree of unavailablity of the enzyme to added substrate. Evidence that the enzyme is indeed adsorbed onto the water-soluble part of the muscle in the kind of sample assayed in this work, was obtained by comparing the aldolase activity of filtrates of homogenates of both post-rigor and frozen stored muscle with the homogenates themselves. It was found that the filtrates had little aldolase activity. On the other hand high activity was found in similar filtrates prepared from pre-rigor cod muscle. It appears that during rigor mortis the enzyme, which is normally water-soluble, becomes attached to the fibrillar part of the muscle so that it can no longer be completely extracted into water.

The question as to whether availability was the cause of declining activity during storage, was tested by homogenizing a sample of cod (stored at −14° for 26 weeks) for periods of 1, 2, and 5 min. Care was taken to maintain the temperature at about 5° throughout the homogenizing periods by means of frequent stops and cooling. Homogenizing for the longer periods resulted in a considerably finer state of subdivision of the muscle, but did not increase the specific enzyme activity. There is thus some evidence that unavailablity of the enzyme to the substrate is not the cause of the decline in activity during storage of the muscle.

The decline in aldolase activity on storage as measured by the method described, is in any event, regular enough to serve as a possible objective test of frozen storage deterioration alternative or supplementary to those already proposed.

REFERENCES

Bruns, F. H., and H. U. Bergmeyer. 1963. "Methods of Enzymatic Analysis." p. 724. Verlag Chemie, Weinheim/Bergstr., and Academic Press, New York.

Connell, J. J. 1960. Changes in the adenosinetriphosphatase activity and sulfhydryl groups of cod flesh during frozen storage. *J. Sci. Food Agr.* **11**, 245.

Connell, J. J. 1964. Fish muscle proteins and some effects on them of processing. *In:* "Proteins and their Reactions." p. 255. Avi Publ. Co., Westport, Conn.

Gomori, G. 1941–42. A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. *J. Lab. Clin. Med.* **27**, 955.

Gould, E. 1965a. "Testing the Freshness of Frozen Fish." Fishing News (Books) Ltd., London.

Gould, E. 1965b. Observations on the behavior of some endogenous enzyme systems in frozen stored fish flesh. *In:* "The Technology of Fish Utilization." p. 126. Fishing News (Books) Ltd., London.

Mackie, I. M. 1965. Unpublished work.

Rambeck, U., and J. J. Connell. 1955. Quoted by: Heen, E., and O. Karsti, 1965; *In:* "Fish as Food," vol. 4, p. 362. Academic Press, New York.

Sibley, J. A., and A. L. Lehninger. 1949. Determination of aldolase in animal tissue. *J. Biol. Chem.* **177**, 859.

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Separation of a Polyphenol Oxidase for Anthocyanin-Degradation in Eggplant

SUMMARY

The anthocyanin-decolorizing enzyme which was previously found to be a polyphenol oxidase in eggplant fruits was further purified by DEAE-cellulose column chromatography. At least two different enzymes were prepared, both of which equally oxidized catechol to its o-quinone as a primary product, indicating their polyphenol oxidase nature, but differed from each other in substrate specificity. One of the two enzymes showed higher activity toward the eggplant anthocyanin and catechol than toward chlorogenic acid. When the anthocyanin was used as a substrate, the pH of maximum enzyme activity was 6.5. The second enzyme was more active on chlorogenic acid, with maximum activity near pH 5.0, and less active on the anthocyanin.

Recent investigations have demonstrated that polyphenol oxidases (PPO's) from eggplants (Sakamura and Obata, 1963), mushrooms, and white potatoes (Sakamura *et al.*, 1965), and also a commercial tyrosinase (Peng and Markakis, 1963) served as an oxidative degradation enzyme on the anthocyanin substrates. Nevertheless, it is still doubtful whether there is a specific enzyme decolorizing anthocyanins among the PPO preparations. The present work was undertaken to separate the anthocyanin-degrading enzyme by DEAE-cellulose column chromatography.

The eggplants used as enzyme source (*Solanum melangena* L.) were purchased at a local market and were identical with those used in the structure determination of the anthocyanin (Sakamura *et al.*, 1963). Enzymatic activities were measured throughout in a reaction mixture (4 ml) containing the enzyme solution (1 ml), $M/10$ phosphate buffer (pH 6.8), and substrates in a certain concentration. Control experiments were run using reaction systems that were complete except for the enzyme preparations. Chlorogenic acid oxidase activity in $5 \times 10^{-5}M$ chlorogenic acid was determined by the spectrophotometric method (Sisler and Evans, 1958). Catechol oxidase activity in $2.5 \times 10^{-3}M$ catechol was measured by the increase of absorbance at 390 mμ one min after the reaction initiated. Decolorizing activity of the anthocyanin in 10 mg% of the anthocyanin, incubated 15 min at 25°C, was measured by the decrease of absorbancy at 530 mμ on the addition of equal volume of 2% HCl in methanol. Tyrosinase activity in $2.5 \times 10^{-3}M$ tyrosine was also observed, by a red pigmentation in the complete systems.

Preparation of the crude PPO was followed by a method essentially similar to that described by Bouchilloux *et al.* (1963). Using 500 g peeled eggplant flesh the technique involves extraction with 30% cold acetone followed by precipitation with addition of 2 volumes of cold acetone (step 1), collection of precipitates in 70% saturation with $(NH_4)_2SO_4$ (step 2), collection of precipitates from 20 to 60% saturation with $(NH_4)_2SO_4$ (step 3). Oxygen uptake was measured by using this enzyme preparation of step 3, showing that the enzyme oxidizes chlorogenic acid at a high rate and to a large extent, oxidizes anthocyanin somewhat less, and does not oxidize p-phenylenediamine and hydroquinone at all. Oxidation of p-cresol was observed after an induction period. These facts suggested that the enzyme preparation contained PPO as well as cresolase and no laccase.

Further purifications were achieved by DEAE-cellulose column chromatography according to the directions of Alberghina (1964). As shown in Fig. 1, the bulk of proteins not absorbed was passed through with $1 \times 10^{-3}M$ phosphate buffer. Activities were eluted with $2.5 \times 10^{-2}M$ (peak A), $5 \times 10^{-2}M$ (peak B), and $7.5 \times 10^{-2}M$ (peak C) of the buffer. Tyrosinase activity was found only in peak A. The activities plots seemed to indicate that the enzyme of peak A had greater affinity toward both anthocyanin and catechol, whereas those of peaks B and C had less affinity toward them than toward chlorogenic acid. In fact, this consideration was reasonable from the purifi-

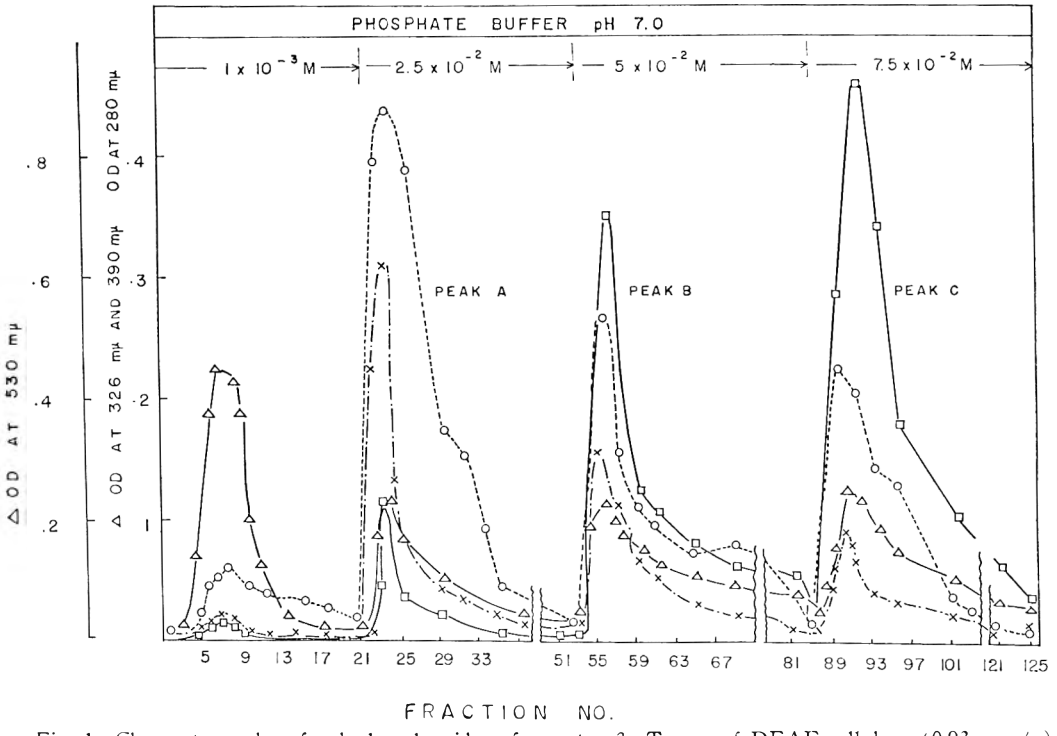

Fig. 1. Chromatography of polyphenol oxidase from step 3. Two g of DEAE-cellulose (0.93 meq/g) in 1-cm-diameter column was equilibrated with $1 \times 10^{-3}M$ phosphate buffer (pH 7.0), and sample was then placed on the absorbent, followed by elution with increasing concentration of the buffer. Each fraction was 4 ml. OD at 530 mμ for anthocyanin-decolorizing activity (○---○---○), OD at 326 mμ for chlorogenic acid oxidase activity (□—□—□), OD at 390 mμ for catechol oxidase activity (X—X—X), OD at 280 mμ for protein (△—△—△).

cation data in Table 1. The enzymes from peaks *A* and *B*, moreover, gave the red dianilino-o-benzoquinone crystal, mp 192°C, equally in oxidizing catechol in the presence of aniline (Claton, 1959). When their substrates were assayed by the colorimetric method (Shiroya, 1964), the Michaelis constant (Km) was found to be $1.60 \times 10^{-3}M$ chlorogenic acid and $0.40 \times 10^{-3}M$ catechol for the enzyme of peak *A*, and was $0.60 \times 10^{-3}M$ chlorogenic acid and $4.0 \times 10^{-3}M$ catechol for the enzyme of peak *B*.

Subsequently, the central fractions from the elution peaks *A* and *B* were respectively dialyzed against water and rechromatographed under the same conditions as in the first column. Each elution peak *A* and *B* were realized on the second chromatographic pattern. The central fractions corresponding to their peaks were pooled and dialyzed against water. The purified enzyme solutions thus obtained were examined for the effect of pH on the activities. The data are presented in Fig. 2. It could be concluded that there are two different enzymes catalyzing the oxidation in favor of either polyphenolics,

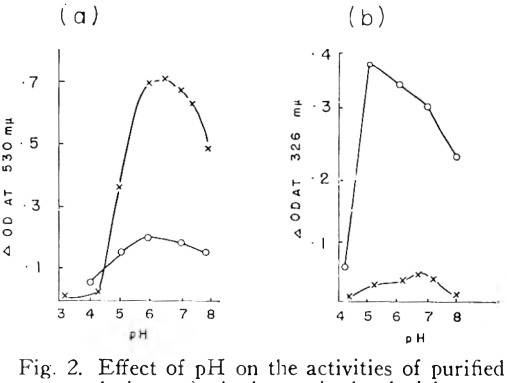

Fig. 2. Effect of pH on the activities of purified enzyme solution. a) Anthocyanin-decolorizing activity; b) chlorogenic acid oxidase activity. Assay conditions were as described in text except that citrate and phosphate buffers were used for pH adjustment. Rechromatographed and pooled fractions from peak *A* (X—X—X). Rechromatographed and pooled fractions from peak *B* (○—○—○).

Table 1. Purification data of eggplant polyphenol oxidase.

Preparations	Volume (ml)	Total protein[a] (mg)		Total units[b]	Specific activity (units/mg)	Purification	Yield
Step I	45	301	CA	19,800	66	1	100
			Ant	6,057	20	1	100
Step II	45	150	CA	16,200	108	1.7	82
			Ant	4,732	32	1.6	79
Step III	39	80	CA	13,800	168	2.6	70
			Ant	3,923	49	2.4	65
Peak A	94	4.2	CA	105	25	0.4	0.5
			Ant	600	143	7.2	10
Peak B	134	3.9	CA	1,700	436	6.7	9
			Ant	315	80	4.0	5
Peak C	103	4.3	CA	950	221	3.4	5
			Ant	200	47	2.3	3

[a] Expressed at N × 6.25.
[b] CA, chlorogenic acid oxidase activity; one unit is 0.01 decrease of OD in 2 min under the assay conditions. Ant, anthocyanin-decolorizing activity; one unit is 0.1 decrease of OD under the assay conditions.

chlorogenic acid and the anthocyanin, both of which occur in eggplants.

REFERENCES

Alberghina, F. A. M. 1964. Chlorogenic acid oxidase from potato tuber slices; partial purification and properties. *Phytochem.* **3**, 65.

Bouchilloux, S., P. Mcmahill, and H. S. Mason. 1963. The multiple forms of mushroom tyrosinase. *J. Biol. Chem.* **238**, 1699.

Clayton, R. A. 1959. Properties of tobacco polyphenol oxidase. *Arch. Biochem. Biophys.* **81**, 404.

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

Sakamura, S., and Y. Obata. 1963. Anthocyanase and anthocyanins occurring in eggplant. II. Isolation and identification of chlorogenic acid and related compound from eggplant. *Agr. Biol. Chem.* **27**, 121.

Sakamura, S., S. Watanabe, and Y. Obata. 1963. The structure of the major anthocyanin in eggplant. *Agr. Biol. Chem.* **27**, 663.

Sakamura, S., S. Watanabe, and Y. Obata. 1965. Anthocyanase and anthocyanins occurring in eggplant. III. Oxidative decolorization of the anthocyanin by polyphenol oxidase. *Agr. Biol. Chem.* **29**, 181.

Sisler, E. C., and H. J. Evans. 1958. Direct spectrophotometric determination of chlorogenic acid oxidase activity. *Biochim. et Biophys. Acta.* **28**, 638.

Shiroya, M. 1964. Polyphenol oxidase in higher plant leaves. (In Japanese). *Repts. Enzyme Chem. Symposium* **16**, 279.

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Pectin Methyl Esterases of the Banana. Purification and Properties

SUMMARY

Earlier, three fractions of pectin methyl esterase (PME) had been obtained from the pulp of the banana by differential extraction. The crude enzyme preparations have now been purified further by ammonium sulfate fractionation and ion-exchange chromatography on columns of DEAE- and CM-cellulose. Each fraction has a unique behavior on the ion-exchange columns. Additional criteria have been established to differentiate the fractions. These include response to cations, inorganic phosphate, nucleotides, and sucrose, as well as differences in initial reaction kinetics, resistance to end-product inhibition, and cold stabilities. The possibility of interconversion between fractions is discussed; Fractions I and II may be converted by treatment with $3.5 \times 10^{-4}M$ sodium dodecyl sulfate to forms resembling Fraction III as determined by pH-activity spectra.

Earlier, we published a report of the preparation from banana of three fractions containing pectin methyl esterase (PME) activity. One fraction was obtained by extraction with water, one with $0.15M$ NaCl, and one with $0.15M$ NaCl after adjustment to an alkaline pH. The fractions were labeled I, II, and III, respectively, and were distinguishable by their pH-activity curves, by the effect of an anionic detergent, sodium dodecyl sulfate (SDS), on their activities, and by differential temperature inactivation (Hultin and Levine, 1963). This paper reports the results of purification studies, as well as additional criteria by which the fractions of banana PME can be distinguished.

EXPERIMENTAL

Materials. Bananas were purchased locally. Both the Gros Michel and Valery varieties were used. The nucleotides were purchased from Pabst Laboratories. The pectin used was Pectin N.F. from Nutritional Biochemicals Corp., and the low-methoxy pectin was a product of Sunkist Growers, Inc. The pectin was purified by the method of Olsen *et al.* (1939) to remove contaminating cations. The ash content generally went from about 3.6% to 0.5%. At this latter level, the pectin would contribute less than 10% of the total ions even when used in our "low-level" studies, described below. The salts used were the purest available, either biological or reagent grade.

Fractionation. Crude preparations of Fractions I, II, and III were obtained as previously described (Hultin and Levine, 1963). The water-soluble extract (Fraction I) was brought to 15% saturation by the addition of solid ammonium sulfate and then centrifuged to remove the brownish mass that came out of solution. This sediment contained little protein and only minor amounts of PME activity. It was necessary to remove this high-molecular-weight non-protein material to prevent its interfering with subsequent chromatographic procedures. The supernatant fraction was dialyzed against several changes of water to remove the ammonium sulfate. It was then concentrated by dialysis against previously dialyzed polyvinyl pyrrolidone (PVP) or a polyethylene glycol of average molecular weight 20,000. Aliquots of the concentrated solution were then chromatogrammed on diethylaminoethyl (DEAE)- or carboxymethyl (CM)-cellulose ion-exchange columns. The eluting solution was a $0.02M$ phosphate buffer at pH 6.8 with a linear concentration gradient of NaCl with a limiting concentration of $1M$. Five-ml fractions were collected automatically, and the OD of these fractions at 280 mμ was determined in a Beckman DU spectrophotometer. Assays for PME activity were carried out as described below.

The PME fraction soluble in $0.15M$ NaCl (II) was partially purified by treatment with ammonium sulfate to a final saturation of 60%. This produced two fractions, one coming out of solution and floating on the surface and one remaining in solution. The former had a pH-activity spectrum closely resembling that of the crude Fraction III, and the latter corresponded roughly to Fraction II. The soluble fraction was then treated as described for Fraction I, including the dialysis, concentration, and chromatography.

The crude Fraction III (soluble in $0.15M$ NaCl at pH 7.5) could not be fractionated by treatment with ammonium sulfate into more than one PME as determined by the pH-activity spectra of treated samples. Therefore, crude Fraction III was just dialyzed against water, without treatment with ammonium sulfate, prior to concentration and chromatography as described for Fraction I. All procedures were performed at 0–4°C.

Assay for PME. The effluent fractions from the columns were qualitatively assayed by incubating

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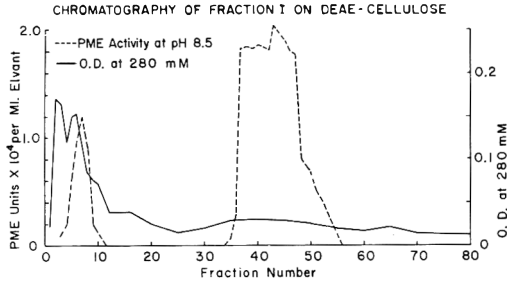
0.1 ml of sample with 1 ml of a pectin solution adjusted to the proper pH and containing a dye that would change color over the range of interest. Bromthymol blue was used as the indicator for the assays at the higher pH (around 8), and bromcresol green was used at the low pH (around 5). The fractions showing activity were assayed quantitatively by the procedure previously described, which was based on maintenance of a given pH with standard base as measured with a glass electrode (Hultin and Levine, 1963). The salt and nucleotide concentrations used in the reaction media, as well as other specific variables, are explained in the text.

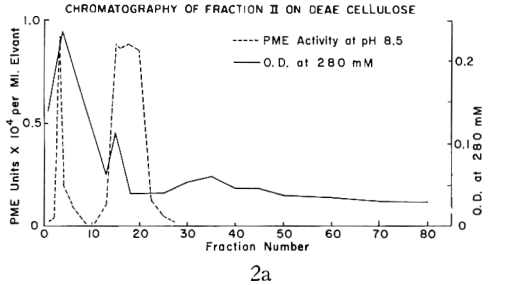
Protein determination. Protein was determined by a modified Folin procedure (Lowry *et al.*, 1951).

RESULTS

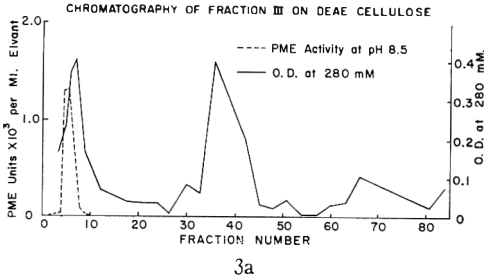
The chromatographic patterns of a water-soluble extract on DEAE- and CM-cellulose ion exchange columns are respectively given in Figs. 1(a) and 1(b). CM-cellulose does not retain any of the water-soluble PME activity, nor does it retain a significant proportion of the protein in the extract (as determined by OD at 280 mμ). Although most of the protein of the concentrated extract also passed through the DEAE-column, the greatest part of the PME activity was held back by the anion-exchange column. This fraction had a pH-activity spectrum similar to that of the crude Fraction I; it represented approximately 85% of the total PME activity assayed at pH 8.5. This second peak is used hereinafter as Fraction I and represents an increase of about 50-fold in the specific



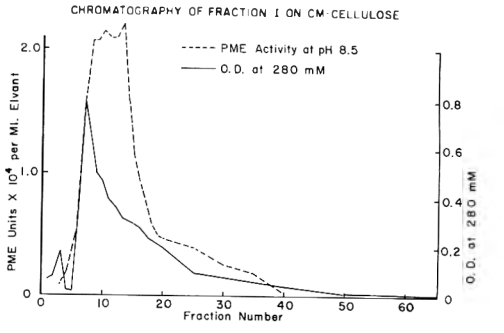
1a



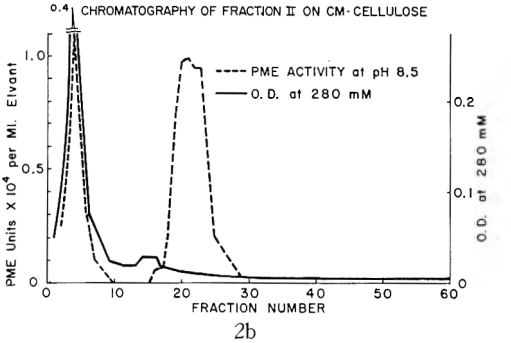
2a



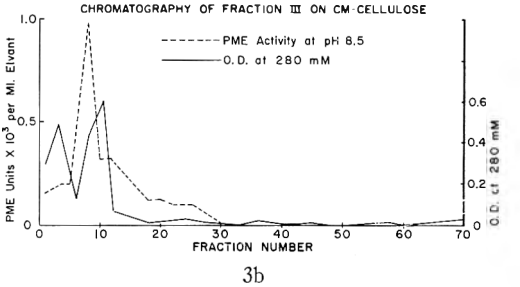
3a



1b



2b



3b

Fig. 1–3. Chromatography of Fractions I, II, and III, respectively, on columns of DEAE- and CM-cellulose. The fractions were previously treated as described in the text. In each case, *a* represents the DEAE-cellulose and *b* the CM-cellulose data. PME assays were performed at pH 8.5 as previously described (Hultin and Levine, 1963) on those fractions which qualitatively showed activity (see text). The OD of each 5-ml fraction was read in a Beckman DU spectrophotometer.

activity of the enzyme over that of the crude extract at pH 8.5. The smaller peak that is not retained by this column is characterized by a very broad optimal pH range closely resembling that of Fraction III. We have observed in some of our preparations an additional peak of activity in the water-soluble fraction (I). This activity, designated Fraction I_A, showed an optimal pH in the crude extract at approximately 5. When we originally obtained pH data on the water-soluble enzyme, this peak was not apparent. We still do not obtain this activity in the crude fractions consistently; the appearance of the activity is related neither to variety (neither the Gros Michel nor the Valery gave consistent results) nor to time of harvest. The fraction of PME activity that was not retained by the column is similar in many respects to the activity at low pH in the crude water extract; hence it is hereinafter referred to as Fraction I_A.

Chromatographic patterns of Fraction II, partially purified by ammonium sulfate fractionation, on columns of DEAE- and CM-cellulose are respectively shown in Figs. 2(a) and 2(b). Two fractions were obtained in both cases, one not retained and one retained by the columns. pH activity data indicated that in both cases the retained fraction, which was the major fraction and represented 80–90% of the activity, corresponded to Fraction II. The unretained fractions were similar to Fraction III. The increase in specific activity of the Fraction II peak was approximately two-fold. This rather small increase in specific activity was due in part to relatively poor recovery from the column (about 35–40%), and in part undoubtedly to the rather selective nature of the extraction procedure. This fraction is used in later experiments as Fraction II.

The chromatographic results with Fraction III are given in Figs. 3(a) and 3(b). Only one peak of activity is observed from either type of column and in both cases it passes without hindrance through the column. The increase in specific activity is only about 1.3 times. Because of this fact and the observation of no differences in kinetic properties between the enzyme before and after chromatography, Fraction III was used as directly extracted from the banana in all subsequent assays. The increases in specific activities of the fractions in a typical run are shown in Table 1.

The very important effect of cations on the activity of pectin methyl esterase has been known and well-documented for some time (Lineweaver and Ballou, 1945; MacDonnell *et al.*, 1945; Kertesz, 1951). This effect of cations is intimately linked to pH and is dependent on the valency of the cation. We assayed our PME fractions under certain specific conditions to see if we could obtain differences in response to various cations. The effect of NaCl

Table 1. Increase in specific activities of the PME fractions.

Fraction	PME units $\times 10^3$ per mg protein[a]		
	Crude extract	After ion-exchange chromatography	Increase in specific activity
I	1.4	53	38×
I_A[b]	0.3	1.3	4×
II	27	51	2×
III	34	43	1.3×

[a] The assays were performed at pH 8.5 and 25°C in 150 m*M* NaCl as previously described (Hultin and Levine, 1963).

[b] Fraction I_A was assayed at pH 5. Other conditions were similar to those of the other fractions.

at three different levels on the activities of the three major fractions of PME was studied. The three levels used were 8, 150, and 408 m*M* NaCl. The lowest level represents the amount of Na^+ that is introduced when the necessary amount of NaOH is added to bring the pH of the purified pectin and the dialyzed enzyme fraction to pH 8.5 for the assay, i.e., this represents our zero added-Na^+ level. Where the enzymic assays were performed at a lower pH (as in the case of Fraction III), enough NaCl was added to the assay medium to maintain the zero level of Na^+ at 8m*M*. The 150m*M* level has approximately the same ionic strength as that of physiological saline and has been reported to give optimal activity (Lineweaver and Ballou, 1945). A level of 408m*M* NaCl was arbitrarily chosen as the high level. Assays were generally carried out at pH 8.5, which is roughly optimal for all fractions. A low pH (6.0) assay was also performed on Fraction III, since this fraction is as active at this pH as at 8.5. In most systems studied it has been shown that the effect of cations is greater at lower pH's and that this should be taken into account in any study designed to relate cation concentration to the physiological role of these enzymes, but it must be remembered here that at this point we were interested only in establishing differences in the enzyme fractions. Any conditions, therefore, that could do this would be pertinent. Fraction II was insensitive to the three salt levels. Whereas Fractions I and III reacted as one might expect, i.e., they showed a decreased activity at both the high and low salt levels, the activities of Fraction II were constant at the three levels. The relative inhibition of the high and low salt levels on Fraction III was greater at pH 6 than at 8.5; this is true for most systems studied, as mentioned above. The effect of low pH was most marked at the low salt level.

A study was performed to determine the effect of K^+ on these fractions. KCl was substituted for the sodium salt, and KOH was used to adjust the

pH and for the enzyme assay itself. Under the conditions described above, no differences were observed when K^+ was substituted for Na^+.

Divalent cations have been shown in other systems to achieve maximal activation of PME at a much lower concentration (approximately 20 m*M*) than is required for the monovalent cations (Lineweaver and Ballou, 1945; MacDonnell *et al.*, 1945). Table 2 presents the data obtained with our three

Table 2. Effect of 20 m*M* Ca^{+2} on pectin methyl esterase activities.[a]

20 m*M* $CaCl_2$	Fraction I (6)	Fraction II (2)	Fraction III (2)
pH 8.5			
+ 130 m*M* NaCl	91	102	87
+ 8 m*M* NaCl	100	118	180
pH 6.0			
+ 130 m*M* NaCl	—	—	71
+ 8 m*M* NaCl			225

[a] Values are compared to controls in which the $CaCl_2$ was replaced with an equal molar concentration of NaCl. The control was taken as 100%. The assays were performed as described previously (Hultin and Levine, 1963). The values in parentheses refer to the number of preparations that were assayed.

enzyme fractions in the presence of 20 m*M* $CaCl_2$. The effect of the $CaCl_2$ was determined at two salt levels; in one, the total salt concentration was 150 m*M*, and in the other it was 28 m*M* (the latter represents the 8 m*M* Na^+ that is introduced in bringing the substrates and enzyme solutions to pH 8.5 plus the 20 m*M* added salt). In the controls the salt used was NaCl, and in the test mixtures it was 20 m*M* $CaCl_2$ plus NaCl to the indicated level. The data in Table 2, then, indicate the relative effect of Ca^{+2} as compared to Na^+. At pH 8.5, the effect of Ca^{+2} differed for each fraction. At 150 m*M* salt, Fractions I and III were inhibited (slightly but consistently), and there was no effect on Fraction II. At the 28 m*M* salt level, Fractions II and III were stimulated, and there was no effect on Fraction I. The same effects were observed on Fraction III at pH 6 as at 8.5 except that they were accentuated. It must be emphasized that the figures in the tables are relative and show the effect of Ca^{+2} as compared to Na^+. The two values which were not predictable on the basis of previous results with other systems are the absence of stimulation of Fraction I at the lower salt level and the lack of inhibition of Fraction II at the high salt level. The latter, however, is in agreement with our results, mentioned earlier, which showed Fraction II to be insensitive over a wide range of NaCl concentrations. In some cases, the NaCl content in the test solutions was adjusted to an equal ionic strength rather than molar concentration, e.g., the lower salt level control was adjusted to 68 m*M* rather than 28 m*M*. The relative trends in activation and inhibition were the same as indicated above, but they were subdued. This was especially true at the lower level of salt. Again this is reasonable, since what is happening is that a greater amount of NaCl is added, which brings the control to a level more nearly like that of the $CaCl_2$ as far as effective activation is concerned (Lineweaver and Ballou, 1945). The primary point illustrated by our data is that, under our specified conditions, the three fractions of PME have different properties.

The effect of 20 m*M* $MgCl_2$ on the activities of the PME fractions was similar in most respects to those obtained with $CaCl_2$. We observed, however, no inhibition of Fraction I at the high salt level at pH 8.5 or of Fraction III at the high salt level at pH 6. As a means of differentiating our PME fractions, the Mg^{+2} effect is not as discriminating as that of Ca^{+2}, but it does indicate differences between Fraction III and Fractions I and II.

Observations of an inhibitory effect of inorganic phosphate on the activity of water-soluble crude extracts at pH 5 (our Fraction I_A) led to a survey of the effect of inorganic phosphate and nucleotides on all of the PME fractions. We were particularly interested in these substances because of their known role in the regulatory mechanisms of metabolic systems, and we wanted to determine if there might be any possibility of the PME fractions being linked to other metabolic processes in the pulp of the banana through these substances. Inorganic phosphate was tested at 20 m*M* and the nucleotides at a level of 5 m*M*. The effect was examined at both high and low levels of salt. The high salt level was made 150 m*M* in Na^+ with NaCl; the low-salt-level controls were adjusted to a concentration commensurate with what was introduced into the assay medium by the sodium salt of the inorganic phosphate or nucleotide used. No adjustment was made for any sodium not dissociated from the phosphates (Batra, 1965). Fractions I and II were assayed at pH 8.5, and Fraction III at 8.5 and 6.0. In general, there was little effect on Fractions I and III by these substances. A slight but significant effect of both ATP and ADP on Fraction I was observed at pH 8.5 at the higher salt level (150 m*M* Na^+). These compounds at 5 m*M* inhibited approximately 10% of the activity of Fraction I under these conditions. Five different preparations showed this inhibition. No inhibition was observed at the lower level of salt. The principal effect of inorganic phosphate and the nucleotides was noted with Fraction II. Inorganic phosphate at a concentration of 20 m*M* inhibited Frac-

tion II at both the high and low salt levels at pH 8.5 by 25–30%. Significant effects of the nucleotides were not observed at the higher salt concentration; however, an inhibition of 15 to 30% was seen at the low salt level. The results obtained with Fraction II with the phosphate compounds were variable, and standard deviations of the averages were large. Nevertheless, the inhibitions were consistently observed at the low salt level and not at the high salt level. This variability of the assays was not observed with any of the other additives used. It is a possibility that we are observing interconvertibility of our enzyme fractions catalyzed by the nucleotides and salt present. Chilson *et al.* (1965) reported a study of the mechanism of hybridization of lactic dehydrogenases on freezing. The rate of hybrid formation was faster if sodium phosphate plus either thiocyanate or a halide ion were present. Jolley and Mason (1965) observed changes in forms of a mushroom tyrosinase if incubated for a few minutes with buffers of moderate ionic strength (0.1–0.2) over the range of pH from 3.8 to 10.4. Instability of Fraction II and its conversion to another form by some means or other is a reasonable explanation of what may be happening in the presence of nucleotides. When Fraction II is first treated with ammonium sulfate to afford a preliminary separation of the impurity that resembles Fraction III, the total activity always amounts to more than the original. It is possible that what we observe here is a conversion of Fraction II to Fraction III under the influence of the salt; this may be an indication of the instability of Fraction II. Kertesz and Zito (1962) converted a one-component tyrosinase into a two-component enzyme by treatment with ammonium sulfate. In any case, there appears to be a definite effect of inorganic phosphate as well as ATP, ADP, DPNH, and DPN^+ on Fraction II which is not observed with either Fraction I or III. Further studies on these compounds at other pH values and at various levels are necessary to determine whether they exhibit the typical characteristics of regulatory substances (Atkinson and Walton, 1965).

A study of sucrose inhibition of papaya pectin methyl esterase has been recently reported (Chang *et al.*, 1965). Those workers found that the efficacy of sucrose in delaying papaya puree gelation was due to its inhibitory action on PME and that the effect of sucrose on a partially purified enzyme preparation was linear up to a concentration of 50% sucrose. Other sugars as well as glycerol displayed similar inhibitory activities. The activities of our PME fractions were assayed at 0, 5, 10, 25, and 50% sucrose concentrations. Fraction I was unaffected at the 5% sucrose level but was at 10%, and the activity continued to decrease up to the 50% level of sucrose in a roughly linear fashion. Fraction II was somewhat more sensitive at low sucrose levels, but did not lose any activity between 25 and 50%. Fraction III was unaffected up to a concentration of 25%, but lost one-half of its activity between the 25 and 50% levels of sucrose. The significance, if any, of these results as they relate to osmotic changes in the banana caused by a conversion of starch to sugar during ripening is not known. However, they do serve once again to illustrate differences in kinetic behavior of our three PME fractions.

When assayed over any period, the rate of reaction catalyzed by Fraction II increased initially, followed by a later decrease. Fig. 4 presents a

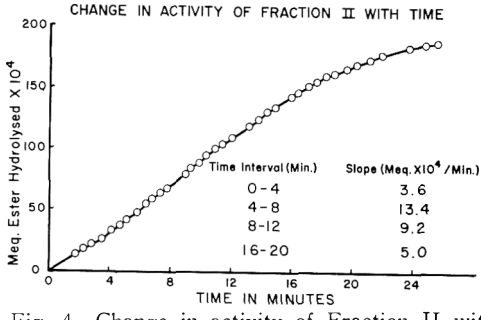


Fig. 4. Change in activity of Fraction II with time. The assay was performed as usual (Hultin and Levine 1963) with frequent readings. This particular assay was carried out at pH 7.0.

typical example of this. The assay shown in this figure was carried out at pH 7.0, but we found this phenomenon to be quite independent of pH over the range 5.5–8.5 and it was consistent. The rate would increase almost by a factor of 4. Neither Fraction I nor Fraction III behaved in this manner. Two possible explanations could account for these observations. One is that Fraction II is converted during the initial stages of the assay to another, more active form (such as one similar to Fraction III). The postulated instability of this fraction as shown by the data with phosphate compounds and ammonium sulfate described above lends credence to this view. The second possibility is that Fraction II is simply more active at a lower methoxy content than is normally present in our substrate. The pectin we used ordinarily was about 40–45% methylated, and a maximal rate with Fraction II was obtained at approximately a 10% lower level.

Although it does not really distinguish between these two possibilities, the data in Table 3 do serve to illustrate the greater tolerance of Fraction II to the use of a low-methoxy (LM) pectin in the assay medium. These assays were performed at a level of 150 m*M* NaCl and pH 8.5 and 6 (Fraction I was not assayed at pH 6, because of its very low activity

Table 3. Inhibition of the pectin methyl esterase fractions by a low methoxy pectin.[a]

0.5% LM Pectin 150 m*M* NaCl	Fraction I (3)[b] % loss	Fraction II (5) % loss	Fraction III (3) % loss
pH 8.5	64	24	67
pH 6.0	—	45	67

[a] Values are compared to a control which contained 0.5% of regular pectin.
[b] The numbers in parentheses refer to the number of samples tested.

at this pH). The regular pectin was replaced in the test media by an LM pectin of approximately 15% methylation. At this methoxy concentration, Fraction II is much less sensitive to inhibition than either Fraction I or Fraction III, losing approximately one quarter of its activity at pH 8.5 and less than one half at pH 6.0, compared to about a 2/3 loss of the other fractions. This resistance to end-product inhibition may have implications relative to the cellular localization of the enzyme and its physiological role. An enzyme that can act on a low-methoxy pectin could conceivably play an important role in the metabolism of pectic acids which have been reported to constitute a major segment of certain parts of plants, e.g., the middle lamella (Zaitlin and Coltrin, 1964). In any case, the data demonstrate a basic difference in response between Fraction II and Fractions I and III.

DISCUSSION

In the preceding section, we have presented evidence which reinforces the conclusions of an earlier paper that the three PME fractions obtained from banana pulp by differential extraction procedures possess different kinetic properties. The variability in extractability of the three major fractions becomes somewhat more clear when the ion-exchange chromatography data are examined, if we make the assumption that the pectin methyl esterase activity that is associated with particulate fractions is bound to the cell wall or related parts (Jansen *et al.*, 1960). Fraction I behaves as an anion on the ion-exchange chromatographic columns, i.e., it is retained by the DEAE-cellulose column. The cell wall and adhering parts could be expected to consist principally of negative charges (one cannot dismiss the possibility of positive charges contributed by such compounds as mucopolysaccharides, proteins, etc., but the major charge-bearing components would be negative). As an anion, Fraction I would not be expected to be retained by a negatively charged particulate structure, and, as we have observed, can be easily extracted with water. That Fraction II can be relatively easily extracted with salt solutions of moderate ionic strength would lead one to predict a simple electrostatic interaction between the enzyme and the cell wall, and that the enzyme should be capable of acting as a cation. The amphoteric properties of Fraction II as determined by its behavior on the CM- and DEAE-cellulose columns are consistent with this view. The situation with Fraction III is considerably more complicated. It is not extensively extracted with a NaCl concentration of 0.15*M*, and appears to be bound in some way dependent on pH. Also, it was not retained by either the cationic or anionic columns used, which may be a reflection of a blocking of its charged groups by subunit interaction, binding to nonactive material, etc. Elaboration of the nature of the binding of Fraction III must await further study.

In addition to obtaining additional evidence for the difference among the three major PME fractions, a fourth component was observed and was located in the water-soluble fraction. This component (I_A), when purified, had a pH-activity curve similar to that of Fraction III, and its chromatographic behavior was also identical with that of this fraction. Although this component has not been studied as extensively as the others, there are certain facts which argue against its being identical to Fraction III. The most significant of these is a susceptibility to inhibition by inorganic phosphate which Fraction III does not show. In addition, Fraction I_A appears to be less sensitive to Ca^{++} inhibition in 150 m*M* NaCl and to be more sensitive to low levels of Na^+ than is Fraction III. There is the distinct possibility, nevertheless, that these differences are due to some component of the Fraction I_A system (which is not purified to any great extent by the procedures outlined and which has a considerably lower specific activity than the other three fractions) which is affecting the response of the enzyme to these compounds. Recent work in our laboratory has shown that a small amount of Fraction III activity could be expected to be extracted under the conditions used for the extraction of Fraction I. Therefore, fur-

ther experiments are necessary to characterize the exact nature of the I_A fraction and its relation to Fraction III. The apparent lack of Fraction I_A activity in some crude extracts appears to be due to the presence of a nondialyzable inhibitor which is removed by DEAE-cellulose chromatography. This inhibitor may be a tannin (Badran and Jones, 1965).

In addition to the differences already mentioned and discussed, one further peculiarity of Fraction II is worth noting. This enzyme is remarkably stable at above-freezing temperatures, retaining approximately all of its activity for 2 weeks at 0°C or for 3 days at room temperature. It is, however, susceptible to inactivation by freezing and will completely lose its activity if frozen and held for 3 days at −30°C. Fractions I and III are not as stable at above-freezing temperatures as Fraction II, nor are they inactivated as easily by freezing.

The principal reasons for the experiments described in this paper were to obtain further evidence to establish differences in the properties of the three enzymes and to find further means of characterizing them. It is entirely possible, however, that some of the observations made may be of significance in understanding the physiological role of these enzymes. The differences in sensitivity of the fractions to various cations, or classes of cations, to sugar concentrations, and to end-product inhibition, as well as the inhibition of Fraction II by phosphate compounds, could be significant as to the action of these enzymes *in vivo*.

A question of great importance is: what is the exact nature of the various PME fractions? Are they different proteins? Do they represent complexes of similar subunits at various stages of aggregation? Do they represent complexes of dissimilar subunits? Are they the same enzymes bound to different cellular components such as inert proteins, phospholipids, polysaccharides, etc.? Is it a combination of these factors? The following observations may be pertinent to this problem. When assayed in the presence of 3.5×10^{-4} *M* SDS, the pH-activity curve of Fraction I became identical to that of Fraction III. A similar change took place when Fraction II was assayed in the presence of SDS. These results are suggestive of a simple relationship existing between the various fractions. The actual significance of these observations is not known, however, since we have not yet examined other properties of the SDS-treated Fractions I and II to establish whether a conversion of these fractions to one identical to that of Fraction III has actually taken place.

A further fundamental question remains unresolved. Is what we are seeing *in vitro* a true picture of the PME enzymic situation in the tissue, or are we creating these fractions by our procedures? The only thing we can say at this point is that we have seen no interconversions between these fractions to any great extent under conditions similar to our normal extraction, purification, and assay procedures.

REFERENCES

Atkinson, D. E., and G. M. Walton. 1965. Kinetics of regulatory enzymes. E. coli phosphofructokinase. *J. Biol. Chem.* **240**, 757.

Badran, A. M., and D. E. Jones. 1965. Polyethylene glycols-tannins interaction in extracting enzymes. *Nature* **206**, 622.

Batra, S. C. 1965. The ionization behavior of some poly (meta) phosphates used in food products. *J. Food Sci.* **30**, 441.

Chang, L. W. S., L. L. Morita, and A. Y. Yamamoto. 1965. Papaya pectinesterase inhibition by sucrose. *J. Food Sci.* **30**, 218.

Chilson, O. P., L. A. Costello, and N. O. Kaplan. 1965. Studies on the mechanism of hybridization of lactic dehydrogenases in vitro. *Biochemistry* **4**, 271.

Hultin, H. O., and A. S. Levine. 1963. On the occurrence of multiple molecular forms of pectinesterase. *Arch. Biochem. Biophys.* **101**, 396.

Jansen, E. F., R. Jang, and J. Bonner. 1960. Binding of enzymes to Avena coleoptile cell walls. *Plant Physiol.* **35**, 567.

Jolley, R. L., Jr., and H. S. Mason. 1965. The multiple forms of mushroom tyrosinase. Interconversion. *J. Biol. Chem.* **240**, PC1489.

Kertesz, Z. I. 1951. The Pectic Substances. Interscience Publishers, New York.

Kertesz, D., and R. Zito. 1962. Phenolase. *In:* Oxygenases. O. Hayaishi, ed. p. 312. Academic Press, New York.

Lineweaver, H., and G. A. Ballou. 1945. The effect

of cations on the activity of alfalfa pectinesterase (pectase). *Arch Biochem.* **6**, 373.

Lowry, O., O. H. Rosenbrough, N. J. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265.

MacDonnell, L. R., E. F. Jansen, and H. Lineweaver. 1945. The properties of orange pectinesterase. *Arch. Biochem.* **6**, 389.

Olsen, A. G., R. F. Steuwer, E. R. Fehlberg, and N. M. Beach. 1939. Pectin studies—relation of combining weight to other properties of commercial pectins. *Ind. Eng. Chem.* **31**, 1015.

Zaitlin, M., and D. Coltrin. 1964. Use of pectic enzymes in a study of the nature of intercellular cement of tobacco leaf cells. *Plant Physiol.* **39**, 91.

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Volatile Carbonyls in Stored Unblanched Frozen Peas

SUMMARY

The volatiles from stored unblanched frozen peas were shown to contain 12 carbonyl compounds: ethanal, propanal, hexanal, pent-2-enal, hex-2-enal, hept-2-enal, oct-2-enal, non-2-enal, hept-2,4-dienal, non-2,4-dienal, dec-2,4-dienal and propan-2-one. Identifications were based on thin-layer partition chromatography and infrared, ultraviolet, and visible spectra of the 2,4-dinitrophenylhydrazones. The origin of these compounds and their probable effect on the flavor of stored and unblanched peas are discussed.

INTRODUCTION

Unblanched peas deteriorate in flavor when held in frozen storage for extended periods, and these changes have been ascribed to the activity of naturally occurring enzymes not inhibited by low temperatures (Joslyn, 1949; Lee and Wagenknecht, 1951). Lee and Wagenknecht (1958) later showed that adding lipase, lipoxidase, or catalase to blanched peas induced the development of a flavor change similar to the off-flavor associated with stored unblanched frozen peas. The compounds responsible for off-flavor, however, have remained unidentified, although efforts have been made to correlate peroxide and carbonyl levels with the development of off-flavors in stored frozen peas (Lee and Wagenknecht, 1951; Lee, 1958). Evidence supporting an association between peroxides and carbonyls was obtained by Siddiqi and Tappel (1956), who showed that pea lipoxidase converts linoleate to a mixture containing unstable hydroperoxides, monocarbonyls, and polymeric products. These findings led Tappel (1961, 1962) to suggest that lipoxidase in intact peas reacts with unsaturated fatty acids to form hydroperoxides which on decomposition yield carbonyl compounds responsible for off-flavor.

Recently, chemical and gas chromatographic studies have been reported on the volatile components associated with the processing and storage of peas. Bengtsson and Bosund (1964) used gas chromatography to evaluate the formation of volatile substances, and Ralls *et al.* (1965) combined gas chromatography and mass spectrometry to identify 40 components in the steam above a commercial pea blancher.

In this Division, work is in progress to identify the compounds responsible for flavor changes in stored frozen peas. The present paper outlines isolation and identification of 12 volatile carbonyl compounds present in unblanched frozen peas after storage.

MATERIALS AND METHODS

Extraction of carbonyls from frozen peas. Peas (Edgell Freezer) were harvested, vined, washed, and held for 6 hr at 18°C prior to freezing. The frozen peas were then stored at −17.8°C for 2 years. The stored product was inedible, causing a burning sensation to the tongue and throat. Samples of the frozen peas (2,500 g) mixed with sodium chloride (1000 g) were rapidly minced, and then vacuum-distilled for 2 hr at 1–3 Torr pressure, the distillate being collected in traps cooled in liquid air. During distillation the temperature of the pea puree, which was continuously stirred while being heated in a water bath at 20°C, increased from −21° to 12°C. The distillate (300 ml), thawed under vacuum, was removed from the distillation unit, treated with a saturated solution of 2,4-dinitrophenylhydrazine in 2*N* hydrochloric acid (150 ml), and stored overnight at 5°C. The filtered-off insoluble mixture of hydrazones was washed with water and dried over phosphorus pentoxide; 17.5 Kg of frozen peas yielded 5.0 g of 2,4-dinitrophenylhydrazones.

Fractionation of the carbonyl 2,4-dinitrophenylhydrazones. A thin-layer partition chromatogram, on Kieselguhr G impregnated with 2-phenoxyethanol and developed with petroleum ether, bp 100–120°C (Urbach, 1963), indicated ethanal 2,4-dinitrophenylhydrazone as the major component of the hydrazone mixture. Chromatography of the mixture (1.5 g) on a nitromethane (150 g) and celite (150 g) partition column (Hughes, 1961) resolved the mixture into fractions *A*, *B*, and *C*. The application of TLPC (thin-layer partition chromatography) as before, followed by treatment of the resolved components with alcoholic sodium hydroxide (Anet, 1962), indicated the following compositions: fraction *A*: 2,4-dinitrophenylhydrazones of C_3 to C_{10} monocarbonyls; fraction *B*: ethanal 2,4-dinitrophenylhydrazone; and fraction *C*: 2,4-dinitrophenylhydrazine and, possibly, dicarbonyl 2,4-dinitrophenylhydrazones. No further investigation was carried out on fraction *C*. Ethanal 2,4-dinitro-

phenylhydrazone accounted for 96% of the hydrazones recovered from the column. Analysis of fraction *A*, by thin-layer chromatography (TLC) on magnesia-celite (Schwartz and Parks, 1963) followed by quantitative separation on a magnesia-celite column (Schwartz *et al.*, 1962), gave four sub-fractions: *A*1: alkanones; *A*2: alkanals; *A*3: alk-2-enals; and *A*4: alk-2,4-dienals. Separate qualitative analysis of these sub-fractions by TLPC on Kieselguhr G impregnated with 2-phenoxyethanol (Urbach, 1963) and on Kieselguhr G impregnated with Carbowax 400 (Badings and Wassink, 1963), using petroleum ether, bp 100–120°C, for development, indicated the presence of one alkanone; four alkanals; six alk-2-enals; and three alk-2,4-dienals. Of these fourteen compounds, eleven (see Table 1) were tentatively identified by TLPC through comparison with authentic specimens on Kieselguhr G whether impregnated with 2-phenoxyethanol or Carbowax 400.

Resolution of components for spectral studies. Samples for spectral studies were obtained by preparative TLPC on Kieselguhr G impregnated with 2-phenoxyethanol and developed with petroleum ether, bp 40–60°C. The hydrazone mixture (5 mg) from each of the above sub-fractions was dissolved in chloroform (minimum quantity) and spotted across a plate (20 × 20 cm) of layer thickness 0.30 mm. The plate was developed 3–5 times to achieve the desired separation. Where the mixture failed to resolve completely, the partially separated bands were removed from the plate, extracted with solvent, and rechromatographed until resolution was complete. The individual bands were then removed from the plate, and the compounds eluted. The solvent was removed by evaporation, and the 2-phenoxyethanol by vacuum sublimation. The residual hydrazone was checked for purity by TLPC and, where possible, was recrystallized from diethyl ether-light petroleum, bp 40–60°C. Hexanal, 2,4-dinitrophenylhydrazone recovered from the impregnated plates, accounted for 3.5% of the hydrazones recovered from the nitromethane-celite column.

Absorption spectra of the 2,4-dinitrophenylhydrazones. For infrared spectra the dried hydrazone was mixed with potassium chloride (0.1:99.9) and was pressed in an evacuable die (Research and Industrial Instruments Co., England). The disc was examined in a double-beam spectrophotometer (Perkin Elmer Corp. Model 221) and the spectrum recorded. Where the quantity of hydrazone was very small, the sample was mixed with potassium chloride, as above, and compressed in a cardboard microframe, window 10 × 2 mm (Perkin Elmer Corp.), the spectrum being recorded with the use of a refracting beam condenser.

The ultraviolet and visible spectra of the 2,4-dinitrophenylhydrazones were recorded in 95% ethanol with a Beckman DB spectrophotometer.

RESULTS AND DISCUSSION

Table 1 lists the volatile carbonyl compounds shown to be present in stored unblanched frozen peas.

Ethanal accounted for 96% of the hydrazones isolated and was by far the major volatile carbonyl component in the stored peas. Its presence in peas has been reported on numerous occasions (see Bengtsson and Bosund, 1964, and references therein). Joslyn (1949) recorded an appreciable increase in ethanal content during the storage of unblanched peas at −17°C and suggested that it arises principally from fermentation processes. Fermentation processes may also account for the formation of propan-2-one, isolated in low yield in the present work and previously detected by Ralls (1960) in blanched frozen peas.

Hexanal, which accounted for 3.5% of the hydrazones isolated in the present work, was detected in unblanched frozen peas by Bengtsson and Bosund (1964), who suggested that it may be formed by reactions following the oxidation of linoleic acid with lipoxidase. Of the remaining aldehydes, only propanal has

Table 1. Carbonyl compounds[a] isolated (as their 2,4-dinitrophenylhydrazones) from stored unblanched frozen peas.

Alkanone	Alkanals	Alk-2-enals	Alk-2,4-dienals
Propan-2-one	Ethanal	Pent-2-enal	Hept-2,4-dienal
	Propanal	Hex-2-enal	Non-2,4-dienal
	Hexanal	Hept-2-enal	Dec-2,4-dienal
	2 not identified	Oct-2-enal	
		Non-3-enal	
		1 not identified	

[a] Tentative identification base on TLPC, confirmed by infrared, ultraviolet, and visible spectral comparison with authentic specimens.

previously been isolated from peas (Ralls, 1960).

In considering the origin of the carbonyls shown in Table 1 it appears that, with the possible exception of ethanal and propan-2-one, these could be derived from either enzymic or autoxidative decomposition of the fatty material in peas. A comparison of these aldehydes with those derived from the autoxidation of linoleate and linolenate (Gaddis *et al.*, 1961) seems to indicate that the carbonyls from peas result from autoxidative degradation of unsaturated fatty acids, which have been shown to be present in peas (Lee and Mattick, 1961). However, since low-temperature autoxidation of fat is negligible in comparison with enzymic oxidation (Siddiqi and Tappel, 1956), enzymic oxidation of the unsaturated fatty acids would be favored by low-storage temperatures. It is known that lipoxidase is present in peas (Lee and Wagenknecht, 1958), and that it promotes the oxidation of linoleic and linolenic acids (Dillard *et al.*, 1961) to hydroperoxides structurally comparable with those obtained by autoxidation processes (Bergström, 1945; Holman, 1946). Decomposition of these hydroperoxides in the peas would be expected to yield the aldehydes listed in Table 1. Though not in itself conclusive, the detection of unidentified carbonyls following the lipoxidase oxidation of linoleate (Siddiqi and Tappel, 1956) provides some support for the view that enzymic oxidation of unsaturated lipids is responsible for off-flavor development.

Aldehydes of the same kind as those isolated from unblanched frozen peas are stated to be responsible for off-flavor in fatty foods (Sulzbacher *et al.*, 1963), and it is likely that such compounds could produce similar off-flavor effects in frozen peas. However, aldehydes represent only a few of the many volatile compounds isolated from unblanched peas and may contribute only partially to the off-flavor noted in such peas after frozen storage.

In view of the distinct possibility that the volatile aldehydes extracted from peas are formed by lipid oxidation, an attempt is being made to confirm this by investigating the composition of the products derived from lipoxidase oxidation of unsaturated pea lipids. Isolation and identification of other volatile compounds associated with the flavor of frozen peas is currently being undertaken by gas chromatography coupled with mass spectrometry and by infrared spectroscopy.

REFERENCES

Anet, E. F. L. J. 1962. Thin-layer chromatography of 2,4-dinitrophenylhydrazone derivatives of hydroxycarbonyl compounds. *J. Chromatog.* **9**, 291.

Badings, H. T., and J. G. Wassink. 1963. Separation and identification of aliphatic aldehydes and ketones by thin-layer chromatography of their 2,-4-dinitrophenylhydrazones. *Neth. Milk Dairy J.* **17**, 132.

Bergström, S. 1945. Oxidation of linoleic acid with lipoxidase. *Arkiv Kemi, Mineral. Geol.* **21A**(15), 1.

Bengtsson, B., and I. Bosund. 1964. Gas chromatographic evaluation of volatile substances in stored peas. *Food Technol.* **18**, 773.

Dillard, M. G., A. S. Henick, and R. B. Koch. 1961. Differences in reactivity of legume lipoxidases. *J. Biol. Chem.* **236**, 37.

Gaddis, A. M., R. Ellis, and G. T. Currie. 1961. Carbonyls in oxidizing fat. V. The composition of neutral volatile monocarbonyl compounds from autoxidized oleate, linoleate, linolenate esters and fats. *J. Am. Oil Chemists' Soc.* **38**, 371.

Holman, R. T. 1946. Spectrophotometric studies of the oxidation of fats. VII. Oxygen absorption and chromophore production in lipoxidase-oxidized fatty esters. *Arch Biochem. and Biophys.* **10**, 519.

Hughes, R. B. 1961. Chemical studies on the herring (Clupea harengus). VI. Carbonyl compounds formed during the heat processing of herring. *J. Sci. Food Agr.* **12**, 822.

Joslyn, M. A. 1949. Enzyme activity in frozen vegetable tissue. *Advances in Enzymol.* **9**, 613.

Lee, F. A. 1958. The effect of blanching on the carbonyl content of the crude lipid during the storage of frozen peas. *Food Research* **23**, 85.

Lee, F. A., and L. R. Mattick. 1961. Fatty acids of the lipids of vegetables. I. Peas (Pisum sativum). *J. Food Sci.* **26**, 273.

Lee, F. A., and A. C. Wagenknecht. 1951. On the development of off-flavour during the storage of frozen raw peas. *Food Research* **16**, 239.

Lee, F. A., and A. C. Wagenknecht. 1958. Enzyme action and off-flavour in frozen peas. II. The use of enzymes prepared from garden peas. *Food Research* **23**, 584.

Ralls, J. W. 1960. Flash exchange gas chromatog-

raphy for the analysis of potential flavour components of peas. *J. Agr. Food Chem.* **8**, 141.

Ralls, J. W., W. H. McFadden, R. M. Seifert, D. R. Black and P. W. Kilpatrick. 1965. Volatiles from a commercial pea blancher. Mass spectral identifications. *J. Food Sci.* **30**, 228.

Schwartz, D. P., and O. W. Parks. 1963. Thin-layer chromatography of carbonyl compounds. Separation of aliphatic carbonyl 2,4-dinitrophenylhydrazones into classes. *Microchem. J.* **7**, 403.

Schwartz, D. P., O. W. Parks, and M. Keeny. 1962. Separation of 2,4-dinitrophenylhydrazone derivatives of aliphatic monocarbonyls into classes on magnesia. *Anal. Chem.* **34**, 669.

Siddiqi, A. M., and A. L. Tappel. 1956. Catalysis of linoleate oxidation by pea lipoxidase. *Arch. Biochem. Biophys.* **60**, 91.

Sulzbacher, W. L., A. M. Gaddis, and R. Ellis. 1963. Oxidative rancidity in meat and meat products. *Proc. Am. Meat Inst. Found. Conf.* **15**th, 111.

Tappel, A. L. 1961. Biocatalysis: lipoxidase and hematin compounds. *In:* "Autoxidation and Antioxidants." Vol. 1, p. 325. (W. O. Lundberg, ed.) Interscience Publishers, New York.

Tappel, A. L. 1962. Hematin compounds and lipoxidase as biocatalysts. *In:* "Symposium on Foods: Lipids and Their Oxidation." p. 122. (H. W. Schultz, ed.) AVI Publishing Co., Westport, Conn.

Urbach, G. 1963. Thin-layer chromatography of aliphatic 2,4-dinitrophenylhydrazones. *J. Chromatog.* **12**, 196.

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Relation of Pectic and Fatty Acid Changes to Respiration Rate During Ripening of Avocado Fruits

SUMMARY

The ripening of a lot of hard mature avocado fruits (*Persea americana* Miller, *cv.* MacArthur) was followed at 15°C by measuring respiration rate and softening. The climacteric rise in respiration was accompanied by rapid softening of the flesh, a drop in content of protopectin and in degree of pectin esterification, and a rise in soluble pectin. Eight fatty acids were identified in the oil, but the oil composition did not change significantly, suggesting that the storage oil of the tissue has no major metabolic role in fruit ripening.

INTRODUCTION

As do most fleshy fruits, mature avocados show during senescence a characteristic respiratory pattern which has been termed the "climacteric." The rise and fall in respiratory rate is accompanied by a complex of other changes in fruit characteristics which, in sum, is called "ripening." In studying fruit ripening, it is useful to relate these changes to this respiratory pattern. While several laboratories have observed compositional changes during avocado fruit growth and development, reports on biochemical changes during ripening are few and limited in scope. In the present work, the ripening of hard mature avocado fruits (*Persea americana* Miller) was examined. Ripening was followed by measurements of respiration and flesh softening. The pectic substances and the fatty acid composition of the oil were analyzed to relate possible changes to the ripening of the fruit.

Basic work on the physiology of avocado ripening, with special reference to the respiratory climacteric, has been carried out by Biale (1941, 1946) and summarized in important reviews (Biale, 1960a, 1964), including attention to biochemical changes (Biale, 1960b). Pectic changes between hard and soft avocado fruits were noted by McCready and McComb (1954). Several laboratories have studied the changes in content and nature of the oil during fruit growth and development, for example: Church and Chace (1922), Appleman and Noda (1942), Davenport and Ellis (1959), and Hatton *et al.* (1964); additional references are provided by those authors. Only Davenport and Ellis (1959) reported on changes in the fruit during a significant postharvest period. Others have reported on the fatty acid constitution of the oil itself, including Jamieson *et al.* (1928), Alvarez *et al.* (1949), French (1962), Prevot and Cabeza (1962), and Mazliak (1965a,b); the source of the avocado oil samples analyzed was not always made clear by those workers.

MATERIALS AND METHODS

Fruit samples. One hundred mature but unripe 'MacArthur' avocados were obtained through a local distributor. Five typical fruits were taken and analyzed for the zero-time sample as soon as the fruits were received. The remainder were sorted into lots of 20 fruits which were put in ventilated jars and held at 15°C for measurement of respiration rate and subsequent sampling. Five fruits were removed from one or another of the jars each day after measurement of the respiration rates (Fig. 1). Each fruit was cut into nine radial longitudinal slices, and the seed discarded. One-third of the slices were used for determination of each of the following: moisture and oil content, fatty acids, and pectins. Moisture and oil were determined on the day of sampling. The portions for fatty acid determination were further sliced, weighed, and frozen in air-tight glass containers at 0° under a nitrogen atmosphere; they were stored at this temperature until analyzed. Slices destined for pectin analysis were heated in boiling 95% ethanol to inactivate the pectic enzymes; the containers were cooled rapidly in running water and then stored at 0°.

Respiration. A constant flow of air (about 25 L per hour) was passed through each jar of fruit, and respiration was determined by measuring the effluent carbon dioxide with the colorimetric method of Claypool and Keefer (1942). The rate is expressed as milligrams of carbon dioxide produced per kilogram of original fresh weight; the weight of each lot was corrected as fruits were removed for analysis.

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Softening. Softening of the flesh of the fruits was determined with a Magness-Taylor pressure tester with a 5/16-inch plunger (Haller, 1941). A patch of skin was cut from the side of the fruit, and the plunger was pressed into the flesh toward the center.

Total pectin. One hundred grams of avocado pulp stored at 0°C in 95% ethyl alcohol were blended until the mixture was homogeneous. The solvent was filtered off through Whatman No. 2 filter paper under slight suction. The pulp (alcohol-insoluble solids) was extracted with two volumes of chloroform to remove the oil, and this extraction was repeated until the pulp was free of oil. The pulp was then washed twice by blending with 50 ml of 95% ethyl alcohol and finally with 20 ml of purified ethyl alcohol. Thereafter, the method described by Postlmayr *et al.* (1965) was followed for the extraction of total pectin. The colorimetric carbazole method (McComb and McCready, 1952; McCready and McComb, 1952) was used to determine the anhydrouronic acid content; color intensity was measured in a Klett-Summerson photoelectric colorimeter with a green filter.

Protopectin. The extraction procedure was the same as that for total pectin except that, after washing with purified ethanol, the moist oil-free alcohol-insoluble solids fraction was mixed with 300 ml of distilled water and a few drops of toluene and kept overnight at 0°C. Seven grams of Supercel were added, and the mixture was filtered through Whatman No. 2 filter paper (lined with a thin layer of moist Supercel) in a Buchner funnel under slight suction. The residue was removed, extracted again with 600 ml of distilled water, sucked dry, and analyzed for remaining pectin as above.

Water-soluble pectin. The difference between the content of total pectin and protopectin represents the amount of water-soluble pectin.

Degree of esterification. Another sample of oil-free alcohol-insoluble solids was prepared from 100 g of avocado pulp as described above, but after the final chloroform extraction the pulp was filtered and pressed as dry as possible. The dried pulp was blended with about two volumes of acidified ethanol (750 ml of 95% ethanol, 200 ml of water, and 50 ml of concentrated hydrochloric acid). After standing about one hour, the solvent was removed as before. The pulp was then blended with two volumes of 70% ethanol and filtered, and the blending treatment was repeated until portions of the filtrate showed a negative reaction for chloride when tested with silver nitrate. The moist chloride-free pulp was washed twice with two volumes of acetone, and this material was dried without heating. The marc was ground in a mortar until it was fine enough to form a homogeneous mixture with ethyl alcohol. Esterification of pectic substances was determined by the method of Gee *et al.* (1959).

Moisture. Approximately 10 g of thin slices of fruit were placed between tared (1 × 4 inch) strips of filter paper and tied with a tared copper wire. This packet was placed in a tared aluminum dish, weighed, and dried in a vacuum oven at 70°C. This sample was considered dry when the percent loss of moisture between two successive weighings was not more than 0.05%.

Oil content. The dried samples from the moisture determinations were extracted with 150-ml portions of Skellysolve B (n-hexane) in Soxhlet extractors. The solvent in the extract was evaporated on a warm bath until the weight was constant.

Fatty acids—extraction of oil. The frozen avocado samples were dried under vacuum for 24 hr in a Stokes freeze-dryer. The plate temperature was kept at 25°C, and the pressure in the chamber at 0.1 mm Hg. Five grams of the dried sample were transferred into a Soxhlet bulb containing reagent-grade Skellysolve B (n-hexane). A continuous flow of nitrogen into the system was maintained until the solvent started to boil. The extraction was carried out for 5 hr. As soon as the heater was turned off, nitrogen was again allowed to flow until the Soxhlet bulb was cold enough to be handled. The solvent was evaporated under a nitrogen atmosphere.

Fatty acids—methylation. The method of Stoffel *et al.* (1959) was followed: Five mg of lipid material were added to 4 ml of 1% dry hydrochloric acid in anhydrous methanol in a 20-ml glass-stoppered test tube. These test tubes were put in a desiccator under a nitrogen atmosphere and kept in an oven at 50°C for 24 hr, then cooled in ice, and mixed with 4 ml of distilled water. The methyl esters of the fatty acids were extracted three times with 3 ml portions of redistilled petroleum ether. One gram of a 4:1 mixture of anhydrous sodium sulfate and sodium bicarbonate was added to the combined methylated fatty acids extract. The dried petroleum ether extract was transferred to a test tube, and most of the petroleum ether was evaporated under a nitrogen atmosphere.

Fatty acids—analysis by gas-liquid chromatography. An Aerograph gas chromatograph (Model A-90-C) was used with two recorders, one ten times as sensitive as the other, eliminating the use of the attenuation controls. A 7-ft column of ethylene glycol succinate polyester (DEGS) (12% by weight on acid-washed 60–80-mesh firebrick) was used at 208°C with helium as the carrier gas (45 ml per min). The amount of each component was calculated by multiplying the peak height by its width at half height. Any peak that was too small on the less sensitive recording was calculated

from the high-sensitivity recorder and converted to the same base by dividing the area by 10. The peak area of a given methyl ester, divided by the sum of the peak areas of the sample and multiplied by 100, gave the percentage by weight of each methyl ester. The percent composition of each component found was multiplied by a correction factor determined from the percentage recovery of a known weight of standard fatty acid methyl ester. These factors were: methyl laurate, 0.92; methyl myristate, 0.96; methyl palmitate and methyl palmitoleate, 0.99; methyl stearate and methyl oleate, 1.00; methyl linoleate, 0.98; and methyl linolenate, 0.97. The components were qualitatively identified by comparing retention times with those of authentic standards (Eastman Organic Chemicals).

RESULTS AND DISCUSSION

Respiration and ripening. The ripening of these lots of fruit is shown by the patterns of respiration and softening (Fig. 1). The

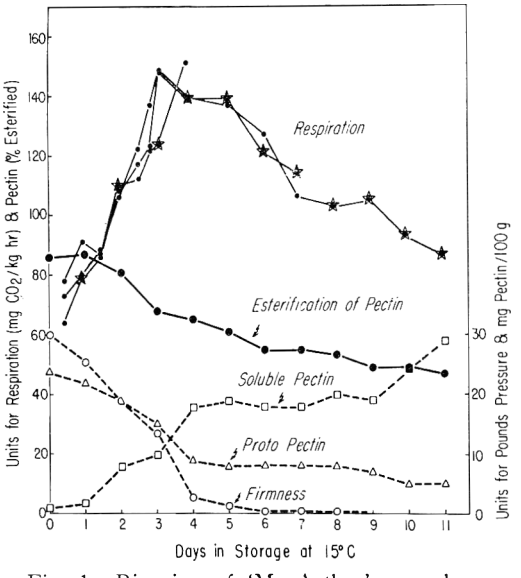

Fig. 1. Ripening of 'MacArthur' avocadoes as illustrated by respiration and pectic changes. The respiration values are given for three jars of fruit, and the stars indicate the removal of fruits for analysis from one or another of the jars. The values for soluble pectin, protopectin, degree of esterification of pectin, and flesh softening were determined on the samples removed after the respiration readings indicated by the respective stars.

'MacArthur' avocado shows the same climacteric rise and fall in respiration as has been reported for the 'Fuerte' (Biale, 1941, 1946). No preclimacteric minimum was observed in this instance, because the fruit was not obtained directly from the tree. However, the fruit was quite certainly in the initial stage of ripening at zero time, when the first samples were taken for analysis. The fruit is at ideal eating ripeness just after the climacteric peak; thereafter, the fruit becomes very soft and mushy and, after 9–11 days in storage, commonly shows brown blotches on the skin.

Pectic changes. The rapid softening of the fruit from the start of the ripening process to the fourth day was accompanied by a rapid decrease in protopectin and an increase in water-soluble pectin (Fig. 1). After the fourth day, the fruit continued to soften, but more slowly, until the firmness could no longer be measured with the Magness-Taylor pressure tester. The content of water-soluble pectin continued to increase, whereas that of protopectin showed relatively little further change. These results are similar to those obtained with other fruits (Postlmayr *et al.*, 1956; de Haan, 1957; Esau *et al.*, 1962). Fig. 1 also shows the decrease in the degree of esterification of avocado fruit pectin during the ripening process; this de-esterification of the pectic substances also contributes to the observed change in fruit texture (Gee *et al.*, 1959).

Composition of oil. The average oil content of the avocado samples was 17% on a fresh basis and 63% on a dry-weight basis. Table 1 gives the relative composition of the oil at each sampling date during the ripening period, as determined by gas chromatography of the fatty acid methyl esters. Eight fatty acids were found, but the content of stearic acid was very low and only traces of myristic and arachidic acids were observed. The composition of this oil was comparable to values reported by others for other cultivars. It is interesting that, although the fatty acid composition of avocado oil changes during growth and maturation of the fruit on the tree (Davenport and Ellis, 1959), there is no significant change in fatty acid composition during postharvest ripening. Important biochemical changes are occurring during ripening, as shown by the changes in respiration rate and pectic constituents, but the "storage" oil fraction appears to have no major metabolic role. Fatty acids of biochemically important

Table 1. Composition of avocado oil samples from fruits stored at 15°C after harvest (mole percent).

Days in storage	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Linolenic	Arachidic
0	Trace	18.90	3.92	0.20	61.80	14.11	0.81	Trace
1	"	19.75	4.25	0.10	61.52	13.37	0.99	"
2	"	18.52	3.09	0.09	64.45	13.18	0.65	"
3	"	19.72	3.58	0.10	61.63	14.10	0.86	"
4	"	16.85	3.47	0.12	64.62	13.90	1.02	"
5	"	18.33	2.58	0.06	61.90	15.87	1.27	"
6	"	16.55	2.87	0.19	65.66	13.73	0.99	"
7	"	21.19	2.67	0.10	61.21	13.90	0.91	"
8	"	19.42	3.69	0.10	62.13	14.01	0.63	"
9	"	19.55	3.89	0.06	61.84	14.06	0.59	"
10	"	17.92	3.11	0.15	63.97	13.87	0.95	"
11	"	16.58	3.90	0.11	62.95	15.41	1.03	"
Av.	Trace	18.77	3.42	0.12	62.81	14.13	0.89	Trace

membrane systems may be changing significantly, but such changes could not be detected by the methods used in this work.

REFERENCES

Alvarez, J., P. Cattaneo, E. Grandolini, G. Karman, and J. P. Rigotti. 1949. Chemical composition of oils from the pulp of the Argentina avocado pear. *Anales asoc. quim. arg.* **37**, 34. (*Chem. Abstr.* **43**, 9489g. 1949.)

Appleman, D., and L. Noda. 1942. Biochemical studies of the Fuerte avocado fruit—a preliminary report. *Calif. Avocado Soc. Yearbook* **1941**, 60.

Biale, J. B. 1941. The climacteric rise in respiration rate of the Fuerte avocado fruit. *Proc. Am. Soc. Hort. Sci.* **39**, 137.

Biale, J. B. 1946. Effect of oxygen concentration on respiration of the Fuerte avocado fruit. *Am. J. Botany* **33**, 363.

Biale, J. B. 1960a. Respiration of fruits. *Handbuch der Pflanzenphysiologie* **12**(2), 536.

Biale, J. B. 1960b. The postharvest biochemistry of tropical and subtropical fruits. *Advances in Food Research* **10**, 293.

Biale, J. B. 1964. Growth, maturation, and senescence in fruits. *Science* **146**, 880.

Church, C. G., and E. M. Chace. 1922. Some changes in the composition of California avocados during growth. *U.S. Dept. Agr. Bull.* **1073**, 22 p.

Claypool, L. L., and R. M. Keefer. 1942. A colorimetric method for CO_2 determination in respiration studies. *Proc. Am. Soc. Hort. Sci.* **40**, 177.

Davenport, J. B., and S. C. Ellis. 1959. Chemical changes during growth and storage of the avocado fruit. *Australian J. Biol. Sci.* **12**, 445.

Esau, J., M. A. Joslyn, and L. L. Claypool. 1962. Changes in water-soluble calcium and magnesium content of pear fruit tissue during maturation and ripening in relation to changes in pectic substances. *J. Food Sci.* **27**, 509.

French, R. B. 1962. Analyses of pecan, peanut, and other oils by gas-liquid chromatography and ultra-violet spectrophotometry. *J. Am. Oil Chemists Soc.* **39**, 176.

Gee, M., R. M. Reeve, and R. M. McCready. 1959. Measurement of plant pectic substances. Reaction of hydroxylamine with pectinic acids. Chemical studies and histochemical estimation of the degree of esterification of pectic substances in fruit. *J. Agr. Food Chem.* **7**, 34.

Haan, I. de. 1957. Pectin conversions in peaches during cold storage. *South African Indus. Chemist* **11**, 26.

Haller, M. H. 1941. Fruit pressure testers and their practical applications. *U. S. Dept. Agr. Circ.* **627**, 22 p.

Hatton, T. T., Jr., P. L. Harding, and W. F. Reeder. 1964. Seasonal changes in Florida avocados. *U.S. Dept. Agr. Tech. Bull.* **1310**, 47 p.

Jamieson, G. S., W. F. Baughman, and R. M. Hann. 1928. Avocado oil. *Oil & Fat Inds.* **5**, 202. (*Chem. Abstr.* **22**, 3545. 1928.)

Mazliak, P. 1965a. Les lipides de l'avocat (*Persea americana*, var. Fuerte). I. Composition en acides gras des diverses parties du fruit. *Fruits* **20**, 49.

Mazliak, P. 1965b. Les lipides de l'avocat (*Persea americana*, var. Fuerte). II. Variation de la composition en acides gras des lipides du péricarpe selon la composition de l'atmosphère autour des fruits en maturation. *Fruits* **20**, 117.

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

McCready, R. M., and E. A. McComb. 1952. Extraction and determination of total pectic materials in fruits. *Anal. Chem.* **24**, 1986.

McCready, R. M., and E. A. McComb. 1954. Pectic constituents in ripe and unripe fruit. *Food Research* **19**, 530.

Postlmayr, H. L., B. S. Luh, and S. J. Leonard. 1956. Characterization of pectin changes in freestone and clingstone peaches during ripening and processing. Food Technol. **10**, 618.

Prevot, A., and F. Cabeza. 1962. Note sur la composition de quelques corps gras peu communs par chromatographie en phase gazeuse. *Rev. franç. corps gras* **9**, 149.

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

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Characterization of Mucoprotein in Bovine Skeletal Muscle

SUMMARY

Mucoprotein was isolated from bovine skeletal muscle by extraction with $CaCl_2$, and characterized. The results indicate that chondroitin sulfate is the sole mucopolysaccharide present in bovine skeletal muscle. It is linked to a protein that contains the same carbohydrate prosthetic grouping, regardless of method of isolation. This carbohydrate portion contains glucuronic acid, glucosamine, and galactose. It appears that virtually all of the mucopolysaccharide is protein bound. The mucoprotein is acted upon by hyaluronidase, papain, and lysozyme. The complex was non-heat-coagulable, a property typical of glycoproteins. A commercial preparation of chondroitin sulfate from bovine tracheal cartilage was also studied. It was found to contain a mucoprotein complex which was similar to that isolated from bovine skeletal muscle.

INTRODUCTION

Mucoprotein was isolated from bovine skeletal muscle by $CaCl_2$ extraction, and purified (McIntosh, 1965). This soluble mucoprotein fraction has been characterized as described here.

Paper chromatographic studies of the mucoprotein hydrolysates showed that galactosamine and uronic acid were invariably present (McIntosh, 1965). This observation suggested the presence of chondroitin sulfate. Glucosamine and galactose were also noted consistently. The galactosamine-containing entity could not be separated from the glucosamine-containing component by any of various classical methods (Meyer *et al.*, 1953; Moore and Stein, 1951; Hoffman *et al.*, 1956; Mathews and Dorfman, 1953). Regardless of the method employed, both the skeletal muscle mucoprotein and the commercial chondroitin sulfate were always recovered in their original form, containing both hexosamines in their original ratios.

The tenacity with which glucosamine and galactosamine seemed bound together suggested the existence of a chondroitin sulfate-protein complex (Mathews and Lozaityte, 1958; Muir, 1958; Partridge and Davis, 1958; Partridge *et al.*, 1961; Castellani *et al.*, 1962; Gregory *et al.*, 1964) similar to that found by Mörner in whale nasal cartilage (Masamune, 1955). This chondromucoid was reported to consist of chondroitin sulfate linked to a carbohydrate-containing protein. The carbohydrate portion of this protein consisted of glucosamine-galactose-sulfate in the ratio of 2:2:1. The results reported here indicate that a comparable mucoprotein complex is present in bovine skeletal muscle.

EXPERIMENTAL METHODS

Chemicals. Cobaltic luteochloride (Mathews and Dorfman, 1953) was prepared in the laboratory (Bjerrum and McReynolds, 1946). The sources of other chemicals used have been given (McIntosh, 1965).

Mucoprotein preparations. Mucoprotein was isolated from bovine skeletal muscle as described (McIntosh, 1965). Several pooled meat samples from animals of the same genetic background were carried identically through the isolation procedure, and the resultant mucoprotein products were analyzed and characterized. The data presented here represent values for a typical product.

Paper chromatography. The separation and preliminary identification of sugars in the mucoprotein hydrolysates were by paper chromatography (McIntosh, 1965). Roughly quantitative determinations of ribose, mannose, and galactose were made with an adaptation of this same technique.

Hexosamine. Total hexosamine was determined as described (McIntosh, 1961). When chromatographic analyses showed both glucosamine and galactosamine present in a given preparation, they were separated quantitatively (Gardell, 1953) and determined colorimetrically (McIntosh, 1961), and the glucosamine-galactosamine ratios were calculated (Table 1).

Uronic acid. A modification of the classical napthoresorcinol reaction was used to determine uronic acid. A preliminary hydrolysis of the mucoprotein sample was necessary, not only to obtain equal liquid aliquots when solid samples were used, but also to obtain maximum uronate values. The sample (20 mg) was hydrolyzed for 20 min in

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Table 1. Analytical values of mucoprotein preparations.[a]

	Skeletal muscle mucoprotein		Commercial chondroitin sulfate[b]		Chondroitin sulfate[g]
	Untreated (Ia)	Alkali-treated (Ib)	Untreated (IIa)	Alkali-treated (IIb)	(Theoretical)
Nitrogen	12.00	2.66	3.91	2.11	2.44
Hexosamine (total)[c]	2.98	30.83	27.02	28.33	37.57
Non-hexosamine nitrogen (by difference)	11.82	0.66	2.14	0.27	.....
Protein	50.94		11.25		
Glucosamine	2.16	.....	3.86	.....	.....
Galactosamine	0.82	30.83	23.16	28.33	37.57
Glucosamine/ galactosamine ratio	2.6/1	.....	1/6	.....	.....
Uronate[d]	3.25	28.00	22.5	24.00	37.57
Sulfate	2.63	35.50	12.25	25.75	16.68
Hydroxyproline	.....[e]	.....[e]	0.70	.....[f]	.....
Galactose	0.33	.....	2.50	.....	.....
Mannose	0.17	0.13	0.42	.....	.....
Ribose	0.5	.....	.....	.....	.....
Relative viscosity	1.26	1.15	1.10	1.08	.....

[a] Values are expressed as percent unless otherwise designated.
[b] Potassium salt.
[c] Expressed as glucosamine HCl.
[d] Expressed as sodium glucuronate.
[e] Less than 0.14%.
[f] Less than 0.5%.
[g] Sodium salt, tetrahydrate.

3 ml 12N H_2SO_4 in a boiling-water bath. Under these conditions, recovery of added glucuronate was complete. After cooling, the hydrolysates were made up to 5 ml in a volumetric flask, and an appropriate aliquot (0–100 μg) was taken for assay. The aliquot, made up, if necessary, to 2 ml with distilled water, was pipetted into a glass-stoppered 18 mm test tube, followed by the addition of 2 ml of 0.2% naphthoresorcinol. (This reagent should be prepared fresh daily. The solution should be shaken well to dissolve pigment, then filtered before use). To this was added, with shaking, 2 ml 18N H_2SO_4. After shaking to mix thoroughly, the tubes were heated, unstoppered, for 90 min in an oil bath (Fishman and Green, 1955) at 92–96°C. The tubes were removed immediately and cooled in cold water. Two ml ethanol were added, and the tubes were stoppered and shaken vigorously, end to end, to dissolve the pigment. It is essential that all of the pigment be dissolved at this step. Ten ml ether were then carefully added, the tubes stoppered again, and the pigment extracted into the ether phase by end-to-end shaking. The ether phase was transferred by decantation to a colorimetric tube at the bottom of which a pinch of anhydrous sodium sulfate had been carefully added to dry the ether. Using the 6-ml well, the tubes were read at 565 mμ in the Evelyn colorimeter set to zero optical density using a water blank that was carried through the entire procedure. A standard curve was run with each assay by using several levels of sodium glucuronate. Values obtained were multiplied by 0.898 to convert from sodium glucuronate to free glucuronic acid. The assay is linear between 0 and 100 μg. The actual degree of color intensity varied somewhat from one bottle of naphthoresorcinol to another. The coefficient of variation for the method was estimated to be about 3.9% for a single sample, or 2.7% for duplicate samples.

Protein. Protein was measured according to the method of Lowry *et al.* (1951), with purified bovine serum albumin used as the standard.

Nitrogen. Nitrogen was determined as described (McIntosh, 1965).

Sulfate. Sulfate was determined colorimetrically with barium chloranilate by a modification of the method described by Bertolacini and Barney (1957). The weighed sample was hydrolyzed for 2 hr in 1 ml 1N HCl in an autoclave at a pressure of 15 psi. After the entire hydrolysate was passed through a Dowex-50 column, the column was washed several times with a total volume of 15 ml water. The sample effluent and combined washings were pooled, adjusted to pH 4, and transferred to a 50-ml volumetric flask. To this was added 5 ml buffer (Bertolacini and Barney, 1957) and 25 ml 95% ethanol. The mixture was diluted to volume with distilled water; 150 mg barium chloranilate

was added, and the remainder of the analysis carried out as outlined by those investigators. A control, which consisted of 1 ml 1*N* HCl, was also carried throughout the entire procedure.

Viscosity. Relative viscosity measurements of the different mucopolysaccharide preparations were taken at 25°C with an Ostwald viscometer. The flow time of 5 ml of a 0.4% solution of mucopolysaccharide in phosphate buffer (Shatton and Schubert, 1954) was compared with that for buffer alone.

Enzyme studies. Hyaluronidase activity was measured by the changes in viscosity that occurred when a 0.2% mucoprotein solution [in sodium acetate buffer, pH 5 (Shatton and Schubert, 1954)] was incubated at 37°C with testicular hyaluronidase (100 μg per ml). Papain activity was measured essentially as described by Muir (1958). The action of lysozyme was studied by the procedure of Smith *et al.* (1955).

COMPLEX HYPOTHESIS

To test the hypothesis that glucosamine and galactose belonged to the protein moiety, and the galactosamine to the mucopolysaccharide (chondroitin sulfate) portion of this proposed complex, attempts were made to devise a method which would split the protein from the mucopolysaccharide portion.

The following procedure was successful for separating the protein from the mucopolysaccharide portion of both skeletal muscle mucoprotein and commercial chondroitin sulfate isolated from bovine tracheal cartilage.

Cleavage of mucoprotein "complex." The mucopolysaccharide portion of the mucoprotein product was freed from the protein portion by digestion with 0.15*N* NaOH (Muir, 1958). A sample of the product (220-500 mg) was hydrolyzed for 4 days in 50 ml 0.15*N* NaOH at room temperature. The digest was neutralized to pH 7 with 1*N* HCl and diluted to 250 ml. The free mucopolysaccharide was obtained by precipitation with aminoacridine hydrochloride (Muir, 1958). Usually, two precipitations with this reagent were sufficient to remove the protein portion of the complex completely. The mucopolysaccharide precipitation must be carried out at pH 7 since, at a lower pH, the split mucopolysaccharide and protein fragment recombine. This recombination results in precipitation of the complex (or a product which is chromatographically similar), rather than precipitation of free mucopolysaccharide.

Products of cleavage reaction. Paper chromatography was used as a preliminary criterion for complete "splitting off" of the protein-carbohydrate entity. After alkali treatment followed by precipitation with aminoacridine at pH 7, chromatograms showed galactosamine and uronate as the only carbohydrates. This composition is indicative of chondroitin sulfate. Glucosamine, galactose, and ribose, which were originally present in the complex, appeared with the protein fragment (obtained by alcohol precipitation of the aminoacridine supernatant). Uronate was also present. Fig. 1 shows a typical chromatogram of hydrolysates of: a) an aminoacridine precipitate; b) aminoacridine supernatant; and c) the untreated product.

Chemical analyses of mucoprotein preparations. Table 1 shows the chemical analyses of mucoprotein products obtained from bovine skeletal muscle and bovine tracheal cartilage, before and after cleavage.

For the untreated skeletal muscle mucoprotein complex (Ia), the relatively high non-hexosamine nitrogen value indicates a high protein content [50.94% by the method of Lowry *et al.* (1951), 73.88% from non-hexosamine nitrogen (× 6.25)]. The glucosamine-galactosamine ratio = 2.6:1. The hexosamine-uronate ratio of approximately 1:1 is in agreement with results of chromatographic studies indicating that uronate was associated not only with galactosamine in the chondroitin sulfate portion, but also with glucosamine as part of the protein moiety.

After alkali treatment (Ib), the skeletal muscle mucoprotein preparation showed a drop in relative viscosity, and anlysis by paper chromatography indicated that glucosamine and galactose were no longer present. Nitrogen content was sharply re-

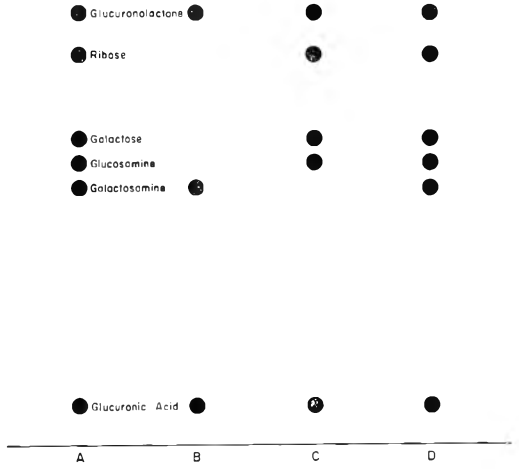


Fig. 1. Splitting of complex into mucopolysaccharide and protein components as shown by paper chromatography.
A) Monosaccharide standards
B) Aminoacridine precipitate of alkali digest (mucopolysaccharide component)
C) Aminoacridine supernatant (protein component)
D) Untreated complex

duced, with very little remaining nitrogen of non-hexosamine origin. These observations lend support to the theory that alkali treatment resulted in virtually complete removal of the protein portion of the complex. The galactosamine-uronate ratio of approximately 1:1 corresponded to theoretical expectations for chondroitin sulfate, which contains equimolar amounts of galactosamine and glucuronic acid.

Except for the absence of ribose (McIntosh, 1965; Table 2), chromatographic studies showed that, qualitatively, the commercial chrondroitin sulfate contained essentially the same carbohydrate components as skeletal muscle mucoprotein. Chemical analyses, however, showed certain definite quantitative differences.

The commercial chondroitin sulfate product (IIa) was much lower in protein content than was the skeletal muscle mucoprotein [11.25% by the method of Lowry *et al.* (1951), or 13.38% from non-hexosamine nitrogen (× 6.25)]. The hexosamine and uronate content is much higher in commercial chondroitin sulfate than in the mucoprotein product from skeletal muscle; again, however, they are present in approximately equimolar ratio. These results lend further credence to the theory that uronic acid is associated with the glucosamine of the protein moiety, as well as with the galactosamine of the mucopolysaccharide portion. The glucosamine-galactosamine ratio of about 1:6 indicated relatively less glucosamine in the commercial chondroitin sulfate product than in the skeletal muscle preparation. The glucosamine-galactose ratio is also considerably lower; total galactose content is higher in the commercial chondroitin sulfate complex (IIa) than in the preparation from skeletal muscle (Ia).

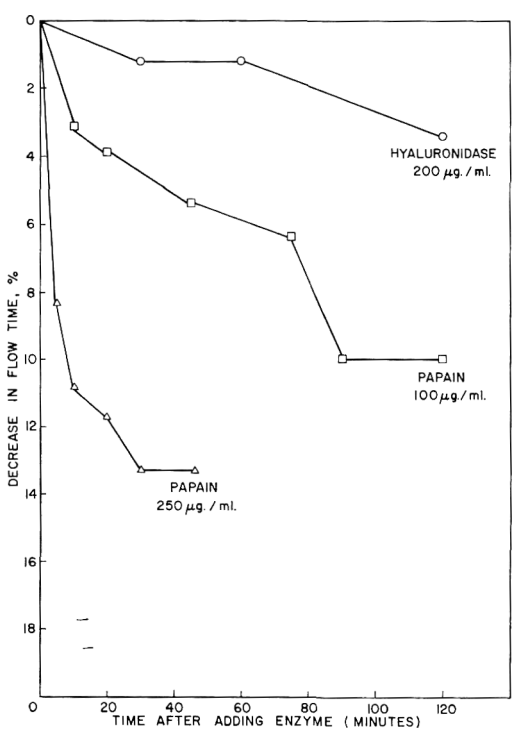


Fig. 2. Effect of hyaluronidase and papain on viscosity of mucoprotein solutions.

Alkali treatment of the commercial chondroitin sulfate product (IIb) effected complete removal of glucosamine and galactose, as judged by paper chromatography. Nitrogen values approached the theoretical value for chondroitin sulfate. Splitting off the protein moiety was virtually complete, as evidenced by the low value for non-hexasamine nitrogen, as well as by the absence of glucosamine and galactose. The galactosamine and uronate values were low; however, they gave the expected 1:1 ratio.

High sulfate values obtained for the chondromucoids from both skeletal muscle and tracheal cartilage indicate that the chondroitin sulfate may exist as a disulfate (Suzuki, 1960) rather than a monosulfate.

The mucoprotein complexes of both skeletal muscle and tracheal cartilage are similar to the complex reported in whale nasal cartilage (Masamune, 1955) in that they contain glucosamine and galactose associated with the protein portion of the complex. However, the preparations studied here contained relatively less galactose, and little or no sulfate of protein origin could be detected. Both of the chondromucoids studied contained uronate as part of the protein moiety, whereas uronate was not reported for whale nasal cartilage.

The consistent observation of glucosamine and galactose had initially suggested that keratosulfate (Meyer *et al.*, 1953) might be present, along with chondroitin sulfate (McIntosh, 1965). However, indications that glucosamine and galactose were associated with the protein portion of the complex, coupled with the finding of little sulfate which was not of chondroitin sulfate origin, seemed to preclude the presence of keratosulfate.

PROPERTIES OF MUCOPROTEIN COMPLEX

Precipitation reactions. The mucoprotein complexes of both skeletal muscle and tracheal cartilage were found to be non-heat coagulable, a property typical of glycoproteins. No coagulation occurred when 0.4% solutions of mucoprotein (in 0.05M phosphate buffer, pH 7) were heated for 10 min or longer in a boiling-water bath.

The skeletal muscle complex was precipitated by trichloracetic acid and by phosphotungstic acid but not by sulfosalicylic acid or picric acid (McIntosh,

1965; Table 3). It thus seemed unlikely that the complex is an albumin or globulin since, typically, these proteins are precipitated by sulfosalicylic acid and are heat coagulable. The complex was precipitable by alcohol. Neither was the complex a carbohydrate-containing "collagen" or "elastin"; these albuminoids are insoluble in neutral salts and contain hydroxypoline, while the complex described here was soluble in neutral 10% $CaCl_2$ and contained no hydroxyproline.

Relative precipitability of the mucoprotein at varying pH levels, coupled with its adsorption behavior toward cationic and anionic exchange resins at different pH values, suggested an isoelectric point near 4.7.

Complete characterization of the protein portion of the complex is difficult since the alkali treatment which effects a cleavage of the complex into its component parts (i.e., protein and free mucopolysaccharide) appeared to result in degradation of the protein component. The alkali digest contained considerable dialyzable material of a nitrogenous nature (along with free chondroitin sulfate) which indicated a degradation of the original protein to smaller fragments.

Effect of enzymes. In the presence of testicular hyaluronidase (200 μg/ml), skeletal muscle mucoprotein lost viscosity slowly at rates of 3.4% in 2 hr (Fig. 2) and 6.8% in 24 hr. Crystalline papain produced a marked drop in viscosity of mucoprotein solutions (McIntosh and Carlin, 1963), as shown in Fig. 2. At a concentration of 100 μg per ml, papain produced a 10% drop in viscosity of a 0.2% mucoprotein solution in 90 min and at a concentration of 250 μg per ml, a 13.3% decrease in 30 min. No further decrease was noted upon further incubation. Papain has been reported to produce decreased viscosity of solutions of mucoprotein isolated from bovine cartilage (Muir, 1958). The susceptibility of the skeletal muscle mucoprotein preparation to testicular hyaluronidase is indicative of chondroitin sulfate *A* or *C*; however, its precipitability by alcohol at a concentration of 50% and above suggests that it is present in the *C* form (Meyer and Rapport, 1951).

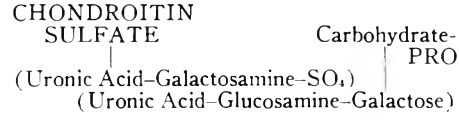
The mucoprotein of skeletal muscle is also affected by lysozyme. At a concentration of 50 μg per ml, lysozyme produced a 22.5% decrease in turbidity of a 0.4% solution of mucoprotein incubated at 39°C. Turbidity was measured at 440 mμ in an Evelyn colorimeter (Smith *et al.*, 1955). There was no further loss in turbidity after 2 hr. The control showed no change in turbidity during this same period.

Behavior toward phosphotungstic acid. Studies with phosphotungstic acid revealed that all of the extractable skeletal muscle mucoprotein was phosphotungstic acid precipitable. No mucopolysaccharide material could be detected in the phosphotungstic acid supernatant. Therefore, virtually all of the skeletal muscle mucopolysaccharide must be protein bound. These observations are similar to the conclusion of Schubert (1958) with respect to bovine nasal cartilage.

Extractability. Less than 10% of the total mucoprotein in skeletal muscle is extractable by $CaCl_2$ (McIntosh, 1965). This observation is similar to the experience of Muir (1958), Shatton and Schubert (1954), and others (Kent and Whitehouse, 1955; Partridge, 1948) who have isolated mucoprotein from other sources by using mild methods of extraction. In all of these cases, most of the mucoprotein remained in the residue, as reported for skeletal mucle (McIntosh, 1965; Table 1). Since the products thus obtained have represented such a small fraction of the total mucoprotein, there has been uncertainty whether they are truly representative of the mucoprotein as a whole (Kent and Whitehouse, 1955). However, work by Malawista and Schubert (1958) resulted in a procedure, using a Vir-Tis homogenizer, whereby extraction of mucoprotein from cartilage was almost complete. Analytical results of the products obtained by the latter technique, compared with those obtained from the earlier method (Shatton and Schubert, 1954), indicated that the products are essentially identical.

STRUCTURE

The observations made in this study suggest that the basic molecule may look something like this:



The structure proposed here is not incompatible with that suggested by Gregory *et al.* (1964) for a chondromucoprotein from bovine cartilage. Those investigators concluded that galactose is not a "fortuitious contaminant" but, rather, serves as a link between chondroitin sulfate and protein.

Observations that the isoelectric point is about 5 initially suggested an electrostatic linkage resulting from attraction between the positively charged protein (at pH values of 5 and below) and the negatively charged anion, chondroitin sulfate. The disruption of the complex at alkaline pH could then be explained by the protein becoming negatively

charged, with subsequent repulsion by the negatively charged chondroitin sulfate. However, if the linkages were completely electrostatic, there would appear to be no union of protein with mucopolysaccharide *in vivo*, since the pH of skeletal muscle is 7.6. Therefore, it seems likely that other bonds exist in addition to electrostatic bonds; e.g., ester linkages between the protein and chondroitin sulfate (Gottschalk and Murphy, 1961). Linkage between the carbohydrate and the hydroxyl group of serine (Muir, 1958; Castellani *et al.*, 1962; Gregory *et al.*, 1964) appears likely. Gregory and co-workers (1964) suggested that chondroitin sulfate chains are joined to galactose by a glucuronidic linkage and that the bond to protein is a a glycosidic one between galactose (or xylose) and the hydroxyl group of serine. Such linkages could explain the existence of a complex *in vivo* at pH 7.6 and yet would be compatible with the observation that the complex is disrupted at alkaline pH.

REFERENCES

Bertolacini, R. J., and J. E. Barney. 1957. Colorimetric determination of sulfate with barium chloranilate. *Anal. Chem.* **29**, 281.

Bjerrum, J., and J. P. McReynolds. 1946. Hexaminecobalt (III) salts. *Inorg. Syntheses* **2**, 216.

Castellani, A. A., B. Bonferoni, S. Ronchi, G. Ferri, and M. Malcovati. 1962. Light chondromucoprotein from metaphysis cartilage of new-born pigs. *Ital. J. Biochem.* **11**, 187.

Fishman, W. H., and S. Green. 1955. Microanalysis of glucuronide glucuronic acid as applied to β-glucuronidase and glucuronic acid studies. *J. Biol. Chem.* **215**, 527.

Gardell, S. 1953. Separation on Dowex 50 ion exchange resin of glucosamine and galactosamine and their quantitative determination. *Acta Chem. Scand.* **7**, 207.

Gottschalk, A. and W. H. Murphy. 1961. Studies on mucoproteins. IV. The linkage of the prosthetic group to the protein core in ovine submaxillary gland mucoprotein. *Biochim. et Biophys. Acta* **46**, 81.

Gregory, J. D., T. C. Laurent, and L. Rodén. 1964. Enzymatic degradation of chondromucoprotein. *J. Biol. Chem.* **239**, 3312.

Hoffman, P., K. Meyer, and A. Linker. 1956. Transglycosylation during the mixed digestion of hyaluronic acid and chondroitin sulfate by testicular hyaluronidase. *J. Biol. Chem.* **219**, 653.

Kent, P. W., and M. W. Whitehouse. 1955. "Biochemistry of the Aminosugers." pp. 74–75. Academic Press, New York.

Lowry, O. H., H. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265.

Malawista, I. and M. Schubert. 1958. Chondromucoprotein; new extraction method and alkaline degradation. *J. Biol. Chem.* **230**, 535.

Masamune, R. 1955. On the mode of combination of carbohydrate with amino acid grouping in mucopolysaccharides and mucoproteins. Proc. Third Intern. Congr. Biochem. Brussels. pp. 72–77.

Mathews, M. B. and A. Dorfman. 1953. The molecular weight and viscosity of chondroitinsulfuric acid. *Arch. Biochem. Biophys.* **42**, 41.

Mathews, M. B. and I. Lozaityte. 1958. Sodium chondroitin sulfate-protein complexes of cartilage. I. Molecular weight and shape. *Arch. Biochem. Biophys.* **74**, 158.

McIntosh, E. N. 1961. Determination of mucoprotein in skeletal muscle. *J. Agr. Food Chem.* **9**, 421.

McIntosh, E. N. 1965. The isolation of mucoprotein in bovine skeletal muscle. *J. Food Sci.* **30**, 986.

McIntosh, E. N., and A. F. Carlin. 1963. The effect of papain preparations on beef skeletal muscle proteins. *J. Food Sci.* **28**, 283.

Meyer, K., A. Linker, E. A. Davidson, and B. Weissman. 1953. The mucopolysaccharides of bovine cornea. *J. Biol. Chem.* **205**, 611.

Meyer, K. and M. M. Rapport. 1951. The mucopolysaccharides of the ground substance of connective tissue. *Science* **113**, 596.

Moore, S., and W. H. Stein. 1951. Chromatography of amino acids on sulfonated polystyrene resins. *J. Biol. Chem.* **192**, 663.

Muir, Helen. 1958. The nature of the link between protein and carbohydrate of a chondroitin sulphate complex from hyaline cartilage. *Biochem. J.* **69**, 195.

Partridge, S. M. 1948. The chemistry of connective tissues. 1. The state of combination of chondroitin sulfate in cartilage. *Biochem. J.* **43**, 387.

Partridge, S. M., and H. F. Davis. 1958. The chemistry of connective tissues. 4. The presence of a non-collagenous protein in cartilage. *Biochem. J.* **68**, 298.

Partridge, S. M., H. F. Davis, and G. S. Adair. 1961. The chemistry of connective tissues.

6. The constitution of the chondroitin sulphate-protein complex in cartilage. *Biochem. J.* **79**, 15.

Schubert, M. 1958. Chondromucoprotein. *Federation Proc.* **17**, 1099.

Shatton, J., and M. Schubert. 1954. Isolation of a mucoprotein from cartilage. *J. Biol. Chem.* **211**, 565.

Smith, E. L., J. H. Kimmel, D. M. Brown, and E. O. P. Thompson. 1955. Isolation and properties of a crystalline mercury derivative of a lysozyme from papaya latex. *J. Biol. Chem.* **215**, 67.

Suzuki, S. 1960. Isolation of novel disaccharides from chondroitin sulfates. *J. Biol. Chem.* **235**, 3580.

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Defatting and Deodorization of Fish Protein Concentrate from Harpoden Nehereus

SUMMARY

In Bombay-duck *(Harpoden nehereus)* fish, petroleum-ether-extractive ("true" lipids) fats, residual fats ("bound" lipids) obtained by acid hydrolysis, and ethanol extractives other than "true" and "bound" lipids were found to contribute to its odor, the last two fractions being more important. Exhaustive extraction with 95% ethanol yielded fish protein concentrate with no lipid or ethanol extractives but with 0.37% "bound" lipids; this concentrate was practically without any fishy odor but developed it on being cooked with water.

INTRODUCTION

Bombay-duck (*Harpoden nehereus*) fish is of economic importance in India. Annual production is 80,000 tons, and 80% of the catch is dried in the sun. Sun-dried fish has its own limitations, and a better way of preserving this fish is to convert it into fish flour or fish protein concentrate. Fish protein concentrate, being a defatted and deodorized product, is expected to have universal appeal, can be utilized in many food preparations without much difficulty, and has improved stability.

In the preparation of fish protein concentrate, the most important operations are dehydration, defatting, and deodorization, and processing methods vary mainly in the method of dehydration, choice of solvent(s), and extraction methods.

The present study was undertaken to find out the optimum conditions for defatting and deodorizing Bombay-duck fish.

Model experiments were used so that conditions could be better controlled.

EXPERIMENTAL

Sun-dried eviscerated Bombay-duck fish (as raw dried fish) and cooked pressed cake with or without drying were used. The fish was cooked with its own weight of water for 30 min, and the cooked meat was drained and pressed. Respective moisture contents were 11.1, 5.5 and 84.4%. The solvents were ethanol of 95% and 80% strength and hexane (67–70°C bp).

Batchwise extraction was carried out in a round bottom glass flask fitted with a water-cooled glass refluxing condenser. Heating was done with a mantle-heater. The period of extraction was generally 30 min, reckoned from the time of boiling of the solvent. Each extraction took about 50–60 min including time for charging, time taken to raise the temperature of the solvent to its boiling point, and time for cooling before draining. In the experiments carried out to fix the period of extraction, the time from the boiling of the solvent was 15, 30, 60, and 120 min. Batch size was 50 g, and the ratio of solvent (ml) to weight of fish material (g) was 5:1. With cooked pressed cake (84.4% H_2O), the volume of solvent (ml) to dry matter of cake (g) was 8:1 in first extraction and 7.5:1 in subsequent extractions. After each extraction, the meal and extract were transferred on a Buchner funnel, and draining was under suction.

In extraction by percolation, only cooked pressed dried meal was used. The inner tube (diameter 3.2 cm) of a jacketed column was filled with 140 g of meal with light tapping; the packed density of the meal was 0.4 g/cc. With pulverized meal, the flow of solvent through the column was too slow. Hot 95% ethanol at 70–74°C was allowed to pass through the meal, and the temperature of the column was maintained by circulating hot water of the same temperature range through the condenser. The rate of flow of solvent through the column was 250 ml/hr. The period of extraction was kept at 5 hr (equal to total time required for 4 extractions in batch extraction process), and 1500 ml alcohol was fed to the column. Of this, 1250 ml alcohol was recovered in the form of extract and the remaining 250 ml was retained by the column. After extraction, the meal of the column was taken out in three sections (top, middle, and bottom) and studied separately.

In hot continuous extraction (Fig. 1, in use at Torry Research Station, U.K.) 28–30 g cooked pressed dried pulverized meal was taken in a thimble and extracted for different periods (2, 4, and 8 hr). The volume of solvent used for refluxing was 180–200 ml (ratio of volume to solvent (ml) to weight of meal (g) was 8.2:1). The rate of refluxing was 5.5–6 ml/min.

The extracted meal was dried for 4–5 hr at 60°C under a vacuum of 29 inches Hg, and organoleptic and other determinations were carried out with the desolventized meal.

Ethyl alcohol extractives were determined by extraction in a Soxhlet apparatus with 95% ethanol

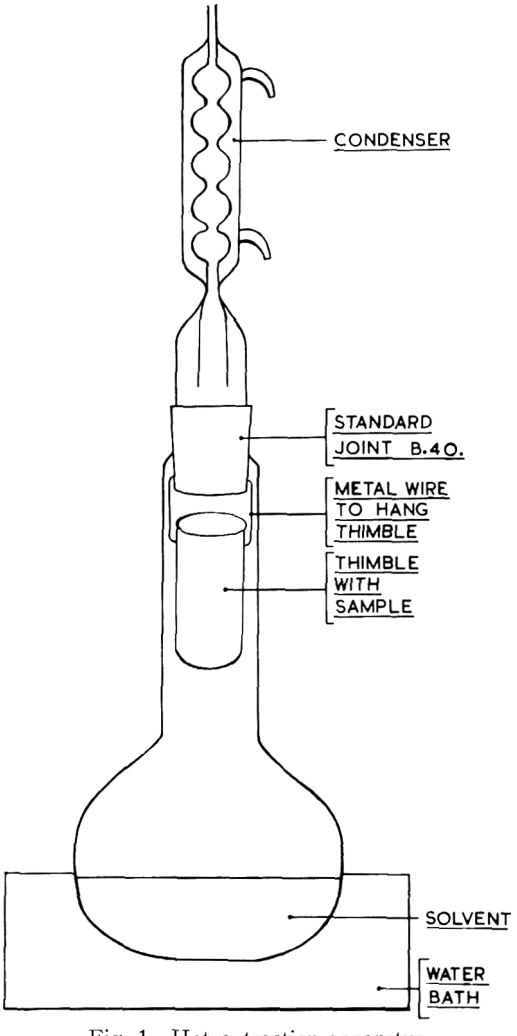

Fig. 1. Hot extraction apparatus.

for 24 hr over 3 days. Petroleum ether extractive ("true" lipids) was determined similarly with petroleum ether (40–60°C). "Bound" lipid was determined in a petroleum-ether-extracted sample after acid hydrolysis by the method given in AOAC (1960, p. 235). The non-lipid fraction of ethanol extractive was determined by removing the lipid fraction by petroleum ether (40–60°C). Pepsin digestibility was determined by the method given in AOAC (1960, p. 286). Available lysine was determined by the method of Carpenter (1960). Scoring for raw and cooked fishy odor was carried out as follows: strongly fishy, 5; fishy, 4–3; slightly fishy, 2; very slightly fishy, 1; doubtful, 0.5; and no fishy odor, 0.0.

Raw-odor and cooked-odor scores give judgment in a general way and should be considered as the indication of a trend.

For cooked-odor score, the meal was mixed with 10 times its weight of water and steam-cooked for 30 min, and samples in warm condition were judged.

RESULTS AND DISCUSSION

Effectiveness of extractives in deodorization. Cooked, pressed, dried meal, on exhaustive extraction with petroleum ether, gave a product with distinctly fishy odor—most of the odor being retained. The 95% ethanol, however, resulted in a product with practically no fishy odor. When cooked, these two samples were respectively fishy and slightly fishy (Table 1). Sample prepared by exhaustive extraction with petroleum ether had 5.4% residual ethanol extractive, 1.2% residual "bound" lipid, and 0.0% "true" lipids, and corresponding figures for samples exhaustively extracted with ethanol were 0.0, 0.37, and 0.0. It seems that a fraction of "bound" lipids is not removable with ethanol.

The general characterization of ethanol extractive (Table 2) indicated that about 48% of the total extractive was "true" lipids, i.e. extractable with petroleum ether;

Table 1. Residual petroleum ether and ethanol extractives, raw odor, and cooked odor of exhaustively extracted fish meal from Bombay-duck. Eviscerated, cooked, pressed and dried meal used for exhaustive extraction. Exhaustively extracted samples were vacuum-dried for 4 hr at 60°C and a vacuum of 29 inches Hg. Extraction done in Soxhlet apparatus for 24 hr in a period of 3 days.

Solvent for exhaustive extraction	% residual ethanol extractive[a]	% residual petroleum ether extractive[a]	% residual "bound" lipids[a]	Raw odor	Cooked odor
95% ethanol	0.0	0.0	0.37	No fishy odor (0.1)	Very slightly fishy (1.0)
Petroleum ether (40–60°C)	5.4	0.0	1.2	Distinctly fishy (3.5)	Fishy (3.4)

[a] Moisture-free basis.

Table 2. General characterization of ethanol extractives (for particulars of starting material, see Table 1).[a]

1) Ethanol extractives	10.94%	(100)
	11.09%	(100)
2) "True" lipids (petroleum ether, 40–60°C extractives)	4.84%	(47.6)
	5.28%	(47.6)
3) "True" lipids and "bound" lipids (petroleum ether 40–50°C) extractives of ethanol extractive	7.24	(66.4)
	7.35	(66.3)
4) "Bound" lipids	2.40[b]	(18.8)
	2.07[b]	(18.7)
	1.20[c]	(10.9)
5) Nonlipids	3.70	(33.7)
	3.74	(33.7)
	4.76	(43.2)

[a] Figures are on moisture-free basis.
[b] 3.2.
[c] Estimated after acid hydrolysis of fat-free samples.

"bound" lipid fraction was 11–19%. The remaining 34–43% was non-lipid—a white solid, sparingly soluble in ethanol but readily soluble in water, with a pungent seashore odor.

It seems that in Bombay-duck fish a substantial part of the odoriferous components are nonlipids and soluble in water. It further appears that fishy odor gets intensified on cooking. Sample exhaustively extracted with ethanol was practically without any fishy odor but was slightly (though distinctly) fishy when steam-cooked with water.

While carrying out investigations on the substance of fish smell, Obata *et al.* (1950) made similar observations.

Deodorization by batch extraction. The data on the successive batch-wise extraction of raw dried fish (Fig. 2) or cooked pressed dried meal (Fig. 3) with 95% ethanol indicate that in these cases, ethanol extractive is a good objective index of deodorization as judged by raw odor. Residual "true" lipid content was less than 0.2% after the second extraction, and cooked odor score showed little or no change after the fourth extraction. Even when "true" lipid content was less than 0.2% (after the second extraction) raw-odor score was 2.8–3.5 (fishy).

The removal of residual ethanol extractive and the extent of deodorization as judged

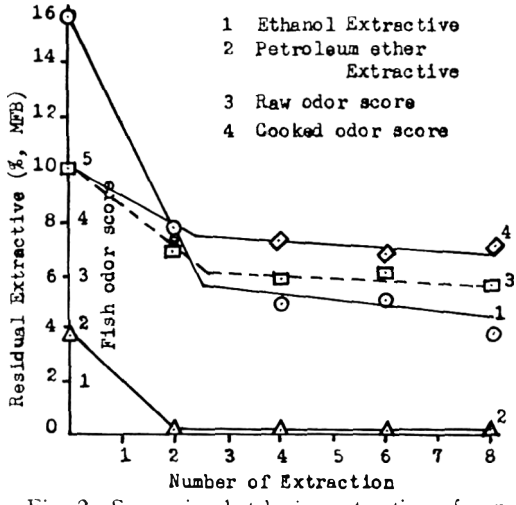

Fig. 2. Successive batchwise extraction of sun-dried fish with 95% ethanol.

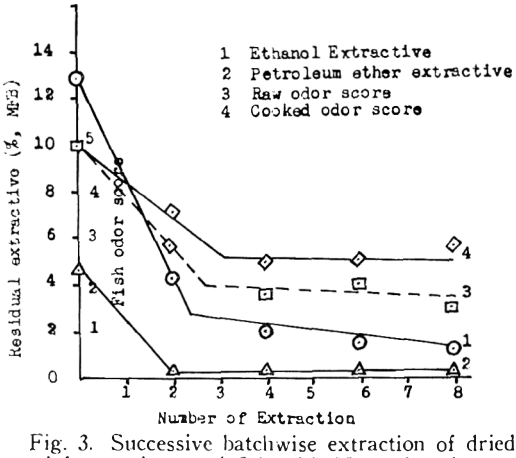

Fig. 3. Successive batchwise extraction of dried meal from eviscerated fish with 95% ethanol.

by raw odor followed two distinct phases: 1) quite rapid during the first three extractions; and 2) very slow beginning with the fourth.

The removal of ethanol extractive (and consequently deodorization) could be effected more rapidly in cooked pressed cake which had not been dried (Fig. 4). Since the non-lipid fraction of the ethanol extractive is soluble in water, the better performance of this case is due to the presence of water derived from press-cake. By this procedure, press-cake can be stored under alcohol till the operations of extraction are undertaken. It has been observed that press-cake with 84% moisture can be stored under

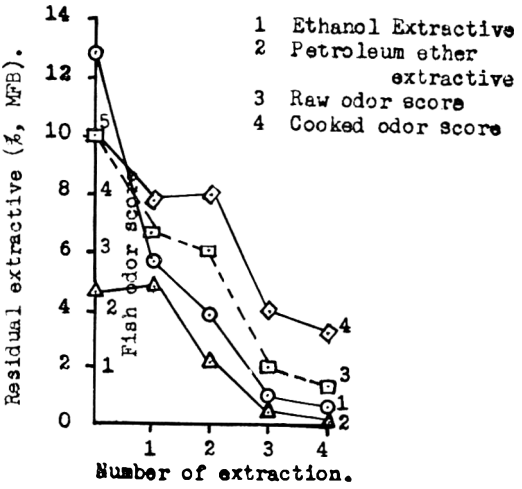

Fig. 4. Successive batchwise extraction of pressed cake from eviscerated fish with 95% ethanol.

alcohol (8 ml of 95% ethanol/g dry matter) for more than six months (Sen, 1964). The alcohol requirement in this case is 30.5 ml/g dry matter and can be cut down to 23 ml, is press cake is dried after first extraction and prior to subsequent extractions with 1:5 for meal to solvent. The product will be equally deodorized.

The efficacy of different solvents. The results obtained with 95% ethanol and cooked pressed dried meal have already been described (Fig. 3). Figs. 5 and 6 give results obtained with: a) hexane followed by 95% ethanol; and b) 80% ethanol. Diluted 80% ethanol was selected since the non-lipid fraction of ethanol extractive was found to be soluble in water.

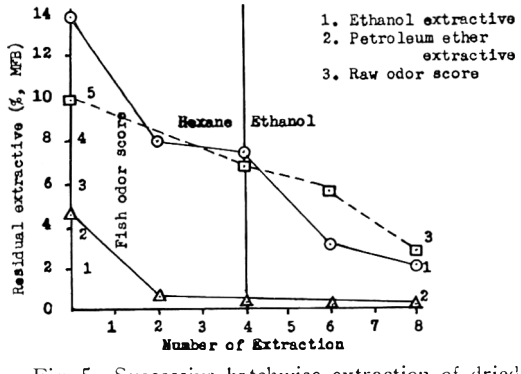

Fig. 5. Successive batchwise extraction of dried meal from eviscerated fish with hexane followed by 95% ethanol.

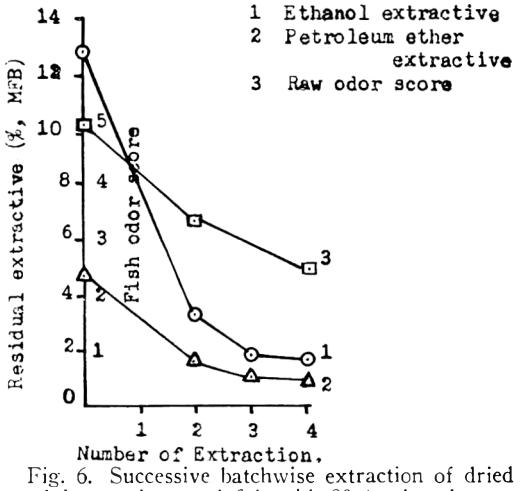

Fig. 6. Successive batchwise extraction of dried meal from eviscerated fish with 80% ethanol.

Using hexane prior to ethanol gave no additional advantage even from the standpoint of removal of "true" lipids, and, as expected, it was a poor solvent to remove odoriferous ethanol extractive. Pariser and Odland (1963), while working on defatting and deodorization of Chilean hake (merluzza), made a similar observation.

Eighty percent ethanol appeared to be superior to 95% ethanol in removing ethanol extractives other than "true" lipids, but was a poor solvent for the removal of "true" lipids. Raw odor score in these cases was higher than that of the corresponding sample extracted with 95% ethanol. The higher odor level might be due to higher "true" lipid contents.

Successive extracts in batch extraction. A study of different extracts obtained with 95% ethanol in batch extraction of cooked pressed dried meal indicated that the 1st, 2nd, 3rd, and 4th extracts respectively contained 1.76, 0.66, 0.27, and 0.17% solids, of which 53, 28, 15, and 12% were soluble in petroleum ether. It seems that the 3rd and 4th extracts can be conveniently utilized for the 1st and 2nd extractions of a subsequent batch.

Comparison of different methods of extraction. With cooked pressed dried meal, four procedures of extractions were compared: 1) batch extraction; 2) batch extraction following partial counter-current principle; 3) extraction by percolation method; and 4) continuous hot extraction.

Table 3. Fish protein concentrate by different procedures, quality of the products and requirement of solvent.

Sl. no.	Raw material	Solvent used	Raw material (MFB) (g to solvent, ml)	Method of extraction	No. of extraction or period of extraction	Residual		Raw odor (score)	95% ethanol (ml/g dry matter)
						Ethanol extractive %	Petroleum ether extractive %		
1.	Sun-dried eviscerated fish	95% ethanol	1 :5	Batch extraction	4	4.97	0.11	Fishy (3.0)	20.0
2.	Eviscerated cooked pressed dried meal	95% ethanol	1 :5	Batch extraction	4	1.82	0.15	Slightly fishy (1.8)	20.0
3.	Eviscerated cooked pressed dried meal	80 %ethanol	1 :5	Batch extraction	4	1.67	1.10	Fishy (2.5)	17.0
4.	Eviscerated cooked pressed dried meal	95% ethanol	1 :5	Batch extraction partial countercurrent	4	2.04	0.13	Slightly fishy (2.0)	10.0
5.	Eviscerated cooked press cake	95% ethanol	1 :8	first extraction	4	0.58	0.17	Doubtfully fishy (0.7)	30.5
			1 :7.5	subsequent extractions					
6.	Eviscerated cooked press cake	95% ethanol	1 :8	first extraction	4	0.51	0.10	Doubtfully fishy (0.8)	23.0
			1 :5*	subsequent extractions					
7.	Eviscerated cooked pressed dried meal	95% ethanol	—	Percolation method	5 hours	2.83	0.04	Slightly fishy (2.0)	13.27
8.	Eviscerated cooked pressed dried meal	95% ethanol	1 :6.5	Hot, continuous extraction	4 hours	1.26	0.15	Slightly fishy (2.0)	8.13

* Material dried after first extraction.

Table 4. Period of extraction and quality of the product in continuous hot extraction with 95% ethanol. Eviscerated cooked dried pulverized meal used; H_2O = 5.51%; ethanol extractive = 12.83%; and petroleum ether extractive = 4.56%. Rate of refluxing = 5.5 ml/min. Meal (g) :solvent (ml) = 1:65–7.0.

Expt. no.	Period of extraction (hr)	Equivalent number of extraction by batch process at 1:5	% residual petroleum ether extractive	% residual ethanol extractive	Raw odor (score)	Available lysine (g/100 g protein)	Pepsin digestibility (%)
1	2	5	2.19	0.13	Slightly fishy (2.0)	.....	.....
2	4	10	1.26	0.15	Slightly fishy (2.0)	9.08	99.5
3	8	20	0.46	0.06	Doubtfully fishy (0.7)	9.54	99.5

The solvent used was 95% ethanol. Results, showing the quality of different products and the alcohol requirements in each case, are given in Table 3. For comparison, certain data from Figs. 2, 3, 4, and 6 are incorporated in the table.

In batch extraction following the partial counter-current principle, the 3rd and 4th extracts of a previous batch were used for the 1st and 2nd extractions of a subsequent batch, and fresh alcohol for the 3rd and 4th extractions. By this method, a product with 2.04% residual alcohol extractive and 0.13% "true" lipid content could be obtained with 50% of the alcohol required by normal batch extraction to bring down residual alcohol extractive to 1.82%. A study of the extracts with respect to total solid contents and the fraction of it soluble in petroleum ether, in three successive partial counter-current extractions, indicated that reuse of the 3rd and 4th extracts could be repeated without affecting the quality of the product as judged by residual ethanol extractive.

In the percolation method, the quality of the product with respect to residual alcohol extractive and fishy odor was different in different layers. In the experiments described, about 66% of alcohol required in normal batch extraction process to bring down the residual alcohol extractive to 1.82%, could yield a product with 2.83% average residual alcohol extractive. No economy in alcohol can be achieved if extraction be carried out to the same extent (1.82% residual ethanol extractive) as obtained by four extractions in a batch process.

In continuous hot extraction, the meal was at the boiling temperature of the solvent during the entire period of extraction. To see whether the quality of protein is damaged by the prolonged exposure to high temperature, the samples extracted for 4 and 8 hr were analyzed as to available lysine and pepsin digestibility. It appears that protein quality is not damaged (Table 4). Of the total ethanol required in a batch extraction process to bring residual ethanol extractive down to 1.82%, 41% as much was required to yield a product with 1.26% residual ethanol extractive, the period of extraction being more or less same in both the cases.

Optimum period in batch extraction. Fig. 7 gives the solid percentage in extracts obtained with different periods of extraction. It appears that 60 min is necessary for the first extraction, and 15 min for subsequent extractions.

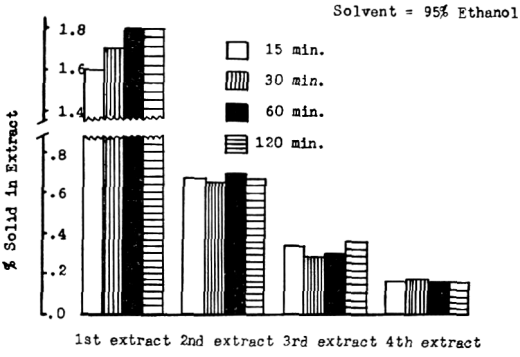

Fig. 7. Period of extraction and amount of solid extracted in batchwise extraction of dried meal.

REFERENCES

AOAC. 1960. Official methods of analysis. 9th ed. Assoc. of Offic. Agr. Chemists. p. 235, 286.

Carpenter, K. J. 1960. The estimation of available lysine in animal-protein foods. *Biochem. J.* **77**. 604.

Obata, Y., T. Yamanishi, and M. Ishida. 1950. Chemical studies on the substance of fish smell. II. Pyridine-group compounds as substances concerned with fishy smell. *Bull. Japan. Soc. Sci. Fisheries.* **15**. 551.

Pariser, E. R., and E. Odland. 1963. MIT-UNICEF studies on the production of fish protein concentrate for human consumption. *Comm. Fisheries Rev.* **25**(10), 6.

Sen, D. P. 1964. Central Food Technological Research Institute, Mysore-2, India (Unpublished data).

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Effects of pH and Temperature on the Carbonyls and Aromas Produced in Heated Amino Acid–Sugar Mixtures

SUMMARY

Solutions of glycine, glutamic acid, lysine, methionine, and phenylalanine adjusted to pH 6.5 were reacted separately with glucose, fructose, maltose, and sucrose at 100 and 180°C. Some of the aromas generated were reminiscent of foods, whereas others were distinctly unpleasant. Ethanal, propanal, acetone, isobutanal, acrolein, butanal, 2-butanone, 2,3-butanedione, crotanal, 2-pentanone, and heptanal were identified as end products in one or more of the 20 mixtures. The glutamic acid–glucose mixture was also heated at 100 and 180°C after being adjusted to pH 5.0 and 8.0. Of the compounds above, isobutanal and heptanal were not formed. Pentanal was formed at 100° but not at 180°C. A synthetic potato medium containing glucose and glutamic acid heated at 100°C yielded ethanal, propanal, acetone, butanal, 2-butanone, 2,3-butanedione, and crotonal. A potato extract heated at 180° differed from the synthetic potato medium heated at 100° in the products formed. Not formed were 2-butanone and 2,3-butanedione; pentanal, heptanal and hexen-1-al were formed. In all, 85 carbonyls were detected in the various mixtures or substrates.

INTRODUCTION

The reactions between individual amino acids and sugars have frequently been studied with reference to color changes, aroma, and taste (Beacham and Dull, 1951; Lewin, 1965; Lento *et al.*, 1960a,b; Herz and Shallenberger, 1960; Rohan and Stewart, 1965; Wiseblatt and Zoumut, 1963), but the identities of the compounds formed have not been as extensively studied. Some of them, being volatile, may logically be expected to affect the flavor of foods. The present work was undertaken to: 1) separate the products formed when sugars and amino acids in a model system were reacted under the influence of temperature; 2) study the effect of pH on the carbonyl compounds formed; and 3) evaluate organoleptically the aroma of the reaction products.

REVIEW OF LITERATURE

Browning occurs in both acidic and alkaline media. The rate of the reaction and the extent of browning increase greatly as the pH increases (Boretti, 1944; Wolfrom *et al.*, 1947; Haugaard *et al.*, 1951; Katchalsky and Sharon, 1953; Underwood *et al.*, 1959). The rate of browning also increases with an increase in temperature (Hendel *et al.*, 1955; Hannan and Lea, 1951; Lea *et al.*, 1951). Zabrodskii and Vitkovaskaya (1954) stated that increasing the temperature increased the quantity of sugars combining with the same quantity of amino acid.

EXPERIMENTAL

Selection of the sugars and amino acids. Selection of the sugars and amino acids used in the pure chemical phases was based on identification of sugars and amino acids present in fresh potato tubers. A water extract of Katahdin variety of potato was used for analysis. A unidimensional chromatographic technique, employing 1-butanol–acetic acid–water (BAW) (4:1:5 v/v) and 80% phenol in water (4:1) was used for the identification of sugars and amino acids. The dry chromatograms were sprayed with 0.32% anisidine to identify sugars and 0.25% ninhydrin in acetone to spot amino acids, followed by heating for 3–5 min in hot-air ovens for development of the color.

Of the amino acids identified, glycine, glutamic acid, lysine, phenylalanine, and methionine were separately reacted with the sugars identified (glucose, fructose, maltose, and sucrose). The amino acids selected were chosen to represent five major classes of amino acids: acidic, basic, neutral, aromatic, and sulfur-containing.

Collection of carbonyl compounds. The reaction between each sugar and amino acid was carried out at 100 and 180°C. The sugars and amino acids (0.2*M* each) were dissolved in 100 ml of 0.028*M* phosphate buffer at pH 6.5. The flasks containing the solution of sugar and amino acid were heated for 60 min in a water bath at 100°C. Each flask was connected to another flask containing dinitrophenylhydrazine (DNPH) reagent. These connections were kept airtight to ensure that all the volatiles bubbled into the reagent. At the end of the heating time, the flasks were removed and cooled to room temperature and poured in the reagent flask to collect traces of carbonyls still in the reaction mixture. The DNPH derivatives were collected according to the procedure of Dornseifer and Powers (1963, 1965), filtered

through Whatman No. 2 filter paper, washed thoroughly with distilled water, and dried in a desiccator containing phosphopentoxide as a desiccant.

For the reaction at 180°C, the sugar and amino acid solution was placed in a special tube capable of withstanding high pressure. The tube was made of a borosilicate glass with an approximate pressure tolerance of 325 psi. A hypodermic syringe inserted through a rubber septum was used to fill the tubes to prevent the loss of solution from back pressure generated by air bubbles. The tubes were connected to a vacuum pump. After complete evacuation was obtained, the tubes were sealed in an oxygen flame and heated in an oil bath for 30 min. At the end of the heating period, the tubes were removed and rapidly cooled to room temperature, and the solutions were poured into DNPH reagent and kept overnight. The crystals were collected and dried as described above.

Effect of pH on the carbonyl compounds generated. Glucose and glutamic acid solutions were prepared as before. Two 100-ml portions were adjusted to pH 5.0, 6.5, and 8.0. One flask at each pH was heated for 60 min at 100°C. The second 100-ml portions were also heated at each pH level, but at 180°C in the borosilicate tubes. The carbonyl compounds were collected as described above.

Generation of carbonyl compounds in potato media. A synthetic potato medium was formulated as closely as possible to diced frozen potatoes (Watt and Merrill, 1963). The mixture was placed in a round-bottom flask and connected to a series of traps. The first two traps contained diluted HCl, and the last three traps DNPH reagent. The mixture was heated 60 min at 100°C. Nitrogen gas was bubbled through the mixture to carry the carbonyls formed to the traps containing the DNPH reagent.

A potato extract was prepared by pureeing a small amount of water containing 130 ppm sodium bisulfite with diced potatoes, followed by filtration under vacuum with a Buchner funnel and Whatman No. 2 filter paper. This extract was heated for 30 min at 180°C. The carbonyl compounds were collected and treated as previously described.

Regeneration of the carbonyl compounds. The method of Ralls (1960) as modified by Dornseifer and Powers (1963, 1965) was used to regenerate the carbonyls. The vapors generated in the tube were withdrawn with a gas-tight syringe and injected directly into the gas chromatograph. An F & M gas chromatograph, model 609, with a flame ionization detector was used. The best of several columns tried were two 10-ft columns, one consisting of 15% butanediol succinate on Diatoport P, and the second of 20% polyethylene glycol 600 on firebrick. The column temperature was 75°C; nitrogen gas flow rate, 57 ml/min; sensitivity, range 1; and attenuation either 15 or 32.

RESULTS AND DISCUSSION

Sugars and amino acids present in potatoes. Identified as being present in the water extract of the Katahdin potatoes were glucose, fructose, maltose, sucrose, glycine, aspartic acid, glutamic acid, proline, lysine, serine, histidine, methionine, valine, and phenylalanine (Table 1).

Habib and Brown (1957) found glucose, fructose, maltose, and sucrose in the Katahdin potatoes they examined; Schwimmer *et al.* (1954) also reported that potatoes contain glucose, fructose, and sucrose, but did not detect the presence of maltose.

Slack (1948), Hirsch *et al.* (1952), Katayama *et al.* (1956), and Furuholmen *et al.* (1964) found arginine, cystine, isoleucine, threonine, tyrosine, and tryptophan, which we did not. The differences are probably a result of the variations in variety of potato examined and of cultural or storage conditions. The composition of potato tubers undergoes changes of varying degrees from year to year, and conditioning of the tubers results in some difference in amino acid content of potatoes, particularly the basic ones (Habib and Brown, 1957).

Table 1. The R_f values of individual sugars and amino acids in the water extract of Katahdin potato.

Compounds	Solvent	
	BAW[a]	PW[b]
Sugars		
Fructose	0.21	0.55
Glucose	0.17	0.40
Maltose	0.10	0.33
Sucrose	0.12	0.37
Amino acids		
Aspartic	0.16	0.19
Glycine	0.19	0.41
Glutamic	0.26	0.31
Proline	0.38	0.90
Lysine	0.45	0.80
Serine	.....	0.40
Histidine	0.18	0.70
Methionine	0.53	0.79
Valine	0.39	0.73
Phenylalanine	....	0.90

[a] BAW = Butanol-acetic acid-water solvent.
[b] PW = Phenol-water solvent.

Table 2. Aromas generated at pH 6.5 by the reaction of various sugars and various amino acids at 100 and 180°C.

	Glycine		Glutamic		Lysine		Methionine		Phenylalanine	
	Aroma	Color	Aroma	Color	Aroma	Color	Aroma	Color	Aroma	Color
At 100°C										
Glucose	Caramelized sugar NO	DB	Old wood small P	Y	Baked sweet potato NO	DB	Overcooked sweet potato NO	B	Rancid caramel candy UP	DY
Fructose	Caramel smell UP	DR	Too weak to describe	YO	Frying butter O	O	Chopped cabbage O	Y	Stinging smell VO	LY
Maltose	Too weak to describe	YO	Too weak to describe	YO	Burned wet wood UP	YO	Overcooked cabbage O	YO	Sweet caramel P	YO
Sucrose	Weak NH_3 O	LY	Caramel smell P	LY	Rotten raw potato UP	LY	Burned wood smell UP	LY	Sweet caramel smell UP	LY
At 180°C										
Glucose	Burned candy NO	DB	Chicken tray smell O	DB	Burned fried potato NO	DB	Cabbage smell O	DB	Caramel smell NO	DB
Fructose	Beef broth P	DR	Chicken manure O	DR	Fried potato NO	DR	Bean soup smell NO	BR	Dirty dog smell VO	DR
Maltose	Beef broth NO	DR	Baked ham NO	DR	Stale potato O	DR	Harsh horseradish UP	OR	Sweet caramel P	YO
Sucrose	Beef broth NO	DR	Charred meat NO	DR	Boiled meat P	DR	Overcooked cabbage VO	BR	Chocolate smell P	BR

ABBR: P = pleasant; O = objectionable; NO = not objectionable; UP = unpleasant; and VO = very objectionable; Y = yellow; O = orange, B = brown; BR = black red; DR = dark red; DB = dark brown; LY = light yellow; OR = orange red; and YO = yellow orange.

Sensory evaluation of aromas generated. Table 2 summarizes the sensory evaluation of the aromas evolved from reacting sugars and amino acids at 100 and 180°C. The pH of all the mixtures was 6.5. The aroma was described by 3 judges with previous experience in evaluating the aroma of foods and chemical mixtures. As anticipated, various odors were produced. The aromas developed when sugars and amino acids were heated at 180°C were different from those produced at 100°C. The aromas produced by the interaction of sugars and amino acids ranged from pleasant to objectionable. Glutamic acid gave a meaty-type odor with the majority of the sugars examined. Methionine was always associated with a sharp and distinctive odor which resembled overcooked cabbage, especially at the higher temperature. This may be attributed to the sulfur compounds released during the reaction. The volatile odors emitted from the reaction of lysine and the sugars were associated with a potato smell. The aroma given off by phenylalanine was described, in most cases, as sweet caramel.

Relative reactivity of individual sugars and amino acids. The amino acids varied greatly in the ease with which they reacted with each sugar. In general, glycine showed the greatest and phenylalanine the least reactivity toward the four sugars used. When the weight of the DNPH crystalline derivatives was considered as a criterion for reactivity, sucrose showed the greatest reactivity, followed by glucose, fructose, and maltose (Table 3). When browning was considered the measure of reactivity, maltose and sucrose were less reactive than glucose and fructose, regardless of the amino acids. The amount of browning varied widely as influenced by temperature, type and the chain length of the amino acid, and kind of sugar present. Hydroxylation of the C-2 position of an aldose is essential for a significant degree of browning (Haugaard *et al.*, 1951; Wolfrom *et al.*, 1947). The Amadori rearrangement is a key reaction for browning (Hodge, 1953). These, coupled with the fact that an elapse of time is required for hydrolysis to occur, might account for the low intensity of browning of the disaccharides. Lysine produced the greatest increase in color formation, followed by glycine, methionine, glutamic acid, and phenylalanine.

Volatile carbonyls formed. Table 4 shows the volatile carbonyls obtained from the reaction of the sugars at 100 and 180°C with the five amino acids. The aldehydes and ketones were identified tentatively by comparing their relative retention times with those of authentic carbonyls on two columns.

A total of 85 compounds were detected. Identified in the 20 combinations of the four sugars and five amino acids were ethanal, propanal, isobutanal, butanal, acrolein, crotonal, heptanal, acetone, 2-butanone, 2,3-butanedione, and 2-pentanone. Because of the unavailability of many authentic carbonyls, the identity of the other compounds could not be determined.

The number of peaks on both columns was significantly greater when the reaction was made to occur at 180°C as compared with 100°C. The volatiles formed from the reactions between the four sugars and methionine at 180°C could not be identified. The instrument did not respond properly when the volatiles from these reactions were injected. In every attempt to resolve them, drift in the base line to the upper part of the chart occurred, apparently from a poisoning effect of the sulfur compounds on the detector.

Table 3. Weight in grams of DPNH derivatives collected from 100 ml of solution at 100 and 180°C.

	Glucose		Fructose		Maltose		Sucrose	
Amino Acid	100°C	180°C	100°C	180°C	100°C	180°C	100°C	180°C
Glycine	.....	0.7010	0.3248	0.8090	.....	1.314	0.5499	1.3880
Glutamic acid	.....	0.7780	0.2396	0.6500	0.1490	0.5395	0.4650	1.2048
Lysine	.....	0.4185	0.3752	0.4196	0.2258	0.9240	0.3825	0.9420
Methionine	.....	0.9776	0.2862	1.3462	0.2392	.....	0.3365	.....
Phenylalanine	0.2707	0.6560	0.1503	0.5640	0.2300	0.7970	0.3285	0.9594

Table 4. Volatile carbonyl compounds formed by reacting various sugars and amino acids at pH 6.5 at 100 and 180°C.

	Glucose					Fructose					Maltose					Sucrose				
	Gly.	Glut.	Lys.	Meth.	Phy.	Gly.	Glut.	Lys.	Meth.	Phy.	Gly.	Glut.	Lys.	Meth.	Phy.	Gly.	Glut.	Lys.	Meth.	Phy.
At 100°C																				
Ethanal	+	+	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Propanal	+	+	+	+	+	(+)	(+)	+	+	+	+	(+)	(+)	+	(+)	+	+	+	+	+
Acetone	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Isobutanal	−	−	−	−	−	−	(+)	−	−	−	−	−	−	−	−	(+)	(+)	−	−	−
Acrolein	+	+	+	(+)	(+)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Butanal	−	(+)	(+)	+	−	−	−	−	−	−	−	−	−	(+)	−	−	−	−	−	−
2-Butanone	+	+	+	(+)	+	(+)	(+)	+	+	+	+	+	+	+	+	+	+	+	+	+
2,3-Butanedione	+	(+)	+	+	+	(+)	(+)	+	(+)	+	+	(+)	+	+	(+)	+	+	+	+	+
Crotanal	+	−	−	(+)	−	−	−	−	+	(+)	(+)	−	−	+	−	−	−	(+)	+	(+)
2-Pentanone	−	−	−	−	−	(+)	(+)	−	−	−	−	−	−	−	−	−	−	−	−	−
At 180°C																				
Ethanal	−	−	−		−	+	+	+		+	+	+	+		+	+	(+)	+		(+)
Propanal	+	+	+		+	+	+	+		+	+	+	+		+	+	+	+		+
Acetone	+	+	+		+	−	−	−		−	+	+	+		(+)	+	+	+		(+)
Isobutanal	−	−	−		−	+	+	+		+	(+)	(+)	(+)		(+)	−	−	−		−
Acrolein	−	−	−		−	(+)	(+)	−		−	−	−	−		−	−	−	−		−
Butanal	+	+	+		+	(+)	−	(+)		−	−	+	+		(+)	+	+	+		+
2-Butanone	+	+	+		(+)	(+)	+	(+)		+	+	(+)	+		+	(+)	+	−		+
2,3-Butanedione	−	−	−		(+)	(+)	(+)	(+)		+	−	−	−		−	(+)	(+)	+		(+)
Crotonal	+	(+)	+		+	(+)	+	+		+	(+)	+	+		+	−	−	−		(+)
2-Pentanone	+	(+)	+		+	(+)	(+)	(+)		(+)	(+)	(+)	+		+	−	(+)	−		+
Heptanal	−	−	(+)		(+)	−	−	−		(+)	−	−	−		−	−	−	−		−

+ Detected on two columns; (+) Detected on one column; − Not detected. Gly = glycine; Glut = glutamic acid; Lys = lysine; Meth = methionine; Phy = phenylalanine.

Table 5. Volatile carbonyl compounds formed by reacting glucose and glutamic acid at 100 and 180°C at pH 5.0, 6.5, and 8.0.

Compound	At 100°C at pH:			At 180°C at pH:		
	5.0	6.5	8.0	5.0	6.5	8.0
Ethanal	+	+	+	+	−	+
Propanal	+	+	+	+	+	+
Acetone	+	−	+	(+)	+	+
Acrolein	−	+	−	(+)	−	(+)
Butanal	−	(+)	−	+	+	(+)
2-Butanone	+	+	+	+	+	+
2,3-Butanedione	+	(+)	+	+	−	+
Pentanal	+	−	+	−	−	−
Crotonal	−	−	(+)	(+)	(+)	(+)
2-Pentanone	−	−	−	−	(+)	−

\+ Appeared on two columns
(+) Appeared on one column
− Not detected

Effect of pH on the volatile carbonyls formed. Table 5 shows the volatile carbonyls generated by the reaction of glucose and glutamic acid at pH 5.0, 6.5, and 8.0 at 100 and 180°C. The amount of browning produced at 100°C increased moderately as the pH increased toward the alkaline range. At 180°C, the respective colors for pH 5.0, 6.5, and 8.0 were orange, deep red, and black. The number of carbonyl compounds produced by the glucose–glutamic acid system at the three pH levels was almost equal at 100°C. When the systems were heated at 180°C, the number of volatiles was greater and increased more drastically as pH increased.

Volatile carbonyls generated in the potato substrates. Table 6 shows the volatile carbonyls formed when the synthetic potato medium was heated at 100°C and the potato extract at 180°C. The difference in the heat treatment was due to the fact that heating the synthetic potato medium at 180°C formed a caramelized mass devoid of liquid. The potato extract was heated at 180°C to simulate the process of frying potato chips. The volatiles isolated confirmed that the sugars and amino acids reacted in mixed systems and that many of the same carbonyls were formed.

For the potato substrates as for the amino acid–sugar mixtures, the number of carbonyls formed increased as the temperature was raised. As already pointed out, the total yield of carbonyls was greater at 180 than at 100°C (Table 3), and browning was greater at the higher temperature. Hodge (1953) has reviewed possible browning mechanisms and end products formed. This study was not designed to elucidate the mechanism of carbonyl formation. However, since all the various amino acid–sugar combinations tended to yield carbonyls differing by only one carbon length, breakdown of the compounds appears to have been a step-wise degradation. The Wohl degradation or thermal decarboxylation (Roberts and Caserio, 1964) might account for the decrease of chain length by one unit. The data suggest that coupling of compounds might have also occurred. Lysine and phenylalanine with glucose or fructose yielded heptanal (Table 4). Of the four compounds, none of the initial reactants contained over 6 carbon units except phenylalanine. An aldol condensation could account for the increase in chain length.

Even though only 14 carbonyls were identified, 85 were detected as judged by gas chromatographic retention times. Some of the carbonyls identified are fairly odoriferous; the others are quite mild. The odors listed in Table 2 probably did not come from the carbonyls alone. In the heating of the amino acid–sugar mixture, noncarbonyl compounds very likely were formed

Table 6. Volatile carbonyl compounds generated upon heating a synthetic potato medium for 60 min at 100°C and a potato extract for 30 min at 180°C.

Carbonyl compound	Synthetic potato medium	Potato extract
Ethanal	+	+
Propanal	+	+
Acetone	+	−
Isobutanal	−	−
Acrolein	−	−
Butanal	+	+
2-Butanone	+	−
2,3-Butanedione	+	−
Crotonal	+	+
2-Pentanone	−	−
Pentanal	−	+
Heptanal	−	+
Hexen-1-al	−	+

A plus sign shows the compound was present, a minus sign shows it was absent.

and they may have contributed to the odors described by the three judges. The fact that particular types of odors were produced by the amino acid–sugar mixtures is important, both basically and for applied reasons. That there are differences in the odor produced by different combinations has been known for some time. This study is important as a first step in relating the composition of the volatiles to odor differences and conditions which result in the generation of odor.

REFERENCES

Beacham, J. J., and M. F. Dull. 1951. Some observations on the browning reaction. *Food Research* **16**, 439.

Boretti, G. 1944. Reaction between amino acids and carbohydrates. *Chem. ind.* (Milan) **26**, 31. *Chem. Abstr.* **40**, 6424.

Dornseifer, T. P., and J. J. Powers. 1963. Change in the volatile carbonyls of potato chips during storage. *Food Technol.* **17**, 1330.

Dornseifer, T. P., and J. J. Powers. 1965. Volatile constituents of potato chips and changes during storage. *Food Technol.* **19**, 195.

Furuholmen, A. M., J. D. Winefordner, R. A. Dennison, and F. W. Knapp. 1964a. Isolation, concentration, separation, and identification of amino acids in potatoes by ion exchange and paper chromatography. *J. Agr. Food Chem.* **12**, 112.

Furuholmen, A. M., J. D. Winefordner, F. W. Knapp, and R. D. Dennison. 1964b. Quantitative analysis of glucose and fructose in potatoes. *J. Agr. Food Chem.* **12**, 109.

Habib, A. T., and H. D. Brown. 1957. Role of reducing sugar and amino acids in the browning of potato chips. *Food Technol.* **11**, 85.

Hannan, R. S., and C. H. Lea. 1951. Reaction between glucose and terminal amino group of lysine. *Nature* **168**, 744.

Haugaard, G. L., L. Turmerman, and H. Silvestri. 1951. A study on the reaction of aldoses and amino acids. *J. Am. Chem. Soc.* **73**, 4594.

Hendel, C. E., V. G. Silveira, and W. O. Harrington. 1955. Rates of non-enzymatic browning of white potato during dehydration. *Food Technol.* **9**, 433.

Herz, W. J., and R. S. Shallenberger. 1960. Some aromas produced by simple amino acid–sugar reactions. *Food Research* **25**, 491.

Hirsch, S. J., A. D. Niles, and A. R. Kimmerer. 1952. The essential amino acid content of several vegetables. *Food Research* **17**, 442.

Hodge, J. E. 1953. Dehydrated foods. Chemistry of browning reaction in model system. *J. Agr. Food Chem.* **1**, 928.

Katayama, O., E. Nomoto, and H. Shioiri. 1956. Free amino acids in vegetables. *Nosan Kako Gejutsu Kaishi* **3**, 10 *Chem. Abstr.* **53**, 9510d.

Katchalsky, A., and N. Sharon. 1953. Kinetics of aldose-amino acid interaction. *Biochim, et Biophys. Acta.* **10**, 290.

Lea, C. H., R. S. Hannan, and D. N. Rhodes. 1951. Studies of the reaction between proteins and reducing sugars in the dry state. IV. Decomposition of the amino-sugar complex and the reaction of acetylated casein with glucose. *Biochim. et Biophys. Acta.* **7**, 366.

Lento, H. G., Jr., J. C. Underwood, and C. O. Willits. 1960a. Browning of sugar solutions. IV. The effect of pH on the steam volatile decomposition products of reducing sugars. *Food Research* **25**, 750.

Lento, H. G., Jr., J. C. Underwood, and C. O. Willits. 1960b. Browning of sugar solutions. V. Effect of pH on the browning of triose. *Food Research* **25**, 757.

Lewin, S. 1957. Some aspects of the effect of the initial pH on the behavior of glucose in the amino acid-glucose interactions. *Biochem. J.* **65**, 30.

Ralls, J. W. 1960. Rapid method of semi-quantitative determination of volatile aldehydes, ketones, and acids. Flash exchange gas chromatography. *Anal. Chem.* **32**, *332.*

Roberts, J. D., and M. C. Caserio. 1964. Basic Principles of Organic Chemistry. W. A. Benjamin, Inc., New York.

Rohan, T. A., and T. Stewart. 1965. The precursors of chocolate aroma: the distribution of free amino acids in different commercial varieties of cocoa beans. *J. Food Sci.* **30**, 416.

Schwimmer, S., A. Bevenue, W. J. Weston, and A. L. Potter. 1954. Potato composition. Survey of major and minor sugar and starch components of the white potato. *J. Agr. Food Chem.* **2**, 1284.

Slack, E. B. 1948. Nitrogenous constituents of the potato. *Nature* **161**, 211.

Underwood, J. C., H. G. Lento, Jr., and C. O. Willits. 1959. Browning of sugar solutions. II. Effect of pH on the color produced in dilute glucose solutions containing amino acids with amino groups in different positions in the molecule. *Food Research* **24**, 181.

Watt, B. K., and L. Merrill. 1963. Composition of foods. Agriculture Handbook No. 8. U. S. Department of Agriculture, Washington, D. C.

Wiseblatt, L., and H. F. Zoumut. 1963. Isolation of a bread flavor constituent. *Cereal Chem.* **40**, 162.

Wolfrom, M. L., L. F. Cavalieri, and D. K. Cavalieri. 1947. Chemical interaction of amino

compounds and sugars. II. Methylation experiment. *J. Am. Chem. Soc.* **69**, 2411.

Zabrodskii, A. G., and V. A. Vitkovskaya. 1954. Conditions affecting the course of reaction taking place between amino acids and sugars. Biokhimiya **19**, 738. Cf. *Chem. Abstr.* **49**, 6337.

Ms. rec'd 8/2/65.

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Analysis for Stereoisomers of Beta-Carotene in Fermentation Preparations

SUMMARY

The potential vitamin A activity of a carotene-containing preparation obtained from mated cultures of *Blakeslea trispora* was investigated by determining the stereoisomers of the β-carotene fraction. The β-carotene extracted from some fresh mycelia contained about 94% of the all-*trans* isomer, the remainder being about 3.5% of neo-β-carotene B and 2.5% of neo-β-carotene U. β-Carotene from stored dried preparations showed about 91% of all-*trans*, 7.5% of neo-β-carotene B, and 1.5% of neo-β-carotene U.

INTRODUCTION

Carotenoid compounds are used in foods and animal feedstuffs and have potential for expanded consumption (Bunnell and Bauernfeind, 1962). Carotene preparations are obtained commercially by chemical synthesis (Isler *et al.*, 1958) and from carrots and palm oil (Pazola and Wenta, 1957; Mosher, 1961). However, a new potentially economical process for carotene production by fermentation utilizing mated cultures of the mold *Blakeslea trispora* has been developed (Anderson *et al.*, 1958; Ciegler *et al.*, 1961, 1962, 1963a,b, 1964).

Because carotene produced by fermentation may be used as a vitamin A source for foods and feedstuffs, the true biological activity of the carotenoids produced is important. The biological value of individual carotenes depends on their stereoisomeric configuration as well as on their basic (carbon-skeleton) chemical constitution. Vitamin A activity of the most important carotenes in the rat is as follows (Bickoff, 1957):

All-*trans*-β-carotene	100% (1,670,000 IU/g)
Neo-β-carotene U	38%
Neo-β-carotene B	53%
All-*trans*-α-carotene	53%
Neo-α-carotene U	13%
Neo-α-carotene B	16%
All-*trans*-γ-carotene	27%

To obtain more exact values of vitamin A potency of the carotene product from the fermentation process, we estimated the total β-carotene and the all-*trans*-β-carotene content of both fresh and dried mycelium.

EXPERIMENTAL METHODS AND RESULTS

Most of the analyses were carried out in a semi-dark room illuminated by red fluorescent bulbs. Illumination at bench top level was 1–4 ft-c or less; since most of this light was at the low-actinic end of the spectrum, photolabile carotenoids were well protected. The carotenoids, which are red, yellow, or orange, were not visible on the columns under the red light; hence, columns had to be examined momentarily with flashlights, desk lamps, or subdued daylight. Although these exposures were kept to the practical minimum, it is not possible to rule out completely some actinic effect. Before the dark room was constructed, earlier work had been done under less protective conditions. Although the column work for the earlier experiments was carried out in subdued light (1 ft-c or less) the extractions preceding the chromatographic steps often had to be conducted in a normally illuminated room (*ca.* 10 ft-c). Some early results are given for com-

Table 1. Stereoisomer content of various fermentation β-carotene preparations.

Preparation	Total carotenoids (mg/g mycelium)	β-Carotene (% of total carotenoid)	Neo-β-Carotene B (% of β-carotene)	All-*trans* β-Carotene (% of β-carotene)	Neo-β-Carotene U (% of β-carotene
1. Fresh	13.8	78	3.4	94	2.6
2. Dried	11.8	85	9.4	87.3	3.3
3. Fresh	11.0	86	7.4	88	4.6
4. Dried (composite)[a]	15.4	88	7.3	91.1	1.6

[a] Stored in freezer; occasionally removed for sampling.

parison (Table 1, line 4); they do not differ much from the values obtained later under the more protective conditions.

It must also be recognized that, even with the precautions described, some isomerization occurs when carotenes are put into solution. Approximately 95% of β-carotene is present as the three isomers all-*trans*, neo-B, and neo-U in equilibrium solution at 25°C (Bickoff, 1957; Bickoff *et al.*, 1949; Polgár and Zechmeister, 1942).

The amounts of β-carotene were determined by reading appropriate dilutions in a Beckman B spectrophotometer and computing from a standard curve, prepared by measuring the absorbance of dilutions of a freshly opened ampoule of all-*trans*-β-carotene from a commercial source. Identity of the stereoisomers was judged by their position on calcium hydroxide columns and confirmed by spectrogram traces on a recording Beckman DB spectrophotometer. Efficiency of recovery from the $Ca(OH)_2$ columns was estimated by comparing the sum of the absorbances of the individual isomers at 450 mμ after chromatography with the absorbance of the mixure at 450 mμ before chromatography. Correction factors were applied to the absorbances of the neo-B and the neo-U fractions. These factors are the ratios of the absorptivity of the all-*trans* form at 450 mμ to the absorptivities of the respective isomers at that wave length, as estimated from the curves (Bickhoff *et al.*, 1948), i.e. 250:176 for neo-B and 250:230 for neo-U.

Cultural methods for microbiological production of β-carotene have been described in previous publications (Ciegler *et al.*, *op. cit.*). Five 100-ml shaken-flask cultures of *Blakeslea trispora* were harvested after 6 days' incubation and filtered onto cheesecloth. The combined mycelia (wet weight *ca.* 100 g) were twice macerated in a Waring blender with 600 ml of methanol:acetic acid (50:50 v/v) and filtered on a Büchner funnel; the filter cake was washed both times with low-boiling petroleum ether (hereinafter referred to as pentane-hexane). Since the mycelium was still colored, it was extracted twice more on a magnetic mixer with 10% acetone-pentane-hexane and finally five times more with pentane-hexane. Water-miscible solvents were removed from the extracts by repeated aqueous washings. Emulsions were broken by salting out with sodium sulfate and by centrifugation. The combined epiphases were concentrated *in vacuo* to 2720 ml. The concentrate contained 149 μg/ml of pigment calculated as all-*trans*-β-carotene. Fifteen ml were put on a Sea-sorb:Hyflo-supercel (1:1) column and developed with 10% acetone-pentane-hexane. (Sea-sorb is an activated magnesium oxide; Hyflo-supercel is a refined diatomaceous earth.) After the rapidly moving orange β-carotene band was eluted (no α-carotene was detected) it contained 117 μg/ml of β-carotene on the basis of the original volume of 15 ml. Hence, the total pigment extract contained 117/149 or 78% β-carotene. The β-carotene solution was evaporated *in vacuo* to dryness and 36 ml of high-boiling (87–98°C) petroleum ether (hereinafter referred to as heptane) was immediately added to the dried material ahead of the incoming air. The heptane solution contained 44.25 μg/ml of β-carotene. One ml of the heptane solution was then added to each of five 1× 10 cm columns of new, freshly opened calcium hydroxide (Mallinckrodt's U.S.P. grade) and developed according to the method of Bickoff (Bickoff *et al.*, 1948, 1949; Bickoff, 1957) with 1½% (v/v) of p-methylanisole in heptane. The effluents were read in the spectrophotometer; results are summarized in Table 1, line 1.

Five more shaken-flask cultures, grown at the same time as those just described, were filtered on cheesecloth in the same way, but the mycelial pads were dried overnight in a vacuum oven at 60°C. The mycelium (average dry weight 5.9 g) was ground to 20-mesh; 200 mg of each preparation were put on a column similar to those above and extracted directly into the adsorbent with 10% acetone-pentane-hexane. The procedure from this point on was identical to that given above. The average results are given in Table 1, line *2*.

In another experiment, four 6-day-old cultures were harvested as before and filtered through cheesecloth. The mycelial pads were combined for wet extraction which was carried out by macerating the mycelium in the blender for 3 min in 300 ml ethanol + 225 ml pentane-hexane. A second extraction was made with 500 ml of ethanol:acetone (1:1) and a third with 500 ml of methanol. A reddish pigment was left in the cells, and an attempt was made to remove this with a blender treatment in 500 ml ethanol:acetic acid (1:1 by vol.). This left some red color, so sonification was resorted to, with methanol as the suspending medium. After addition of pentane-hexane and water, followed by centrifugation, the cells still had a reddish pigment. It was believed, however, that substantially all the β-carotene had been extracted, so all extracts were combined, diluted with pentane-hexane, and washed free of water-miscible solvents with 20% NaCl solution and then repeatedly with water. The pentane-hexane solution (1620 ml, 159 μg total pigment per ml) was treated on a Sea-sorb column as before. Fifteen ml of influent (equivalent to *ca.* 2385 μg total pigment) yielded a fraction containing 2050 μg β-carotene (86% of total pigment). It was evaporated under N_2, made up to 24 ml with heptane, and analyzed on 5 $Ca(OH)_2$ columns as before. The average recovery from the

columns was 97%, and the isomer composition is shown in Table 1, line 3. The earlier analyses mentioned above, averaged in Table 1, line 4, were done on composite dried material, stored in a freezer, but occasionally taken out and exposed to light and air at room temperature.

DISCUSSION

The results of these and other analyses indicate that the two principal neo-β-carotenes are found in the fresh mycelium, although the possibility is not completely excluded that only the all-*trans* isomer is present in the living organism and that isomerization to the neo-β-carotenes takes place almost immediately during extraction. Irradiation with visible light tends to decrease the content of *cis* isomers, whereas heating promotes the formation of these isomers (Thompson *et al.*, 1951). We have studied the effects of ordinary temperature and lighting on the total carotenoid content of the fermentation product (Ciegler *et al.*, 1961) but not on its stereoisomer content.

In considering the vitamin A value of the fermentation product, we calculate as follows, using Bickoff's table (see Introduction) for the β-carotene in our better dried material (Table 1, line 4):

100%	× 91.0%	× 1,670,000	=	1,520,000 IU/g
53%	× 7.3%	× 1,670,000	=	65,000 IU/g
38%	× 1.6%	× 1,670,000	=	1,000 IU/g
		Total		1,586,000 IU/g

Hence our product has about 95% of the theoretical vitamin A value for all-*trans*-β-carotene. This product, having been stored and exposed occasionally to light and air, represents somewhat more nearly the conditions that a commercial preparation to be used in feeds would encounter than do the other preparations above. Carotene could be extracted from fresh mycelium for use as a food additive.

REFERENCES

Anderson, R. F., M. Arnold, G. E. N. Nelson, and A. Ciegler. 1958. Microbiological production of *beta*-carotene in shaken flasks. *J. Agr. Food Chem.* **6**, 543.

Bickoff, E. M., L. M. White, A. Bevenue, and K. T. Williams. 1948. Isolation and spectrophometric characterization of four carotene isomers. *J. Assoc. Offic. Agr. Chemists* **31**, 633.

Bickoff, E. M., M. E. Atkins, C. G. Bailey, and F. Stitt. 1949. Stereoisomeric analysis of *beta*-carotene. *J. Assoc. Offic. Agr. Chemists* **32**, 766.

Bickoff, E. M. 1957. Determination of carotenes. *In*: "Methods of Biochemical Analysis," Vol. 4. pp. 33–36. (D. Glick, ed.) Interscience Publishers, N. Y.

Bunnell, R. H., and J. C. Bauernfeind. 1962. Chemistry, uses, and properties of carotenoids in foods. *Food Technol.* **16**(7), 36.

Ciegler, Alex, George E. N. Nelson, and Harlow H. Hall. 1961. Microbiological production of carotenoids. Stabilization of β-carotene in dried fermentation solids. *J. Agr. Food Chem.* **9**, 447.

Ciegler, Alex, George E. N. Nelson, and Harlow H. Hall. 1962. Microbiological production of carotenoids. VIII. Influence of hydrocarbon on carotenogenesis by mated cultures of Blakeslea trispora. *Appl. Microbiol.* **10**, 132.

Ciegler, Alex, George E. N. Nelson, and Harlow H. Hall. 1963a. Enhancement of β-carotene synthesis by citrus products. *Appl. Microbiol.* **11**, 128–131.

Ciegler, Alex, A. Lagoda, V. E. Sohns, H. H. Hall, and R. W. Jackson. 1963b. Carotene production in 20-liter fermentors. *Biotechnol. Bioeng.* V, 109–121.

Ciegler, Alex, Zdzislaw Pazola, and Harlow H. Hall. 1964. Stimulation of carotenogenesis by microbial cells. *Appl. Microbiol.* **12**, 150–154.

Isler, O., A. Ofner, and G. F. Siemers. 1958. Industrial synthesis of carotenoids for use as food colors. *Food Technol.* **12**, 520.

Mosher, R. C. 1961. New techniques offer lower-cost natural carotene. *Food Eng.* **33**, 5, 89.

Pazola, Z., and J. Wenta. 1957. Production of carotene concentrates from carrots in the Poznan Food Products Manufacturing Company. *Przemysl Spozywczy* **11**, 439. *Chem. Abstr.* **52**, 20756b (1958).

Polgár, A., and L. Zechmeister. 1942. Isomerization of β-carotene. Isolation of a stereoisomer with increased adsorption affinity. *J. Am. Chem. Soc.* **64**, 1856.

Thompson, C. R., E. M. Bickoff, and W. D. Maclay. 1951. Formation of stereoisomers of *beta*-carotene in alfalfa. *Ind. Eng. Chem.* **43**, 126.

Ms. rec'd 5/29/63.

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Quantitative Aspects of the Interaction of Carrageenan and Other Hydrocolloids with Polyvalent Cobalt Complexes

SUMMARY

Carrageenan, other sulfated polysaccharides, and carboxylic acid polysaccharides react with hexol nitrate, hexamine cobaltic chloride, and tris-(ethylenediamine)-cobalt III chloride in aqueous solution to give precipitates which exhibit differential solubilities in solutions of simple salts. The turbidity (optical density) produced when the hydrocolloids are mixed with these cobalt complexes is proportional to the amount of hydrocolloid present. Salts inhibit the reaction, and, generally, the order of inhibition is: monovalent cations < divalent cations < trivalent cations.

The temperature and pH of the reaction medium greatly influence the final turbidity developed. Generally, the complexes formed through the reaction of the sulfated polysaccharides and the cobalt complexes are less readily dissolved by solutions of simple salts than those formed by the carboxylic polysaccharides and the cobalt complexes. Neutral polysaccharides are not precipitated by the cobalt complexes, hence three component systems containing a sulfated, a carboxylic, and a neutral polysaccharide can be resolved and the individual components determined quantitatively. Good resolution was obtained with a mixture such as carrageenan (sulfated) plus algin (carboxylic) plus locust bean gum (neutral).

Quantitative assay of those polysaccharides which form precipitates (turbidity) with the complexes can be done either by measuring the turbidity developed or by determining spectrophotometrically the amount of cobalt complex cation bound. These colored complexes have sharp absorption maxima: hexol nitrate, max = 490 mμ; hexaminecobalt chloride, max = 475 mμ; and tris-(ethylenediamine)-cobalt III chloride, max = 470 mμ.

INTRODUCTION

Carrageenan is a sulfated polysaccharide which is widely used in the food and pharmaceutical industries as an emulsifier and stabilizer. The mechanism of its action, especially with the proteins of milk, has not yet been fully elucidated, partly because of a lack of rapid and precise methods for its separation and quantitative determination in complex milieu, particularly in the presence of other hydrocolloids such as sodium carboxymethylcellulose, pectin, gum tragacanth, locust bean gum, and algin.

Several schemes have been advanced for the identification of food gums (Bundensen and Martinek, 1954; Smith and Montgomery, 1959; Ewart and Chapman, 1952). However, apart from the method of Hansen and Whitney (1960) and the methylene blue method (Graham, 1960), methods for the quantitative determination of carrageenan have not been forthcoming.

The trivalent cation $[Co(NH_3)_6]^{+++}$ and the hexavalent complex cobalt ion $[Co(OH)_6(Coen_2)_3]^{+6}$ have been used to precipitate chondroitin sulfate from aqueous solution (Mathews and Dorfman, 1953; Vouras and Schubert, 1957). The polyvalent cobalt complexes, as well as tris-(ethylenediamine)-cobalt III chloride, $[Co(en)_3]\ Cl_3$, have also been used to precipitate hyaluronate, nucleic acid, and mucoproteins from solutions and to aid in the study of physiological and pathological conditions involving the above-named biochemical entities (Shatton and Schubert, 1954; Weissmann *et al.*, 1959; Weissmann 1960; Harris and Schubert, 1955; Harris and Teodoru 1961; Mathews, 1964).

The precipitates formed through the interaction of these polyvalent cobalt complexes with chondroitin sulfate, mucoprotein, DNA, and hyaluronate dissolve readily in solutions of simple neutral inorganic salts such as calcium chloride (Vouras and Schubert, 1957) and this has facilitated the isolation of the hydrocolloids. In addition, preliminary experiments indicated that the precipitates formed through interaction of the cobalt complexes and the proteins of milk were highly insoluble in saturated sodium sulfate, sodium chloride, magnesium sulfate, and magnesium chloride.

Although hexol nitrate has been used as an identifying agent for several hydrocolloids, including carrageenan (Smith and Montgomery, 1959), several other sulfated hydrocolloids, such as furcellaran, hypnean, fucoidan, and iridophycan, have gained sig-

nificance recently. Secondly, carrageenan is related to chondroitin sulfate both structurally and physiologically. Therefore, in view of the reported selective precipitation of sulfated mucopolysaccarides by these polyvalent cobalt ions and the facile solubility of the precipitates in solutions of simple salts, it was considered of interest to study the solubility characteristics of the complexes formed through the interaction of such polyvalent cations with several classes of hydrocolloids. This approach poses the possibility of resolving complex mixtures of hydrocolloids commonly used in the food and pharmaceutical industry, but, as far as is known, it has not been investigated.

The colored nature of the cobalt salts in aqueous solution, with sharp absorption maxima, enables rapid quantitative determinations of the amount of hydrocolloid present. This communication summarizes the results of such investigations and outlines application of the techniques developed to the separation and quantitative determination of carrageenan and other hydrocolloids in milk and other complex mixtures.

EXPERIMENTAL

Materials. The carrageenan and other hydrocolloids used are listed in Table 1. Stock solutions were prepared and dialyzed as previously outlined (Graham, 1960). Hexamine cobaltic chloride (luteocobaltic chloride)—$[Co(NH_3)_6]\ Cl_3$—was prepared according to the method of Bjerrum and McReynolds (1946). It may also be obtained from the K and K Laboratories, 177-10, 93rd Avenue, Jamaica 33, New York, N. Y. A 20 m*M*/L stock solution was made up in double distilled water and stored in an amber bottle.

Hexol nitrate—$[Co(OH)_6(Coen_2)_3]\ (NO_3)_6$—was prepared according to the method of Werner (1907). A 20 m*M*/L stock solution was prepared as described for hexamine cobaltic chloride. Tris-(ethylenediamine)-cobalt III chloride—$[Co(en)_3]Cl_3$—was prepared according to the method of Work (1946).

The purity of all three cobalt salts was checked by the Kjeldahl nitrogen determination and by the constancy of their absorption spectra after recrystallization. The absorption spectra are shown in Fig. 1.

Inorganic salts, C. P. grade, were used without further purification. Isopropyl alcohol and ethyl alcohol were Fischer certified reagent grade.

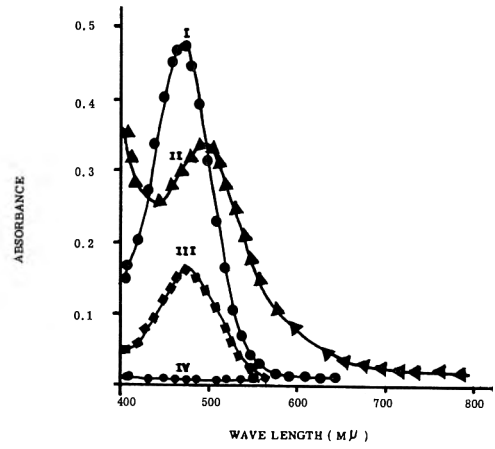


Fig. 1. Absorption spectra of polyvalent cobalt complexes and carrageenan (Seakem type 5) in distilled water.
I, tris-(ethylenediamine)-cobaltic chloride
II, hexol nitrate
III, hexamine cobaltic chloride
IV, carrageenan (Seakem type 5)

Ion-exchange resin—Dowex 50W-X8 resin, H^+ form, 50–100 mesh—was used.

DEVELOPMENT OF THE METHOD

General procedure. Two to 10 mg of the particular hydrocolloid dissolved in distilled water was added to a series of 30-ml Pyrex test tubes. If necessary, the volume was made up to 8 ml with distilled water or any other solution being investigated. The test tubes were preincubated for 10 min at 30±1°C. Two ml of the stock solution of the cobalt complex was added dropwise to each tube, shaking the tube well after each drop. The tubes were then incubated 30 min at 30±1°C, cooled 10 min in an ice-water bath, and allowed to stand 15 min at room temperature, and the turbidity developed was measured at 600, 600, and 700 mμ for tris-(ethylenediamine)-cobalt III chloride, hexamine cobaltic chloride, and hexol nitrate, respectively, against a reagent blank. The appropriate reagent blank consisted of 2 ml of the cobalt complex plus all the other ingredients except the hydrocolloid. When the influence of variables or some other aspect of the problem was being studied, suitable controls were included as required.

Factors influencing the reaction. To assess the influence of several variables which may affect the reaction, the turbidity developed through the interaction of 10 mg of carrageenan (Seakem type 5) with 2 ml of each of the cobalt complexes was measured as outlined in the general procedure, but varying the factors under investigation between the desired limits.

Table 1. Summary of reaction of hydrocolloids dispersed in distilled water with cobalt complexes at 29±1°C.

Hydrocolloid tested	Reagent[a] A	B	C
Group I. Sulfated polysaccharides			[b]
Carrageenan (8 samples)	+	+	+
Furcellaran	+	+	+
Hypnean	+	+	+
Fucoidan	+	+	+
Iridophycan	+	+	+
Agar	−	−	−
Group II. Carboxylic polysaccharides			
Sodium alginate	+	+	+
Sodium carboxymethylcellulose	+	+	+
Pectin	+	+	+
Mucin	+	+	+
Gum Karaya	+	±	±
Gum arabic	−	−	−
Gum tragacanth	+	±	±
Gum ghatti	+	±	±
Quince seed mucilage	+	+ (Gels)	+ (Gels)
Polysaccharide B-1459 (NRRL)	+	+	+
Group III. Phosphorylated polysaccharides			
Phosphomannans Y-2448 and Y-2154	+	±	±
Group IV. Neutral Polysaccharides			
Locust bean gum	−	−	−
Gum guar	−	−	−
Potato starch	−	−	−
Tamarind seed mucilage	−	−	−
Group V. Proteins			
Casein	−	−	−
Lactalbumin	−	−	−
Gelatin	−	−	−
Group VI. Other Compounds			
Polygalacturonic acid	+	+	+
Polyglucose sulfate	+	+	+
Dextran sulfate	+	+	+
Chondroitin sulfate	+	+	+
Mepesulfate[c]	+	+	+
Heparin	+	+	+
Sodium lauryl sulfate	+	+	+
Sodium lauryl sulfonate	+	+	+

[a] Reagent A, hexol nitrate.
Reagent B, hexamine cobaltic chloride.
Reagent C, tris-(ethylenediamine) cobalt III chloride.

[b] + Precipitation or turbidity.
− No visible reaction.
± Doubtful reaction.

[c] Mepesulfate—Sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside.

Table 2. Solubility of precipitates of hydrocolloid-polyvalent cobalt complexes in various salt solutions at 30°C. Concentration (normality) of salt necessary to reduce OD of systems to ½ of its value.

	System tested				
Salt tested	A	B	C	D	E
Li_2SO_4	0.065	0.04	0.045	0.028	0.05
NaCl	0.51	0.26	0.14	0.110	0.22
KCl	0.69	0.50	0.28	0.09	0.30
LiCl	0.52	0.15	0.10	0.06	0.12
NH_4Cl	0.6	0.30	0.2	0.10	0.27
HCl	0.9	0.33	0.14	0.002	0.05
$MgSO_4$	0.16	0.11	0.10	0.05	0.10
$MgCl_2$	0.70	0.30	0.10	0.06	0.20
$CaCl_2$	0.95	0.12	0.12	0.06	.....
$(NH_4)_2SO_4$	0.11	0.10	0.09	0.35	0.08
$ZnSO_4$	0.15	0.12	0.10	0.10	.....
Na_2SO_4	0.075	0.05	0.05	0.03	0.06
NaSCN	0.6	0.45	0.30	0.125	0.28
$K NO_3$	0.6	0.38	0.25	0.07	0.30
K_2SO_4	0.115	0.18	0.085	0.038	0.11
$NaNO_3$	0.6	0.22	0.24	0.10	0.20

A = carrageenan (Seakem type 5)—hexol nitrate.
B = carrageenan (Seakem type 5)—hexamine cobalt chloride.
C = carrageenan (Seakem type 5)—tris-ethylenediamine cobaltic chloride.
D = sodium carboxymethylcellulose—hexamine cobaltic chloride.
E = sodium alginate—hexamine cobaltic chloride.

The influence of pH was studied by adding various amounts of 0.01–0.1*N* phosphate buffer to the hydrocolloid-cobalt salt mixture and following the general procedure outlined above. The pH of each system was checked before and after incubation. Tubes containing only the cobalt salts were treated similarly in order to assess any possible effect of pH on the absorption spectrum of the cobalt salts themselves.

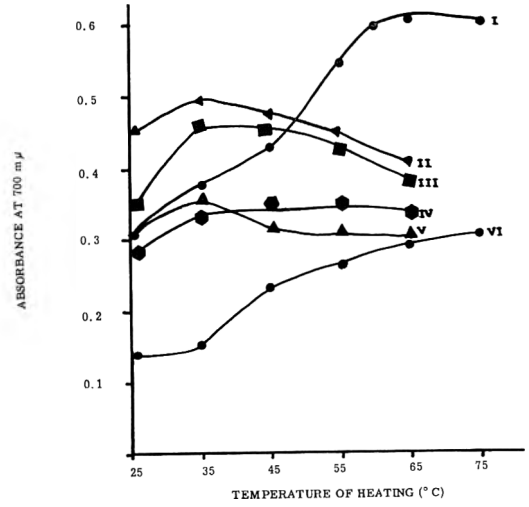


Fig. 2. Influence of temperature on the turbidity developed through the interaction of hydrocolloids with hexol nitrate.
I, carrageenan
II, gum tragacanth
III, sodium carboxymethylcellulose
IV, pectin
V, sodium alginate
VI, furcellan

The influence of temperature on the development of turbidity (Fig. 2) and on the spectrum of the cobalt salts was also studied.

Solution of precipitates by various salts. To test the solubility of the precipitates formed by various hydrocolloids and the cobalt complexes, 0–4 ml of a solution of the salts listed in Table 1 was added to each tube. The tubes were shaken well and the mixture in each tube was measured as outlined in the general procedure. A plot of the optical density vs. the final normality of the salt indicates the ability of each salt to dissolve a particular precipitate. Comparisons were made by noting the concentration of salt needed to reduce the amount of precipitate formed to 50% of the value obtained when no salt was added to the system (Vouras and Schubert, 1957). For convenience, this has been dubbed "the ½-OD value." The results are summarized in Table 2 and Fig. 3.

The influence of aging of the precipitates on their solubility was also checked.

Influence of temperature of reaction on the solution of precipitates by solutions of various salts. Since preliminary observations indicated that, apparently, as the temperature of reaction increased, the solubility of the precipitate in salt solu-

Table 3. Influence of variables on determination of carrageenan by methods I and II.

Variable investigated	Range investigated	Value selected
Procedure No. 1		
1. pH adjustment before heating	pH 7-11	pH 9
2. Alkalis used	NaOH,KOH NH_4OH	NaOH
3. Time of heating	0–3-min	10 min
4. Temp. of heating	60–100°C	100°C
Procedure No. 2.		
1. As above	As above	9
2. As above	As above	NaOH
3. Final normality of NaOH	0–1.0	0.5
4. Cobalt complex used	All three	Hexamine cobaltic chloride
5. Salt used for extraction	Na_2SO_4 NaCl $MgSO_4$ $MgCl_2$	$MgCl_2$

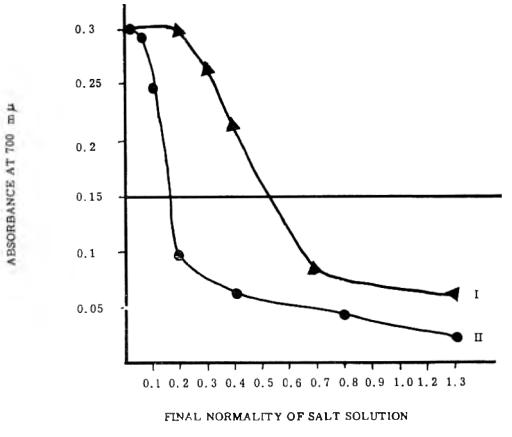


Fig. 3. Comparative solubility of carrageenan-hexol nitrate precipitate in sodium chloride and magnesium sulfate. Demonstration of calculation of "½-OD value."
I, sodium chloride
II, magnesium sulfate

tions decreased, this aspect was investigated further. For this, precipitation was done at 30, 45, and 60°C. The precipitates formed were treated with increasing amounts of salts as described above, and the ½-OD value was calculated.

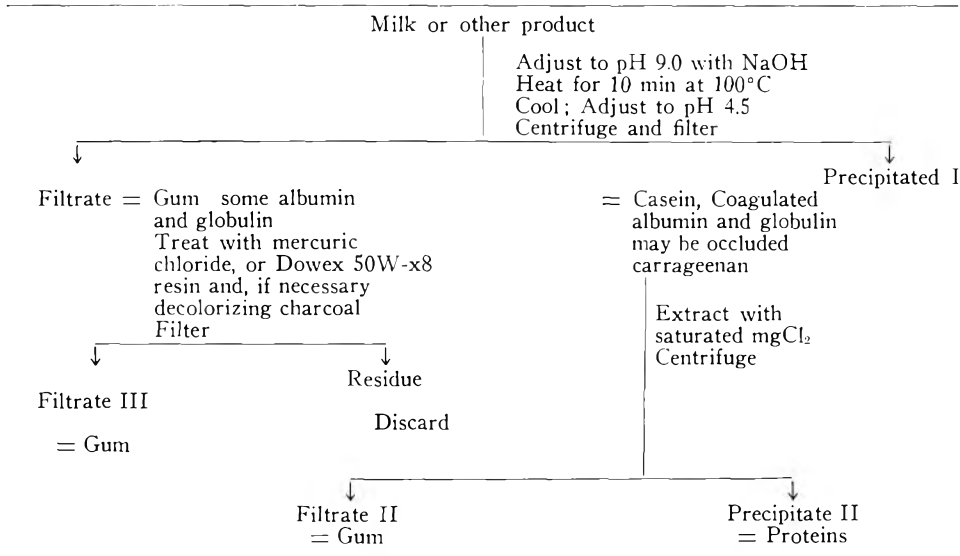
Application of the method. After the several factors affecting the reaction of carrageenan with the cobalt complexes were delineated, the proposed method was applied to the determination of carrageenan in milk and other mixtures. Two different procedures were followed in isolating the hydrocolloid from mixtures.

Procedure No. 1. Coprecipitation of albumin and globulin with casein at pH 4.5. Fifty g of milk or other material, weighed to the nearest gram, was placed in a stoppered 100-ml centrifuge bottle. Where necessary, 10–20 ml of warm distilled water was added to disperse the material. The pH of the mixture was adjusted to 9 by adding, dropwise, 5*N* NaOH and following the procedure with a Beckman Zeromatic pH meter. Then the mixture was treated as shown in the outline. Filtrates II and III were collected in a 100-ml volumetric flask and the volume made up to mark with distilled water. The contents were mixed thoroughly, and 8 ml was used for determination turbidimetrically as outlined in the general procedure.

Procedure No. 2. Simultaneous precipitation of carrageenan and milk proteins with cobalt complex and extraction of the carrageenan with saturated magnesium chloride. For this, the milk or other material was weighed out, and, if necessary, warm water was added as described under procedure No. 1. Enough 5*N* NaOH was added to give a final concentration of 0.5*N* and the mixture was treated as shown in the outline. The carrageenan content of filtrate III was determined on a suitable aliquot by the anthrone-thiourea method (Graham, 1965) or by the barium chloride procedure (Hansen and Whitney, 1960).

Resolution of mixtures of hydrocolloids. Three component systems consisting of 100 mg each of carrageenan, sodium carboxymethylcellulose, and locust bean gum were resolved using hexamine cobaltic chloride and sodium sulfate. This was done on "untreated" mixtures and on mixtures

Determination of carrageenan in milk and other products, procedure No. 1.



added to milk. Hexamine cobaltic chloride (0.1 g) was added to the mixture dispersed in 40 ml of distilled water and the mixture was incubated for 30 min at 30±1°C and then centrifuged for 15 min at 2500 rpm. The supernatant was carefully poured off onto Whatman No. 2 filter paper and the filtrate collected. This filtrate contains the locust bean gum (neutral polysaccharide); any precipitate on the filter paper was returned to the centrifuge tube. The precipitate contains the carrageenan (sulfated) and the sodium carboxymethylcellulose (carboxylic hydrocolloid). Ten ml of 0.05*N* sodium sulfate was added to the precipitate, and the mixture was shaken well and left at room temperature (28±1°C) for 15 min. It was then centrifuged for 10 min at 2500 rpm and the supernatant poured carefully over Whatman No. 2 filter paper. The filtrate contains the carboxylic polysaccharide, and the precipitate contains the carrageenan. The carrageenan was dissolved in 0.2*N* Na_2SO_4. Each hydrocolloid in the mixture was assayed by an established method (Bundesen and Martinek, 1954; Cameron *et al.*, 1948; Helbert and Brown, 1957, 1961). Prior to analysis of any of the fractions, it was shaken with 5 g of mercuric chloride and filtered, and the filtrate was used for analysis. This clarifies the solution through precipitation of the cobalt salt by mercury. Dowex 50W-8 resin, H^+ form, 50–100 mesh, may also be used (Mathews and Dorfman, 1953). The results are summarized in Table 4. Mixtures containing carrageenan, sodium alginate or any other carboxylic polysaccharide and any neutral polysaccharide may be fractioned in the same way.

Determination of carrageenan in milk and other products, procedure No. 2.

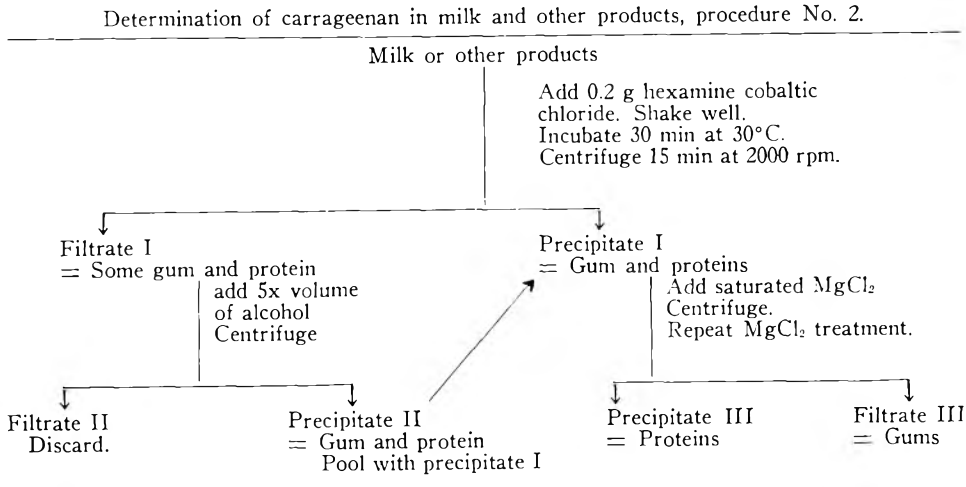


Table 4. Resolution of mixtures of hydrocolloids with hexamine cobaltic chloride and sodium sulfate.

Hydrocolloid added	Quantity Added (mg)	Recovered (mg)	% recovery [a]
Isolated hydrocolloids			
Carrageenan	100	99.4	99.4
Sodium carboxymethyl-cellulose	100	99.6	99.6
Locust bean gum	100	101.2	101.2
Hydrocolloids added to milk system and then isolated according to the method I			
Carrageenan	100	95.8	95.8
Sodium carboxymethyl-cellulose	100	97.2	97.2
Locust bean gum	100	98.3	98.3

[a] Each value represents the average of 3 different determinations.

Recovery of carrageenan added to milk and milk products. The recovery of carrageenan from milk and milk products was tested by adding 0–100 mg (dry-weight basis) of the hydrocolloid to various systems. Isolation was done by the proposed methods, and quantitative determination was carried out as outlined in the general procedure. The results are summarized in Table 5.

RESULTS AND DISCUSSION

Fig. 1 shows the absorption spectra of the cobalt complexes in distilled water. By making turbidity measurements at 700 mμ for hexol nitrate and at 600 mμ for hexamine cobaltic chloride and tris-(ethylenediamine)-cobalt III chloride, minimum interference due to absorption by the cobalt complexes themselves will be encountered. After precipitation of the hydrocolloid the residual cobalt complex can be measured. Alternately, the precipitate formed can be dissolved in sodium sulfate or some other salt and the absorption of the released cobalt complex measured. In both cases, measurement is facilitated by the sharp absorption maxima of the highly colored cobalt salts.

When dissolved in distilled water, the absorption maxima of all three complexes were the same between the temperatures of 25 and 65°C. Over this same temperature range their absorption at their respective absorption maxima differed by only 1–2%, thus indicating that, under the experimental conditions, temperature essentially does not affect the absorption spectra.

At 30±1°C, pH had a profound effect on the cobalt complexes, especially at the lower and higher levels. Between pH 4.2 and 8.0, no essential difference was found. Below pH 3.8, absorption was lower, while above pH 8.5 it increased because of a darkening of the solution. This was especially true for hexol nitrate.

The reactivity of several hydrocolloids and some related materials with the polyvalent cobalt complexes in aqueous suspension at 30±1°C is shown in Table 1. The sulfated polysaccharides all reacted with the three cobalt cations. Several other sulfated compounds, listed under group VI, were also precipitated by all the complexes, indicating that, in general, sulfated and sulfonated compounds are active.

Table 5. Recovery of carrageenan from milk and other products.[a]

System	Method No. 1 Added (mg)	Recovered (mg)	Av.	Range	Method No. 2 Added (mg)	Recovered (mg)	Av.	Range
Milk	50	47.8	95.6	94.6–96.2	50	48.1	96.2	94.6– 98.2
(commercial sample)	12.5	11.6	92.8	90.8–94.5	12.5	11.9	95.2	92.8– 97.1
Chocolate milk	50	47.0	94	92.9–95.8	50	47.2	94.4	91.2– 95.6
(3% chocolate in commercial milk)	12.5	11.0	88	85.5–90.6	12.5	11.5	92.0	90.8– 94.0
Beer	50	49.2	98.4	97.9–99.2	50	49.5	99.0	96.2–101.6
(commercial sample)	12.5	12.1	96.8	92.4–98.4	12.5	12.0	96.0	93.6– 98.2
Orange juice	50	49.4	98.8	97.6–99.4	50	49.6	99.0	98.4– 99.8
(canned)	12.5	12.0	96.0	94.5–98.7	12.0	12.2	96.4	94.5– 98.0

[a] Average of 5 separate determinations.

Among the carboxylic polysaccharides, the most active were sodium alginate, sodium carboxymethylcellulose, mucin, pectin, quince seed mucilage, and the bacterial polysaccharide B-1459 (NRRL) (Rogovin *et al.*, 1961; Sloneker and Jeanes, 1962; Sloneker and Orentas, 1962). Gum tragacanth, gum ghatti, and gum karaya reacted strongly with hexol nitrate, but much less with the other two cobalt cations. This reflects the greater activity of the hexavalent cobalt cation of hexol nitrate over that of the other two trivalent cobalt cations. Gum arabic did not react with any of the cobalt cations.

The two phosphorylated polysaccharides (Anderson *et al.*, 1960; Jeanes *et al.*, 1961b; Slodki, 1962) investigated reacted strongly with hexol nitrate, but only weakly with the other two cobalt cations. As expected, none of the neutral polysaccharides were precipitated by the cobalt complexes.

Under the conditions of the screening tests, the proteins did not respond although all the cobalt reagents, when added to milk, caused coagulation of the proteins.

The coagulation of several hydrocolloids by hexol nitrate is listed by Smith and Montgomery (1959, p. 44). Data included in this report supplement those given by the above-mentioned authors, since two trivalent cobalt complexes and several different polysaccharides are included.

The turbidity developed through the reaction of any sulfated or carboxylic hydrocolloid with any of the cobalt complexes, at a specific temperature, is directly proportional to the amount of hydrocolloid present. For carrageenan, a straight-line relationship was obtained when optical density was plotted against hydrocolloid concentration. This held true for concentration ranges of 2–16 mg of the hydrocolloid per 10 ml of the reaction mixture.

Temperature has a profound effect on the turbidity developed, as shown in Fig. 2. Maximum turbidity occurred with carrageenan and furcellaran at 60–75°C, while with sodium carboxymethylcellulose, pectin, sodium alginate, and tragacanth it occurred at about 35–38°C.

When tested in a distilled-water system at 30±1°C, pH also affected the reaction. Maximum turbidity occurred between pH 4.2 and 8.8.

Buffer molarity also affected the turbidity developed with maximum turbidity occurring at and below 0.02 molar phosphate buffer.

Several salts, mainly as chlorides, were tested for their ability to inhibit the precipitation of carrageenan by the cobalt complexes. In general, the order of activity was: monovalent cations < divalent cations < trivalent cations. Trivalent cations such as Fe^{+++} and Al^{+++}, precipitate the hydrocolloid in the absence of the cobalt complexes.

Inhibition of the formation of precipitates by cations is due mainly to competition for the anionic binding sites of the hydrocolloid (Harris and Schubert, 1955; Harris and Teodoru, 1961; Mathews, 1960, 1964). The nature of the accompanying anion of the salt also has some influence since, generally, the sulfates were more active than the chlorides and nitrates.

The most interesting feature of the precipitates formed in interaction of the hydrocolloids with the cobalt complexes is the solubility of such precipitates in solutions of simple salts. The differential solubility of the hydrocolloid-polyvalent cobalt cation precipitate can be exploited to separate mixtures of hydrocolloids.

Table 2 shows the final normality of several salts necessary to reduce the turbidity of various systems to one-half that developed when no salt was added. This "index of activity" was chosen since the concentration of any salt necessary to completely dissolve the precipitate was, at times, too high for practical experimentation. This method has been found by Vouras and Schubert (1957) to give good comparisons. The sulfates were the most effective salts in this respect in the following approximate order: $LiSO_4 > Na_2SO_4 > (NH_4)_2SO_4 = K_2SO_4 = MgSO_4 > ZnSO_4$. Among the chlorides, the approximate order of activity was: $NaCl > LiCl > NH_4Cl > MgCl_2 > KCl > CaCl_2 > HCl$.

Aging seemed to increase the "1/2-OD" values of the salts. Precipitates were formed at 30±1°C and left at this temperature for 6, 12, 24, and 48 hr. After 6 hr, the "1/2-OD" value for sodium sulfate and so-

dium chloride increased 10–15%. This may be attributed, partially, to the tendency of the precipitate to form clumps on standing.

Apparently, the temperature of precipitation also influenced the "1/2-OD" value, since, as the temperature of precipitation increased, the "1/2-OD" value also increased. A portion, but not all, of this increase can be ascribed to the greater precipitation with increased temperature. However, other factors may be involved since, when systems developed at 60°C were diluted to the same turbidity as those developed at 30°C and 45°C and were treated with sodium sulfate and sodium chloride, "1/2-OD" values were relatively higher for the precipitates formed at the higher temperatures.

The method used for the separation of proteins from the mixtures deserve some comment. In method 1, the alkali added to the milk presumably dissociates any complexes formed between carrageenan and the milk proteins. Woodman (1941, p. 168) mentions the use of "a few drops of ammonia" in describing the isolation of food gums from milk and ice cream. Partridge and Elsden (1961) reported that the chondroitin sulfate-protein complex could be dissociated with alkali. Although more critical evidence is necessary for unequivocal proof of the dissociation, the great decrease in the viscous nature of the milk-carrageenan mixture on the addition of alkali indicates that the hydrocolloid-milk protein interaction was disrupted. The alkali added prevents the thermal destruction of carrageenan since this hydrocolloid is most stable at around pH 9.0 and is not readily destroyed at high temperatures in suspensions above pH 6.0. Heating for 10 min at 100°C denatures the albumin and globulin, which are then coprecipitated with casein at around pH 4.7 (Rowland, 1937, 1938).

Determination of the carrageenan content of any sample by the proposed method depends on the use of a standard curve which should be constructed with a sample of the same "type" of the hydrocolloid being determined.

In method 2, the carrageenan polyvalent cobalt complex and, presumably, to some extent the carrageenan-protein complex, are dissociated by the saturated magnesium chloride, in which the carrageenan but not the protein is soluble. As in method 1, the initial addition of alkali dissociates the carrageenan-protein complex. Although sodium or lithium sulfate is more active in dissociating the complex, the use of magnesium chloride obviates the introduction of inorganic sulfate into the sample. This is important where a sulfate analysis on the isolated sample is to be done. Magnesium chloride is also very soluble in alcohol. Determination of the amount of carrageenan present by the anthrone-thiourea-sulfuric acid test (Graham, 1965) is a rapid method for isolated samples. It is particularly advantageous since proteins, sugar alcohols, and ascorbic acid do not react, as in the classical anthrone-sulfuric acid test. Moreover, the reagent is stable for as long as six weeks if stored in an amber bottle under refrigeration.

Recovery of added carrageenan from milk and milk products was somewhat low at low levels of added carrageenan. This could have been due to incomplete dissociation of the carrageenan-protein complex and to incomplete extraction with the magnesium chloride. Removal of the fat by the Röese-Gottlieb method prior to treatment of the sample did not lead to higher recoveries.

When compared with the method of Hansen and Whitney (1960), the recoveries from milk and milk products were somewhat

Table 6. Comparison of determination of carrageenan in milk by proposed method No. 2 and by the ester sulfate method of Hansen and Whitney (1960).[a]

Carrageenan added (mg)		Carrageenan recovered (mg)		% recovery	
Method used		Method used		Method used	
No. 2	H-W	No. 2	H-W	No. 2	H-W
100	100	95.8	99.4	95.4	99.4
50	50	47.4	51.2	94.8	101.4
12.5	12.5	11.6	12.2	92.8	97.6

[a] Average of 3 determinations for each value.

low. The proposed method, though much more rapid than theirs and of approximately the same reproducibility, is less specific. However, by calibrating the proposed method against the ester sulfate method, it does lend promise as a rapid procedure for the detection and determination of carrageenan in food products. The specific food gum present can be ascertained by a preliminary test (Bundensen and Martinek, 1954; Smith and Montgomery, 1959; Helbert and Brown, 1957–1961; Cameron *et al.*, 1948).

Carrageenan and the sulfated polysaccharides formed precipitates which dissolved much less readily in the salt solutions than the carboxylic polysaccharides.

The only exception to this rule was the bacterial polysaccharide B1459 (Jeanes *et al.*, 1961a). The complexes formed by this hydrocolloid with the polyvalent cations, especially that formed with hexol nitrate, were much less soluble in sodium sulfate and the other salts than the carrageenan complex.

In a few experiments, pectin was readily extracted and determined by the procedures outlined.

REFERENCES

Anderson, R. F., M. C. Cadmus, R. G. Benedict, and M. E. Slodki. 1960. Laboratory production of a phosphorylated mannan by Hansenula holstii. *Arch. Biochem. Biophys.* **89**, 289.

Bjerrum, J., and J. P. McReynolds. 1964. Hexaminecobalt (III) salts. *In*: "Inorganic Syntheses" **2**, 216.

Bundensen, H. N., and M. J. Martinek. 1954. Procedure for the separation, detection and identification of the more common vegetable gums in dairy products with special reference to alginates. *J. Milk and Food Technol.* **17**, 79.

Cameron, M. C., A. G. Ross, and E. G. V. Percival. 1948. Routine estimation of mannitol, algenic acid and combined fucose in seaweeds. *J. Soc. Chem. Ind. (London)* **67**, 161.

Ewart, M. H., and R. A. Chapman. 1952. Identification of stabilizing agents. *Anal. Chem.* **24**, 1460.

Graham, H. D. 1960. Quantitative aspects of the interaction of carrageenan with cationic substances. I. Interaction with methylene blue. *Food Research* **25**, 720.

Graham, H. D. 1965. Critical evaluation of the stabilized anthrone-thiourea reagent for the analysis of carbohydrates. *J. Food Sci.* (to be submitted).

Hansen, P. M. T., and R. Mc L. Whitney. 1960. A quantitative test for carrageenan ester sulphate in milk products. *J. Dairy Sci.* **43**, 175.

Harris, A. F., and M. Schubert. 1955. Inhibition of calcification *in vitro* by leutecobaltic chloride. *Proc. Soc. Exptl. Biol. Med.* **90**, 714.

Harris, A. F., and C. V. Teodoru. 1961. Effect of chlorpromazine upon calcification *in vitro*. Interaction with isolated epiphyseal cartilage of rachitic rats. *Proc. Soc. Exptl. Biol. Med.* **107**, 953.

Helbert, J. R., and K. D. Brown. 1957. Color reaction of anthrone with monosaccharide mixtures and oligo and polysaccharides containing hexuronic acids. *Anal. Chem.* **29**, 1464.

Helbert, J. R., and K. D. Brown. 1961. Identification of aldohexuronic acids free and in polymers. *Anal. Chem.* **33**, 1610.

Jeanes, A., J. E. Pittsley, and F. R. Senti. 1961a. Polysaccharide B-1459: A new hydrocolloid polyelectrolyte produced from glucose by bacterial fermentation. *J. Appl. Polymer. Sci.* **5**, 519.

Jeanes, A., J. E. Pittsley, P. R. Watson, and R. J. Dimler. 1961b. Characterization and properties of the phosphomannan from Hansenula hostii NRRL Y-2448. *Arch. Biochem. Biophys.* **92**, 343.

Mathews, M. B. 1960. Trivalent cation binding of acid mucopolysaccharides. *Biochim. Biophys. Acta* **37**, 288.

Mathews, M. B. 1964. Structural factors in cation binding of anionic polysaccharides of connective tissue. *Arch. Biochem. Biophys.* **104**, 394.

Mathews, M. B., and A. Dorfman. 1953. The molecular weight and viscosity of chondroitin-sulfuric acid. *Arch. Biochem. Biophys.* **42**, 41.

Partridge, S. M., and D. F. Elsden. 1961. Dissociation of the chondroitin sulfate-protein complex of cartilage with alkali. *Biochem. J.* **79**, 26.

Rogovin, S. P., R. F. Anderson, and M. C. Cadmus. 1961. Production of a Polysaccharide with Xanthomonas campestris. *J. Biochem. Microbiol. Technol. Eng.* **3**, 51.

Rowland, S. J. 1937. The heat denaturation of albumin and globulin in milk. II. Denaturation and degradation of protein at temperatures of 75–120°. *J. Dairy Research* **8**, 1.

Rowland, S. J. 1938. The precipitation of the proteins in milk. IV. Albumin and proteose-peptone. *J. Dairy Research* **9**, 40.

Shatton, J., and M. Schubert. 1954. Isolation of a mucoprotein from cartilage. *J. Biol. Chem.* **211**, 565.

Slodki, M. E. 1962. Phosphate linkages in phos-

phomannans from yeast. *Biochim. et Biophys. Acta* **57**, 525.

Sloneker, J. H., and A. Jeanes. 1962. Exocellular bacterial polysaccharide from Xanthomonas campestris NRRL B-1459. I. Constitution. *Can. J. Chem.* **40**, 2066.

Sloneker, J. H., and D. G. Orentas. 1962. Pyruvic acid, a unique component or an exocellular bacterial polysaccharide. *Nature* **194**, 478.

Smith, F., and R. Montgomery. 1959. "Chemistry of Plant Gums and Mucilages." Reinhold Publishing Corp., New York.

Vouras, M., and M. Schubert. 1957. The outer sphere association of chondroitin sulfate by polyvalent complex cations. *J. Am. Chem. Soc.* **79**, 792.

Weissmann, G. 1960. Inhibition of hexaminecobaltic chloride DNA interaction by sera of patients with disseminated Lupus erythematosus. *Proc. Soc. Exptl. Biol. Med.* **103**, 469.

Weissmann, G., J. L. Potter, R. T. McCluskey, and M. Schubert. 1959. Turbidity produced by hexaminecobaltic chloride in serum of rabbits injected intravenously with papain. *Proc. Soc. Exptl. Biol. Med.* **102**, 584.

Werner, A. 1907. Polynucleated metal-amines. *Ber.* **40**, 2103.

Woodman, A. G. 1941. "Food Analysis," McGraw-Hill Book Company, New York, London. Page 168.

Work, J. B. 1946. Tris-(ethylene diamine) cobalt (III) chloride. *In*: "Inorganic Syntheses." **2**, 221.

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Stress-Strain and Creep Relationships of Pectin Gels

SUMMARY

The rheological properties of sugar-acid-pectin gels were studied by measuring the stress-strain relationships under three types of loading (impact, compression, and static). These gels are viscoelastic. When the applied stress was less than .25 lb per sq in., a permanent deformation ("plastic flow") was observed, while with larger stress, the gel responded elastically and failure was "brittle." Both these responses were highly time-dependent. The data obtained indicate that the impact and compression method of testing cannot clearly identify small differences in chemical composition. Creep tests indicate, however, that disaccharide gels supported larger stresses for longer times than did gels containing monosaccharides.

INTRODUCTION

The rheological properties of sugar-acid-pectin gels, although of considerable economic importance, have been studied largely by empirical methods. It was felt that a more comprehensive study, using recently developed methods of measuring impact and creep stress-strain relationships, would provide a better insight into these rheological properties.

A variety of devices have been developed for measuring gel strength. However, the rheology of these gels is complex and therefore has been poorly understood. Consequently, the testing methods were often based on unsound premises, commonly taking the form of procedures intended to duplicate as closely as possible the actual commercial use of the material. Matz (1962) reported that "tests may be classified as: a) those in which the elastic limits (breaking strength) of the jellies are exceeded and the jelly is ruptured; and b) those measuring deformation (sag) of jellies without exceeding the elastic limit. In the first category are: Sucharipa's jelly disc method, the Fellers-Clague penetrometer, the Luers-Lochmuller Pektinometer, and the Delaware jelly tester of Tarr and Baker, with its several modifications. Sag methods include the Bloom gelometer, the Cox and Higby sag method with its variations, the B.A.R. jelly tester, and Saverborn's cylindrical torsion method." The F.I.R.A. jelly tester described by Campbell (1937–38) and the Exchange Ridgelimeter (IFT, 1959) also depend upon sag measurements.

Cheftel and Mocquard (1947) investigated the rheological properties of pectin gels with a high sugar concentration. Their results indicated that previous data on the elasticity of gels were in error. These conclusions were disputed by Owens *et al.* (1954), who pointed out that the deformation of high-methoxy pectins is linear with respect to small stress, and that recovery is complete.

A detailed review of methods for determining gel properties was made by Joseph and Baier (1949).

Hinton (1950) pointed out that the setting temperature of a jelly is a determinate physical property, whereas the setting time is a secondary property dependent upon temperature.

Christensen (1954) showed that the relation between "sag" value and "breaking strength" was not constant, but was related to the molecular weight of the pectin. It was therefore impossible to find a simple conversion factor for the two types of test.

Solms (1960) outlined the relationship between the chemical constitution and gelation properties of pectin.

Harvey (1960) reported that sugar-acid-pectin gels are viscoelastic. Viscoelastic materials exhibit the properties of both liquids and solids. The theory of viscoelasticity is discussed by Bland (1960), Reiner (1960), and Van Wazer *et al.* (1963).

Matz (1962) stated: "In the evolution of the various procedures for testing the strength of pectin gels it was frequently found that the results obtained on the same sample by different laboratories did not agree as closely as was desired. Differences were attributed to unrecorded variations in procedures, materials, or equipment. As a result specification of these factors became more and more detailed. The Pectin Jelly

Test (IFT, 1959) of the Pectin Standardization Committee of the Institute of Food Technologists is an interesting example of the rigid procedure produced in the evolution of this empirical type of test."

Although work on the rheological properties of pectin gels (when fully set) has progressed farther than work on other gels, Harvey (1960) pointed out that this work has not included extensive rheological evaluation. The correlation of rheological properties with sensory evaluation of gel texture has not yet been achieved. Because the rheological properties of gels are time-dependent, the empirical nature of much of the previous "rheological-type" measurements has led to some confusion. As each method introduces its own time base, it is not surprising that differing results are obtained by different test procedures. Furthermore, although pectin is one of the oldest hydrocolloids known, many features of its structure and properties remain enigmatic (Anon, 1960). Intensive rheological studies will be required to correlate the various characteristics of the gel with one another and with the gel structure.

APPARATUS

To establish rates of strain between 0.2 and 50 in. per min, a Bellows Hydrocheck and air-motor (in-line assembly) was used (Fig. 1). This unit is available commercially and is described by Mohsenin (1963).

The static (creep) and impact loads were applied to the samples by means of a 2-ft balance arm mounted on knife-edge supports (Fig. 2). The beam was carefully balanced so that it would remain at rest on the knife edges. The desired weight was placed on the platform at the end of the beam. The beam, raised to the required height (½ in.) above the sample, was held by a pin. Upon removal of the pin, the beam moved downward and the plunger head struck the sample. Heads used on the plunger were of 1-inch and 1½-inch diameter.

The force (stress) exerted on the sample was measured by an assembly of four cantilever beams (Fig. 1). The deflection of each cantilever beam was sensed by two Tatnall "Metalfilm" foil strain gages. The eight gages from the four beams were wired to form a Wheatstone bridge. The stress applied to the sample, and hence to the cantilever beams, was sensed by the strain-gage bridge, amplified and recorded.

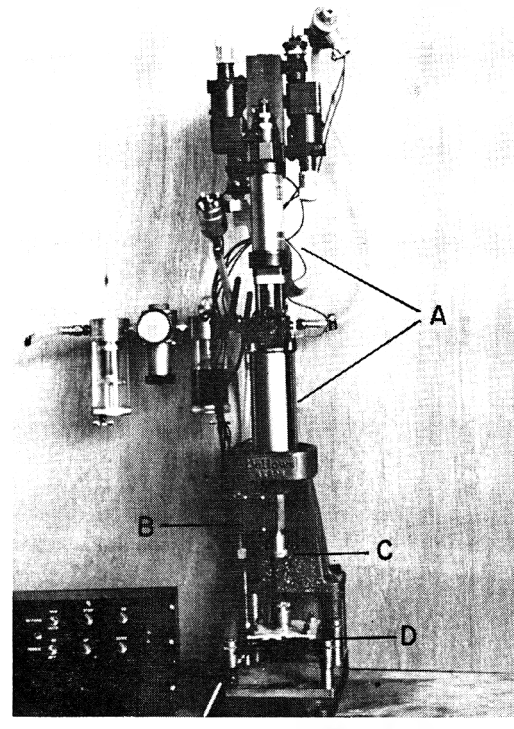

Fig. 1. Bellows hydrocheck compression.
Unit: A, Hydrocheck cylinders
B, Linear variable differential transformer (LVDT)
C, Loading plunger
D, Cantilever beams supporting specimen holder

A Brush "Metresite" linear-variable differential transformer (LVDT) attached to the crosshead of the loading mechanism measured the deflection (or strain) on the sample by sensing the relative position of the crosshead and the plate of the stress-sensing assembly.

A two-channel dual-beam oscilloscope with camera or an X-Y recorder was used to amplify and record the signals from the LVDT and strain-gage transducers.

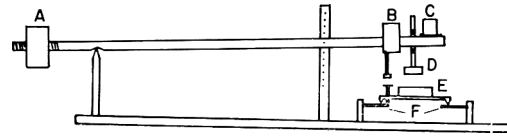

Fig. 2. Static and impact loading beam.
(A) counterweight
(B) LVDT
(C) Loading platform
(D) Loading plunger
(E) Specimen
(F) Cantilever beams

An Exchange ridgelimeter (IFT, 1959) was used as a control instrument to measure the sag occurring in each batch. The soluble solids and pH of all batches were determined according to the AOAC methods of analysis (1960), respectively sections 29.011 and 10.125.

TEST PROCEDURE

Preparation of gels. The procedure of the IFT Committee on Pectin Standardization (IFT, 1959) was followed in preparing the gel samples. A standard batch could be boiled in about 10 min, but a few batches were made smaller in order to decrease the boiling time. Upon completion of the boiling operation (1015 g), the batch was set aside and allowed to cool to 205°F. No skimming was required. After cooling, the required amount of standard citric acid was added to the batch, which was then poured into the standard ridgelimeter jars, and/or aluminum moisture dishes 2 in. in diameter by ⅞ in. deep. All samples were held at room temperature for 36–48 hr before testing.

Most of the test batches were prepared with sucrose at a soluble solids content of 65%. Gels containing sucrose at 62% and 68.5% SS were also tested.

Citrus pectin (150-grade) was used in all tests. Slow-set pectin from one well mixed pectin lot was used in all of the tests, except for the few tests using rapid-set citrus pectin.

The "standard" pectin batches contained the amount of 150-grade pectin required to gel the quantity of sugar in the batch (i.e., in the proportion of 1 lb of pectin to 150 lb of sugar). A series of batches were also prepared with ¾ or 1½ of the "standard" amount of pectin.

For most batches the pH was 2.6, although batches were made with pH's from 2.2 to 3.0 by varying the quantity of citric acid used.

Gels were made up wherein 25% of the sweetener was Cerelose or glucose, the balance of the sweetener being sucrose. Also, batches were made up using only Cerelose or glucose as sweetener.

Measurement of rheological properties. The samples in the dishes were trimmed and turned out (unmolded) onto a steel plate which rested on the cantilever beam assembly, or onto the glass plate supplied with the Exchange ridgelimeter. Each sample was tested at room temperature (70°F). All samples tended to sag from their own weight when unmolded.

Compression loading was done with Bellows "Valvair" unit (Fig. 1). The force was applied at a number of speeds to the gel through a circular plunger 1 in. or 1½ in. in diameter.

A series of tests were performed where a gel sample was subjected to rapid loading and unloading using the impact beam (Fig. 2) and the 1-in.-diameter plunger.

Impact stresses were applied by releasing a weighted beam (Fig. 2). The theoretical velocity at impact for a body in free fall would be 1200 in. per min. The average velocity from motion pictures was found to be 155 in. per min. This value agrees with the terminal velocity of 300 in. per min at impact estimated from the oscillograph records. Therefore it is assumed that the impact tests were done at an impact velocity of 300 in. per min. The deformation under static loads (creep) was measured after carefully lowering the weighted beam (Fig. 2) upon the sample so that the plunger head contacted the center of the test specimen. After contact was made, the beam was released.

RESULTS

The stress-strain curves calculated from the force-deformation relationships obtained at various rates of loading on gels containing 65% sucrose and standard pectin at pH 2.6 are shown in Fig. 3. Each curve represents the average values obtained from at least five different test batches. A typical curve representing the average of five tests is shown in Fig. 4. Figs. 5 and 6 illustrate the variation in stress-strain response when pH or sucrose content is varied.

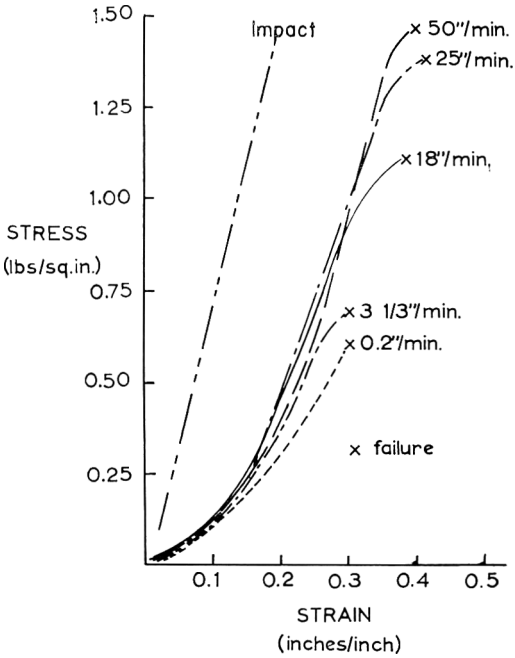

Fig. 3. Stress-strain relationships at various rates of strain. Each curve represents the average values for five tests on 65% sucrose gel pH = 2.6. Standard pectin.

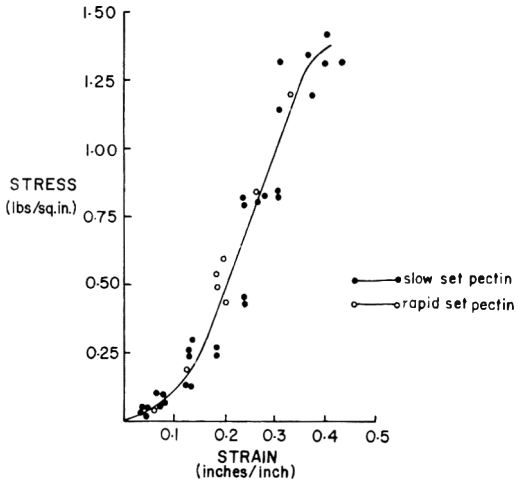

Fig. 4. Stress-strain relationships for five 65%-sucrose gels, pH = 2.6. Standard pectin, rate of strain = 25 in. per min.

In an ideal elastic material, the relationship between applied force (stress) and deformation (strain) can be expressed in the form:

$$E = \frac{\sigma}{\epsilon}$$

where E = Young's Modulus

σ = stress, in lb per sq in.

ϵ = strain, in inches per inch.

Since no allowance has been made for the stress concentration occurring under the plunger, the

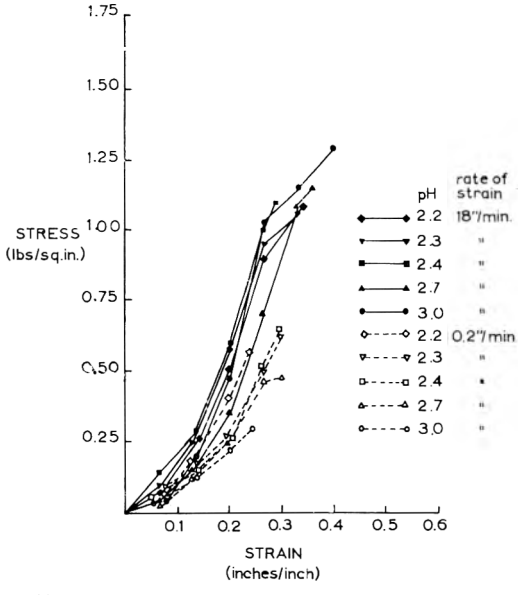

Fig. 5. Stress-strain relationships for 65%-sucrose gels. Standard quantities of slow set pectin.

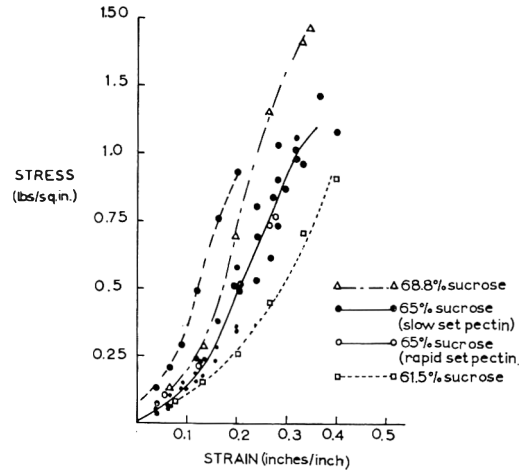

Fig. 6. Stress-strain relationships for sucrose gels at a rate of strain of 18 in. per min, pH = 2.6. Standard quantities of slow-set pectin except where otherwise noted.

term "apparent modulus" is used to describe the ratio of stress over strain. Fig. 7 shows the apparent modulus at failure (cosecant modulus) for various rates of loading as the pectin content is varied. Similar curves for varying sugar content and pH levels can readily be obtained from Figs. 3, 5, and 6.

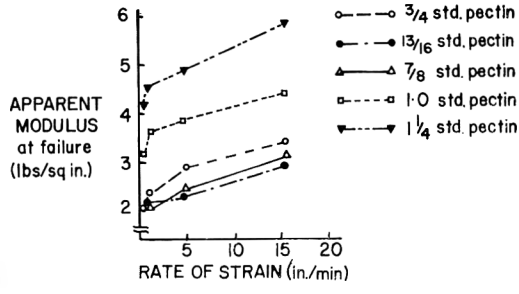

Fig. 7. Apparent modulus at failure for 65%-sucrose gels, pH = 2.6.

The stress-strain response to repeated loading and unloading (with increasing stress) for a 65% sucrose gel is illustrated in Fig. 8.

Fig. 9 illustrates a typical static loading curve of deformation and recovery with time for a 65% sucrose pectin-sugar-acid gel, at a constant loading of 0.34 lb.

Fig. 10 shows typical static loading deformation (to failure) with time for various gel compositions when subjected to constant stress. The strain at failure was remarkably constant. The average values for these creep tests are tabulated in Table 1.

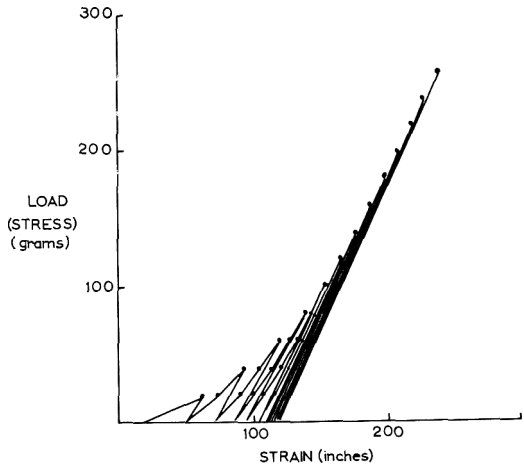

Fig. 8. Typical loading-unloading stress-strain relationship for 65%-sucrose gel.

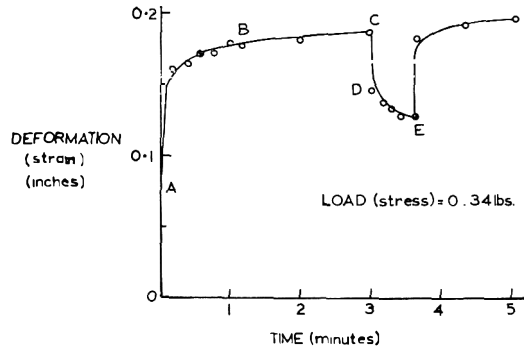

Fig. 9. Creep deformation for 65%-sucrose gel. Standard quantity of slow-set pectin, pH = 2.6.
OA, Instantaneous deformation
AB, Delayed elasticity
BC, Viscous flow
C, Unloaded
CD, Instantaneous recovery
DE, Delayed recovery
E, Reloaded

In Fig. 11, the lengths of time required for various gels to deform (creep) to failure under constant stress are plotted against the applied stress.

Fig. 12 illustrates the sag (%) as measured by the Exchange ridgelimeter for various gel compositions and conditions.

DISCUSSION

Over the range of loading rates studied, all gel samples when loaded at less than .2 lb per sq in. underwent deformations up to .15 inch per inch. This would be equivalent to an apparent modulus of approximately 1.25 lb per sq in. Although it is not obvious from the compression test results, it was later observed in the loading and unloading tests at small stress that the deformation was not completely recoverable, and therefore was not elastic in character. Some sort of "plastic flow" was occurring (at these very low stresses).

At stresses greater than .25 lb per sq in. the response of the gel becomes quite elastic (Figs. 3, 4, 5, 6) although very dependent

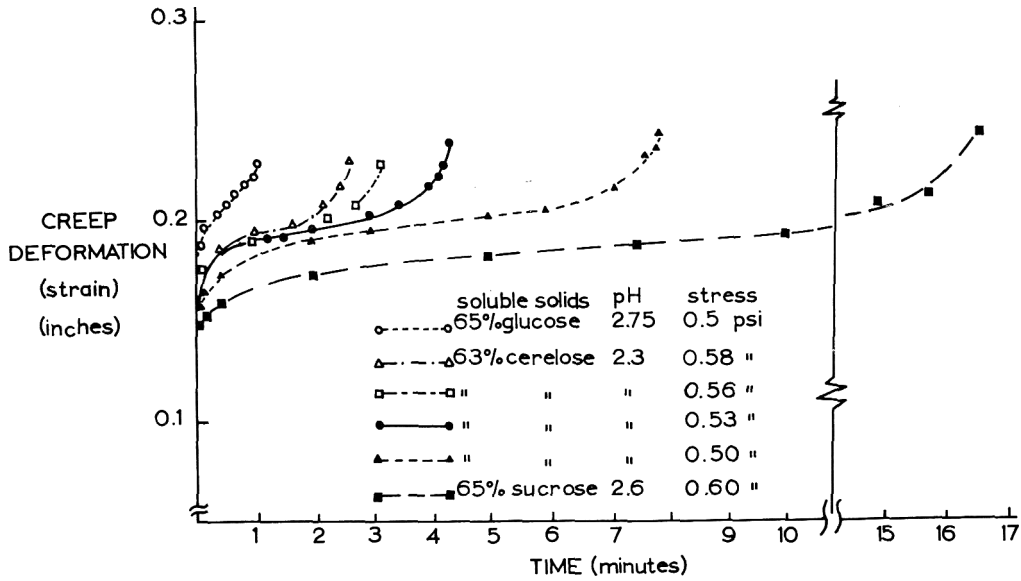

Fig. 10. Typical creep deformation (to failure) of gels containing standard quantities of slow-set pectin.

Table 1. Average values found in the creep test for several gels.

Type of sugar	% sugar	Slow set pectin content	pH	No. of batches tested	Av. strain at failure (in.)	Constant stress (psi)	Av. creep time (min to failure)
Sucrose	65.0	Standard	2.6	5	0.25	0.61	21.0
Sucrose	65.0	Standard	2.58	7	0.27	0.64	4.5
Glucose	65.0	Standard	2.7	9	0.22	0.50	3.6
Cerelose	63.0	Standard	2.3	10	0.25	0.55	4.3

upon the rate of strain. The value of the apparent modulus at failure is also highly dependent on the rate of strain (see Fig. 7). The failure was quite brittle in most cases. In the compression tests, the variability of the results (see Figs. 3, 4, 5, 6) makes it very difficult to distinguish small differences in strength between individual batches of gel. Therefore the test as presently conducted would not be suitable for evaluating gel strength on a commercial basis.

Impact. Frictional resistance in the apparatus, as well as other variables, caused the results of the impact tests to be variable. It was observed that the apparent modulus, as measured by the impact tests, ranged from 6 to 8 lb per sq in., which is considerably higher than the values obtained at lower rates of strain (see Figs. 3, 6). This is to be expected, since the slope of the stress-strain curve is greatly influenced by the rate of strain.

Creep. The average values in Table 1 indicate that, for a given batch composition, the time for creep to failure varies from a few seconds to many minutes. It is interesting to note the tendency of all batches to fail at approximately .25 in. of deformation. Although the batch compositions are not strictly comparable, it is strongly indicated that the disaccharides are able to support greater loads for longer times than the batches containing a monosaccharide sweetener.

Fig. 9 would tend to confirm the "plastic flow" characteristics found in the compression tests, since, on unloading after 3 min

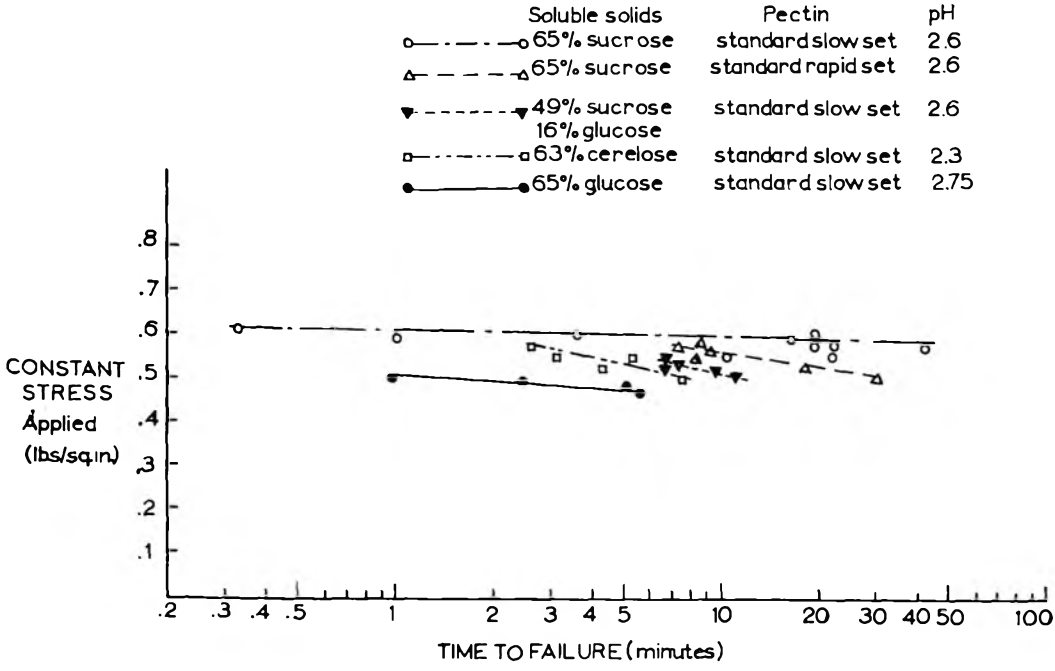

Fig. 11. Time for various gels to fail when subjected to a constant stress.

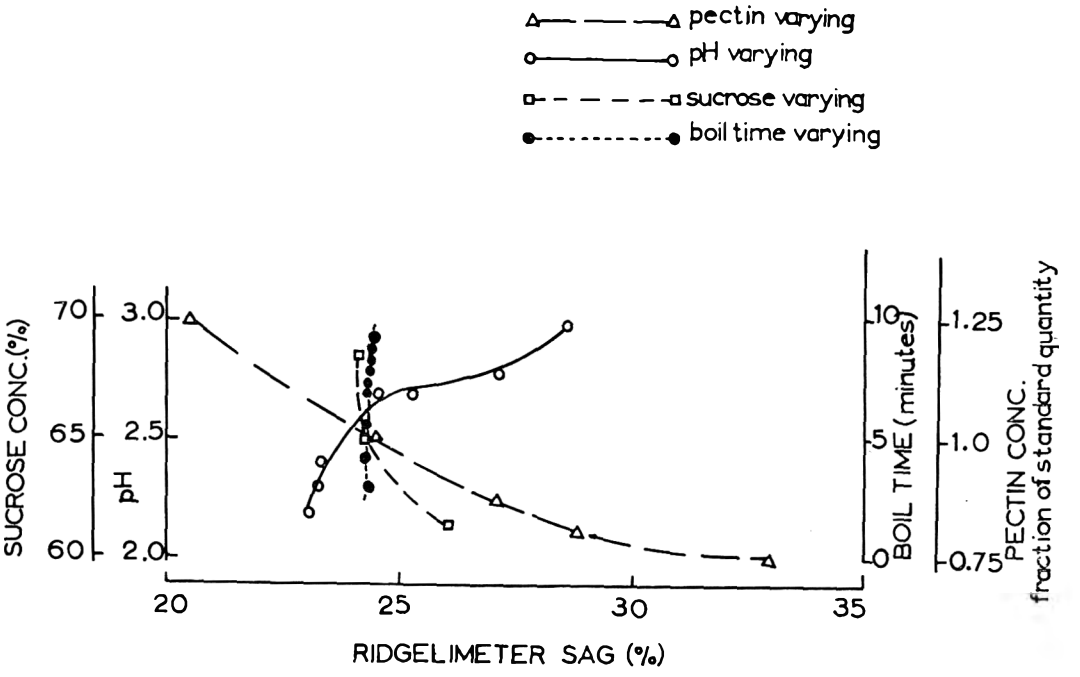

Fig. 12. Variation in percent Ridgelimeter sag when one factor is varied at a time. Basic gel contains 65% sucrose, standard quantity of slow-set pectin with pH = 2.6.

of loading, both the instantaneous and transient gel recovery indicate a permanent deformation of approximately .13 in. The slope of the viscous-flow portion of the curve would indicate that deformation due to viscous flow would be 0.01 in. in 3 min. Therefore, 0.12 in. of this permanent deformation could not have been caused by viscous flow.

Ridgelimeter. The variation of the ridgelimeter (sag readings) with the different variables (Fig. 12) indicates good correlation of the test variables with the ridgelimeter results.

General observations. It was observed that gel samples are subject to "work-hardening" (Figs. 8, 9). The effect of this "work-hardening" on test results merits considerable more study. This probably means that a gel can be meaningfully tested only once. It is possible that the amount of handling the gel receives before testing influences the results obtained.

The diameter of the test plunger influenced the results of the tests; the moduli appeared to be about 10% higher for the tests done with the 1½-in.-diameter plunger than for the 1-in.-diameter plunger. This was not expected, since Timbers *et al.* (1965) found that stress concentration under the plunger should yield larger apparent moduli under the smaller-diameter plunger.

CONCLUSIONS

The rheological properties of sugar-pectin-acid gels measured in this study exhibited considerable variability. The most likely causes of this variability are: 1) the sag of the unconfined samples; 2) the time-dependency on this sag; 3) the "work-hardening" of the samples before testing; 4) the friction and inertia in the measuring apparatus (particularly in the impact tests); 5) the experimental errors in batch preparation; and 6) the zeroing of measuring equipment, etc. The experimental and measuring errors are not considered to be a major factor contributing to the variability.

Certain attributes of the sugar-acid-pectin gels were quite definitely indicated:

1) The presence of a "plastic flow" (nonrecoverable strain) at low stress.

2) The dependence of strength criteria upon the rate of strain when tested in both the "plastic flow" and elastic regions.

3) The presence of only small differences between mono- and disaccharide gels when tested by either compression or impact.

4) The existence of rather substantial differences in creep behavior between gels containing mono- and disaccharides, the latter supporting greater stresses for longer times.

5) The presence of a typically "brittle" failure for all types of gels.

6) The apparently insignificant effect of pectin degradation during boiling upon subsequent rheological properties.

The deformation of rubber and some cross-linked plastics has been interpreted by Howard (1949) and Treloar (1958) as an extension of a randomly jointed chain molecular network. The deformation of gels has been interpreted by Anon (1960) and Matz (1962) in the light of these theories with the addition of water and sugar, acting to swell and plasticize the basic gel-forming chains. However, this work shows that the initial deformation of a gel is not recoverable, and hence is not strictly comparable to rubber or plastic deformation curves displaying similar patterns. Reiner (1960) reported that a similar permanent deformation has been associated with gelatin solutions.

On the basis of these results, it appears that at least two types of bonding are involved in the pectin-acid-sugar gel structure. One type of bond is very weak, and capable of re-forming after disruption. The other bonds would be stronger, probably randomly jointed, and could impart the elastic properties exhibited by the gels at stresses above 0.25 lb per sq in.

A number of characteristics were indicated, but require verification. They were:

1) The sugar-pectin-acid gels appear to have the ability to absorb the greater portion of the energy applied to them. On this basis such gels would likely have a low relative elasticity.

2) They appear to require at least two relaxation constants.

3) They appear to be highly pseudoplastic and thixotropic.

REFERENCES

Anon. 1960. Physical functions of hydrocolloids. *Advances in Chem. Sci.* **25.**

AOAC. 1960. Methods of analysis. 9th ed. Assoc. offic. Agr. Chemists. p. 135 and 419.

Bland, D. R. 1960. The theory of linear viscoelasticity. Pergamon Press, N. Y.

Campbell, L. E. 1937–38. The calibration of jelly testers. *J. Soc. Chem. 2nd* (Trans) **56–57**, 413.

Cheftel, H., and J. Mocquard. 1947. The rheological properties of pectin jellies. *J. Soc. Chem. 2nd.* **66**, 297.

Christensen, P. E. 1954. Methods of grading pectin in relation to the molecular weight of the pectin. *Food Research* **19**, 163.

Harvey, H. G. 1960. Gels—with special reference to pectin gels. *Texture in Foods. London Soc. Chem. Ind. Mono. No.* **7.**

Hinton, C. L. 1950. The setting temperature of pectin jellies. *J. Sci. Food Agr.* **1**, 300.

Howard, K. N. 1949. The strength of plastics and glass. Interscience Publishers, N. Y.

IFT. 1959. Pectin Standardization, Final Report of the IFT Committee. *Food Technol.* **13**(9), 496.

Joseph, G. H., and W. E. Baier. 1949. Methods of determining firmness and setting time of pectin test jellies. *Food Technol* **3**, 18.

Matz, S. A. 1962. Food Texture. Chapt. 4. Avi. Publ. Co., Westport, Conn.

Mohsenin, N. 1963. A testing machine for determining the mechanical and rheological properties of agricultural products. *Penn State Univ. Agr. Expt. Sta. Bull.* **701.**

Owens, H. S., H. A. Swenson, and T. H. Schultz. 1954. Factors influencing gelation with pectin. *Advances in Chem. Ser.* No.**11**, 10.

Reiner, M. 1960. Deformation, strain and flow. H. K. Lewis & Co., London. p. 150.

Solms, J. 1960. Some structural aspects of gel formation of pectins and related polysaccharides. *Advances in Chem. Ser.* No. **25**, 37.

Timbers, G. E., L. M. Staley, and E. L. Watson. 1965. Determining modulus of elasticity in agricultural products by loaded plungers. *Agr. Eng.* **46**(5), 274.

Treloar, L. R. G. 1958. The physics of rubber elasticity. 2nd ed. Clarendon Press, Oxford. p. 120.

Van Wazer, J. R., J. W. Lyons, K. Y. Kim, and R. E. Colwell. 1963. Viscosity and flow measurement. Chapt. 6. Interscience Publishers, New York, N. Y.

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The Thermal Degradation of Sugars. II. The Volatile Decomposition Products of Glucose Caramel

SUMMARY

Twenty-two compounds in the volatile fraction of glucose caramel were separated and identified. The material of the neutral volatile fraction for gas chromatography was prepared by the adsorption method on an active charcoal, and separation of the volatile acids by appropriate pH adjustment, steam distillation, and extraction.

The volatile compounds tentatively identified on the basis of their retention data included 2 furan compounds, 2 alcohols, 11 carbonyl compounds, and 7 fatty acids. In addition, 6 peaks in the neutral fraction and 1 peak in the acidic fraction were found but not identified.

INTRODUCTION

The thermal degradation of sugars during processing at high temperature is one of the important problems of food manufacture. The chief concern in the author's laboratory has been to determine the thermal degradation products of sugars. In the previous paper (Sugisawa and Edo, 1964), it has been reported that α-D-glucose has gone to higher oligosaccharides by heating at 150°C, and some of these oligosaccharides were isolated from glucose caramel by charcoal column chromatography.

Interest in the volatile decomposition products in caramel has always been high, but many workers have been discouraged by the arduous methods of separation that were necessary before the development of gas chromatography. As the first step in elucidating this problem by chemical means, separation of the thermal decomposition products from glucose caramel has been attempted. The separation of volatile compounds was carried out by using the adsorption method on an active charcoal described by Strackenbrock (1961).

There is considerable precedent for this work, since the thermal decomposition products of carbohydrates (Greenwood *et al.*, 1961) and the volatile decomposition products of starch (Bryce and Greenwood, 1963) have been determined by gas chromatography, although these pyrolyses were examined at higher temperature (about 300°C) than that of caramelization of sugar.

EXPERIMENTAL

Preparation of condensable volatiles from glucose caramel. Dried glucose (100 g) was heated for 2.5 hr at 150°C in the retort connected with the adsorption tower of volatiles as shown in Fig. 1. The tower was packed with 130 g of granular active charcoal and served to adsorb the volatile fraction passed through a series of cold traps cooled with ice and water. After pyrolysis of glucose, the charcoal was taken out from the tower, and transferred to the desorption apparatus (see Fig. 2). The charcoal in the desorption apparatus was heated with refluxing of boiling water for 1 hr. The flow rate of nitrogen was adjusted to 20 ml/min, and the volatile compounds desorbed from charcoal were received into the sample collecting tube for gas chromatography (Sugisawa, 1965) kept at about −70°C. After centrifugation of the tube (3000 rpm, 3 min), 0.5 ml of condensable volatile compounds was recovered.

Acidic fraction. The most convenient method for separating the acidic fraction from glucose caramel is by steam distillation and extraction with solvent, after adjustment of pH. The total acidity and the volatile acidity were determined by the method described by Ruck (1956). Total acidity was 0.052% (as succinic acid), and volatile acidity 0.014% (as acetic acid).

The acidic fraction was prepared as follows: The pyrolysate was dissolved into 1 L of water, and the solution (pH 3.4) was adjusted to pH 8.3 by addition of 10% sodium carbonate solution. The neutral volatile fraction was removed by steam distillation for 30 min. The residue was extracted with ether to remove the neutral nonvolatile frac-

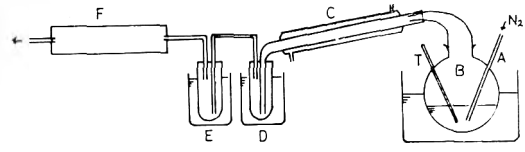


Fig. 1. Schematic drawing of the apparatus of pyrolysis. A = nitrogen inlet, flow rate 20 ml/min, B = 1 L retort, C = condenser, D and E = cold traps, F = the charcoal tower (4 × 30 cm) packed with granular active charcoal and one end of the tower was connected with gentle suction, T= thermometer.

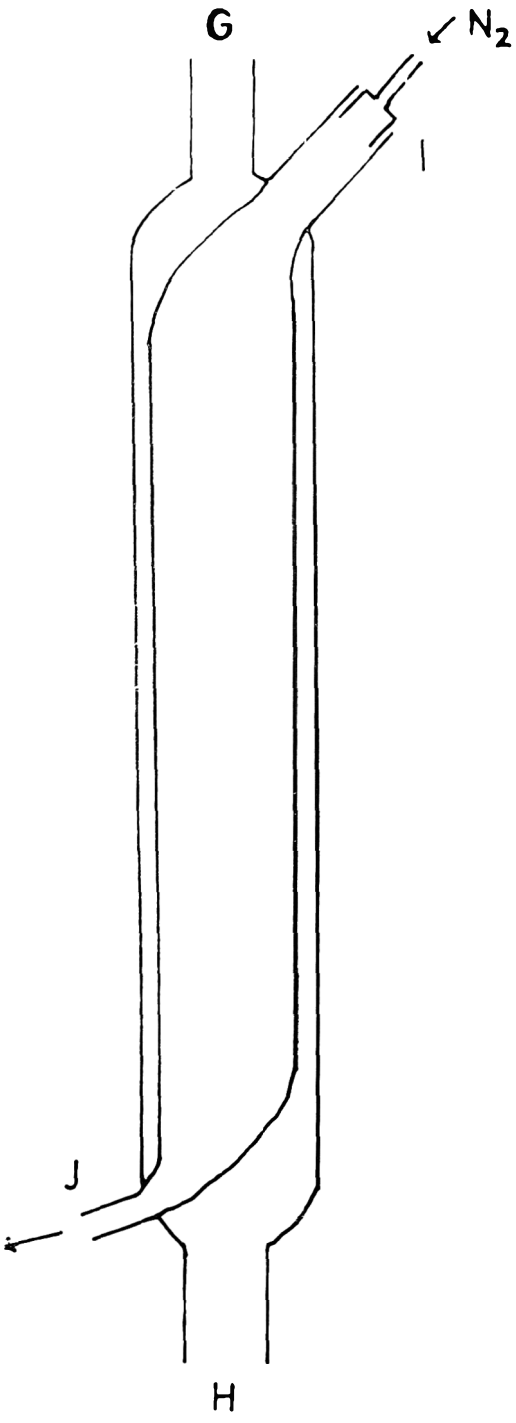


Fig. 2. Drawing of the desorption apparatus. G = reflux condenser, H = 1 L flask, I = charcoal and nitrogen inlet, desorption tube 5 × 52 cm, J = outlet of volatiles. Inlet and outlet were packed with glass-wool.

tion. After extracting with ether, the volatile acidic fraction was recovered from the alkaline aqueous solution by adjusting the pH to 1.0 with dilute sulfuric acid and then distilling with steam. The condensate was saturated with sodium chloride, and the volatile acids were extracted with 100 ml of ether for 30 min on a mechanical shaker. About 0.2 ml of a slightly yellow-colored viscous liquid with a strong acidic odor was obtained from 1 L of the pyrolysate solution. This extract was used as the sample of volatile acidic fraction. After distillation of volatile acids, the residue was continuously extracted with ether for 24 hr, and about 1 ml of the extract was used as the sample of nonvolatile fatty acids.

Gas chromatography of volatile acids. The volatile acids thus obtained were methylated by using the ether solution of diazomethane prepared from nitrosomethyl-urethan (Arndt, 1943). The ether solution of methylated sample was injected into gas chromatography, and the composition of the methyl esters was examined with two types of column packings: 20% Carbowax-400 on Chromosorb W, and 5% diethyleneglycol succinate on Shimalite C. A chromatogram of the methyl esters is shown in Fig. 3.

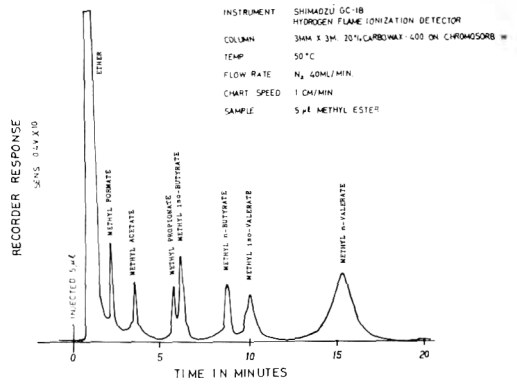


Fig. 3. Chromatogram of methyl esters of the volatile acidic fraction.

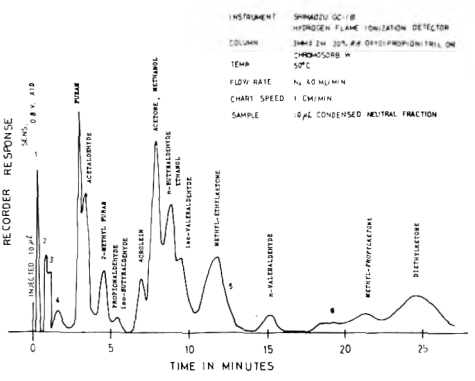
Paper chromatography of nonvolatile fatty acids. The components of the nonvolatile fatty acid fraction were determined by paper chromatography irrigated in a solvent system of formic acid–n-butanol–water (1:4:1 v/v). The chromatogram was developed with 0.04% alcohol solution of bromphenol blue.

Gas chromatography of neutral volatile fraction. The sample was prepared by the adsorption method on an active charcoal as described above. The sample contained not only neutral volatile compounds but also traces of volatile fatty acids, but the latter did not interfere with gas chromato-

graphic analysis, since the fatty acids remained in the column under the conditions employed herein.

The neutral volatile components were analyzed with a Shimadzu GC-1B gas chromatography equipped with a hydrogen flame ionization detector. Many types of solid and stationary-phase materials were tested before it was found that the following three columns gave adequate separation of the volatiles for analytical work: a) 20% Carbowax-400 on Chromosorb W; b) 20% β,β'-oxydipropionitril on Chromosorb W; c) 10% (Carbowax-400 and dibutylphthalate 9:1) on Chromosorb W.

Fig. 4 shows a typical separation when only 10 μl of sample was injected. The exact experimental parameters of operation—temperature, flow rate of carrier gas, etc.—accompany the figures and tables.



Chromatogram of the neutral volatile fraction.

RESULTS AND DISCUSSION

This adsorption method on an active charcoal is very suitable for collecting a small amount of the volatile compounds. Neutral volatile compounds could be separated by appropriate pH adjustment and steam distillation. However, the neutral fraction obtained by steam distillation was not suitable for gas chromatographic analysis, because of the loss of easily volatile material during steam distillation.

Strackenbrock (1961) studied the flavors of apples by the adsorption method on the charcoal and reported that the desorption of volatiles was carried out by distillation under vacuum at 250°C, the volatiles being condensed in a tube kept at −70°C, and tests with pure substances showed that these were not chemically changed by adsorption and subsequent desorption of the charcoal.

Tables 1 and 2 give the relative retention volumes of the volatile compounds of glucose caramel and authentic compounds. Methyl ethylketone was used as the reference compound for the neutral volatile fraction, and methyl propionate for the volatile acidic fraction.

Two furan compounds, two alcohols, and eleven carbonyl compounds were tentatively identified by using three different columns. These volatile compounds of glucose caramel are qualitatively identical to those from glucose pyrolysate at 300°C described by Bryce and Greenwood (1963), it is found that these volatiles were already formed during caramelization at 150°C. Peaks 1 through 6 in Fig. 4 were not identified. Four peaks before acetaldehyde and furan were detected with every column, but these peaks could not be declared whether these were really the volatiles from glucose or not. Most of water formed by thermal dehydration was collected by using a series of cold traps under reduced pressure, although water and permanent gases in the volatile fraction of glucose caramel could not be detected by this gas chromatographic setup, and also the presence of formaldehyde was not examined. 2-Methyl furan and acrolein could not be separated with column *A* or column *C*, but were separated very well with column *B*.

In the volatile acidic fraction, seven volatile fatty acids were identified by comparison of their relative retention volumes with those obtained for standard compounds under identical conditions. Methyl propionate and methyl iso-butyrate could not be separated satisfactorily with column *E*, but the separation of these compounds was achieved with column *D*. One unknown compound could be separated by means of only column *E*, but this peak did not show up when the sample was chromatographed with column *D*.

Succinic, fumaric, levulinic, and furan carboxylic acids as the nonvolatile acids were identified by paper chromatography.

5-Hydroxymethyl furfural was obtained by distillation of the neutral nonvolatile fraction and identified by measuring of ultraviolet spectrum, λ_{max} 283 mμ in methanol. Further it was identified as derivative of 2,4-dinitrophenyl hydrazone (Shriner *et al.*, 1956) by the melting point of an admixture of the derivative and authentic component,

Table 1. Relative retention volumes of volatiles in glucose caramel and authentic compounds.

Name	Column *A*		Column *B*		Column *C*	
	Standard	Sample	Standard	Sample	Standard	Sample
Unknown 1	.....	0.07	.....	0.02	.....	0.02
Unknown 2	.....	0.10	.....	0.06	.....	0.12
Unknown 3	.....	0.13	.....	0.08	.....	0.14
Unknown 4	.....	0.19	.....	0.11	.....	0.18
Acetaldehyde	0.27	0.27	0.27	0.26	0.22	0.22
Furan	0.40	0.41	0.23	0.23	0.31	0.31
Propionaldehyde	0.46	0.45	0.45	0.46	0.37	0.37
iso-Butyraldehyde	0.50	0.50	0.46	0.46	0.45	0.44
Acetone	0.57	0.57	0.70	0.66	0.53	0.54
2-Methyl furan	0.64	0.63	0.37	0.37	0.53	0.54
Acrolein	0.64	0.63	0.61	0.60	0.53	0.54
n-Butyraldehyde	0.77	0.77	0.74	0.76	0.69	0.70
Methyl-ethylketone	1.00	1.00	1.00	1.00	1.00	1.00
Unknown 5	.....	.....	.....	1.08	.....	1.05
iso-Valeraldehyde	1.02	1.02	0.87	0.86	1.16	1.17
Methanol	1.38	1.39	0.70	0.66	0.93	0.94
Unknown 6	.....	1.60	.....	1.62	.....	1.50
Methyl-propylketone	1.68	1.66	1.86	1.86	1.80	1.80
Diethylketone	1.68	1.66	2.10	2.12	1.80	1.80
Ethanol	1.86	1.86	0.79	0.79	1.42	1.42
n-Valeraldehyde	2.00	2.05	1.18	1.16	1.82	1.84

Retention volumes relate to methyl-ethylketone.
Column *A*: 20% Carbowax-400 on Chromosorb W, 50–80-mesh, 3 mm × 2.25 m, 50°C.
Column *B*: 20% β,β′-Oxydipropionitril on Chromosorb W, 80–100-mesh, 3 mm × 2 m, 50°C.
Column *C*: 10% (Carbowax-400 and dibutylphthalate, 9:1) on Chromosorb W, 80–100-mesh, 3 mm × 3 m, 30°C.

Table 2. Relative retention volumes of the methyl esters of volatile fatty acids in glucose caramel and authentic compounds.

Name	Column *D*		Column *E*	
	Standard	Sample	Standard	Sample
Methyl formate	0.47	0.46	0.50	0.51
Methyl acetate	0.66	0.65	0.69	0.70
Methyl propionate	1.00	1.00	1.00	1.00
Methyl iso-butyrate	1.08	1.08	1.03	1.00
Methyl n-butyrate	1.54	1.53	1.50	1.52
Methyl iso-valerate	1.86	1.85	1.78	1.80
Unknown	.....	.....	.....	2.50
Methyl n-valerate	2.82	2.80	2.64	2.65

Retention volumes relate to methyl propionate.
Column *D*: 20% Carbowax-400 on Chromosorb W, 60–80-mesh, 3 mm × 3 m, 50°C.
Column *E*: 5% DEGS on Shimalite C, 60–80-mesh, 3 mm × 3 m, 50°C.

mp 200–201°C. However, it is hoped that the separation of other components in neutral nonvolatile fraction still in progress will determine at least some of them.

It has been known that cross plotting or two-dimensional plotting is one of the simplest ways of obtaining a second parameter for peak identification (Sugisawa *et al.*, 1962; Bryce and Greenwood, 1963). A cross plot was made by plotting the log of the relative retention volumes of the sample run on one column against the log of those of the same sample on a second column. When the values for the relative retention volumes of carbonyl compounds obtained from glucose caramel were plotted in this manner, the identities of the compounds given in Fig. 5 were confirmed. Acetone-acrolein and n-valeraldehyde–methyl propyl-ketone respectively line up on a vertical line; it

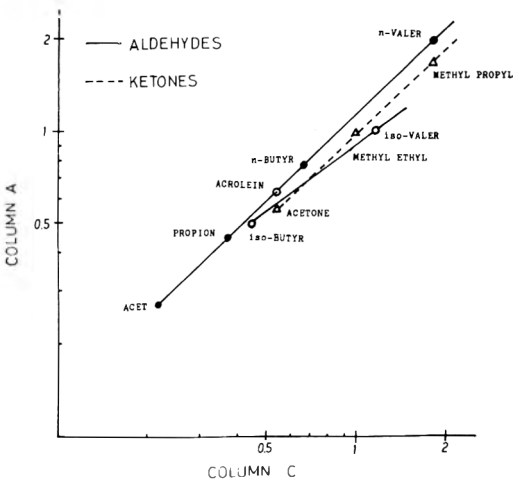


Fig. 5. Cross plot of log relative retention volumes of carbonyl compounds on two different columns.

means that these compounds cannot be separated with column *C*. Similarly, isovaleraldehyde and methyl ethylketone line up on a horizontal line; these two cannot be separated with column *A*.

REFERENCES

Arndt, F. 1943. Diazomethane. "Organic Syntheses." Collection, Vol. 2, p. 165. John Wiley & Sons, New York.

Bryce, D. J., and C. T. Greenwood. 1963. The thermal degradation of starch. II. The identification by gas chromatography of the minor volatile products produced at 300°C. *Stärke* **15**, 285.

Greenwood, C. T., J. H. Knox, and E. Milne. 1961. Analysis of the thermal decomposition products of carbohydrates by gas chromatography. *Chem. & Ind. (London)*. **1961**, 1878.

Ruck, J. A. 1956. Chemical method for analysis of fruit and vegetable products. *Can. Dept. Agr. Chem. Sci. Service*, Summerland, B. C. Contrib. No. 350, p. 8.

Shriner, R. L., R. C. Fuson, and D. Y. Curtin. 1956. "The Systematic Identification of Organic Compounds." 4th ed. p. 219. Maruzen Co., Tokyo.

Strackenbrock, K. H. 1961. Nachweis sortenspezifishe apfelaromen. *Erwerbsobstbau* **3**, 147.

Sugisawa, H., J. S. Matthews, and D. R. MacGregor. 1962. The flavor spectrum of apple wine volatiles. II. Volatile fatty acids. *J. Food Sci.* **27**, 435.

Sugisawa, H., and H. Edo. 1964. Thermal polymerization of glucose. *Chem. & Ind. (London)*. **1964**, 892.

Sugisawa, H. 1965. The design of sample collecting tube for gas chromatography. *Chem. & Ind., Chem. Soc. Japan* **18**, 417.

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Reactivities of Lipid Solvents with Thiobarbituric Acid

SUMMARY

Methyl alcohol, ethyl alcohol, chloroform, diethyl ether, petroleum ether, cyclohexane, and carbon tetrachloride were tested for reactivity with thiobarbituric acid. Pigments with absorption maxima at 450 and 532 mμ were readily formed from petroleum ether and diethyl ether; methyl alcohol, ethyl alcohol, chloroform, and hexane reacted, but to a lesser extent; carbon tetrachloride was unreactive. The production of these malonaldehyde-like compounds, presumably from contaminants in the solvents, was increased by heating.

Kohn and Liversedge (1944) observed that animal tissues which had been incubated aerobically gave a pink chromogen with 2-thiobarbituric acid (TBA). Bernheim *et al.* (1948) found this red color to be the result of a complex formed from oxidation products of unsaturated fatty acids and 2-thiobarbituric acid. The TBA reaction thereafter became an expirical assay of lipid autoxidation and has been widely used in studies of various foods and animal tissues (Simnhuber and Yu, 1958; Tarladgis *et al.*, 1960). The red pigment with an absorption maximum at 532 mμ has been identified as a condensation product of one molecule of malonaldehyde (MA) and 2 molecules of TBA (Simnhuber and Yu, 1958). Tarladgis *et al.* (1962, 1964) have shown that acid and heat used in the TBA reaction are important factors in color development. Unknown substances in acetic acid, which is a solvent commonly used for the TBA reagent, have been shown to be responsible for the formation of an interfering yellow pigment (Yu and Simnhuber, 1964). In fact, it has been found that TBA can form red and yellow pigments, having absorption peaks in the vicinity of 532 and 450 mμ, with ethyl alcohol, methyl alcohol, and formic acid (de Koning and Silk, 1963); glyceraldehyde and epihydrin aldehyde (Patton, 1960); and certain pyrimidines (Shepherd, 1948; Simnhuber *et al.*, 1958).

The TBA assay for lipid oxidation often involves the use of lipid solvents (Dahle *et al.*, 1962. Tarladgis *et al.*, 1960). In experiments using the TBA reaction we noted that a variety of solvents would, alone, react with TBA. This report gives detail in that regard.

EXPERIMENTAL

TBA reagent was prepared in either HCl (Simnhuber and Yu, 1958) or acetic acid (Tarladgis *et al.*, 1962). Seven solvents tested for reactivity with TBA were: methyl alcohol, ethyl alcohol, chloroform, diethyl ether, petroleum ether, cyclohexane, and carbon tetrachloride. Each solvent was distilled until the "pot" volume was about one-tenth of the starting material. The distilled solvent and the "pot" concentrate were both tested for reactivity with TBA. Equal volumes of sample and TBA reagent were reacted in a water bath for 30 min. The absorption spectra against glass-distilled water were recorded in a Cary Model 11 spectrophotometer.

RESULTS AND DISCUSSION

Petroleum ether and diethyl ether, heated with TBA-acetic acid, yield products which absorb very strongly at 532 and 450 mμ. Methyl alcohol, ethyl alcohol, cyclohexane, and chloroform, similarly treated, produce lesser, though readily detectable, amounts of chromogen (Fig. 1). When petroleum ether

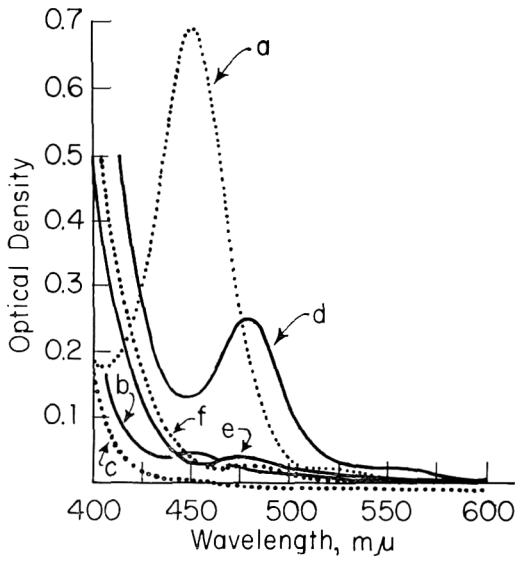

Fig. 1. Absorption spectra of complexes prepared by heating thiobarbituric acid in acetic acid with: a) petroleum ether; b) cyclohexane; c) carbon tetrachloride; d) diethyl ether; e) methyl alcohol; f) chloroform.

and diethyl ether were distilled, the residual fractions contained more TBA-reacting substances that did the distillates (Fig. 2, 3). However, more TBA-reacting material was present in either the residue or distillate than in the original material. That implies that some unknown substance or substances capable of reacting must have been produced by the heating. When HCl was used instead of acetic acid for the reaction, absorption of the chromagen at 532 mμ was lowered, and the peak at 450 mμ disappeared. However, another peak appeared at 495 mμ.

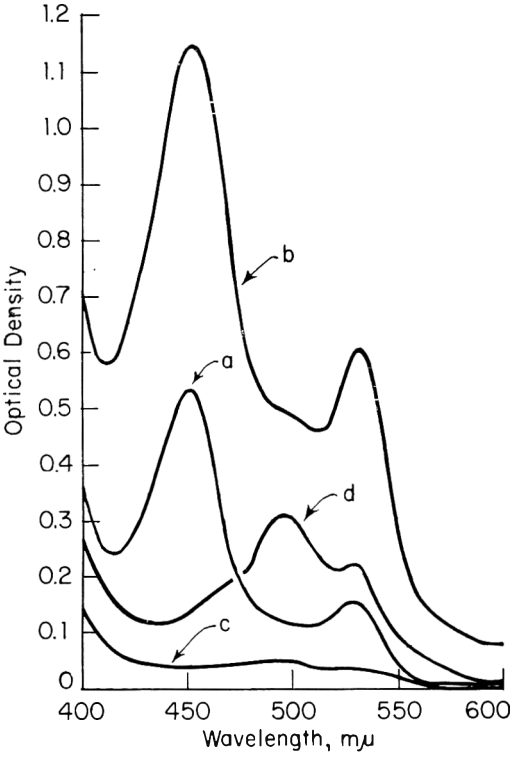

Fig. 2. Absorption spectra of complexes prepared by heating thiobarbituric acid: a) in acetic acid with distillate of petroleum ether; b) in acetic acid with residual fraction of distilled petroleum ether: c) in HCl with distillate of petroleum ether; d) in HCl with residual fraction of distilled petroleum ether.

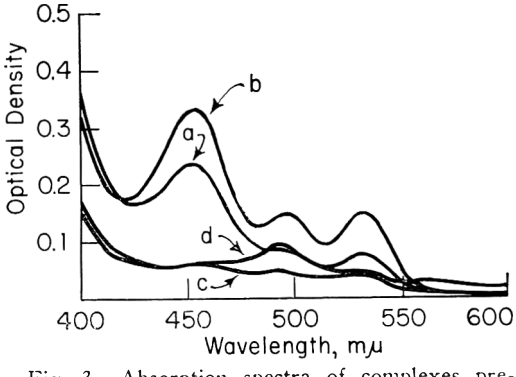

Fig. 3. Absorption spectra of complexes prepared by heating thiobarbituric acid: a) in acetic acid with distillate of diethyl ether; b) in acetic acid with residual fraction of distilled diethyl ether; c) in HCl with distillate of diethyl ether; d) in HCl with residual fraction of distilled diethyl ether.

It has been shown (Yu and Sinnhuber, 1964) that H_2O_2 or other peroxides can react with TBA in acetic acid to form a yellow pigment with an absorption maximum at 450 mμ. Tarladgis *et al.* (1962) also observed that H_2O_2 can react with TBA-acetic acid system, the resulting product having absorption maxima at 450 and 532 mμ. Since ethers are well known to be contaminated with small amounts of peroxides, our results with ether may be due to peroxide contamination. Yu and Sinnhuber (1964) found that TBA-acetic acid reagent, when heated alone, produced a pigment, and showed that acetic could be purified by reaction with 2,4-dinitrophenylhydrazine or with potassium dichromate. In agreement with his observation, we found more pigment formed when acetic acid was used; however, substantial amounts of pigment were formed even in the absence of acetic acid, i.e., in the HCl systems.

In TBA-HCl system, petroleum ether and diethyl ether were, again, most reactive. An absorption peak at 532 mμ (but not at 450 mμ) was present in the distillate and residual fractions of ethyl alcohol, methyl alcohol, chloroform, and cyclohexane. Of the seven solvents tested, only carbon tetrachloride was completely free from any TBA-reacting material, regardless of whether HCl or acetic acid was used.

TBA is a very reactive compound. It is clear that every precaution should be taken in use of the TBA reaction where any lipid solvent is used, especially if a heat treatment is involved. For the quantitative determination of lipid oxidation by the TBA method, not only acetic acid but also lipid solvents should be purified. Preliminary results show that refluxing methyl alcohol or chloroform with TBA effectively traps TBA-reacting material, and the distillates from such systems are free of the chromagen.

REFERENCES

Bernheim, F., M. L. C. Bernheim, and K. M. Wilbur. 1948. The reaction between thiobarbituric acid and the oxidation products of certain lipids. *J. Biol. Chem.* **174**, 257.

Dahle, L. K., E. G. Hill, and R. T. Holman. 1962. The thiobarbituric acid reaction and the autoxidations of polyunsaturated fatty acid methyl esters. *Arch. Biochem. Biophys.* **98**, 253.

de Koning, J. A. and M. A. Silk. 1963. The 2-thiobarbituric acid reagent for determination of oxidative rancidity in fish oils. *J. Am. Oil Chemists' Soc.* **40**, 165.

Kohn, H. I., and N. Liversedge. 1944. On a new aerobic metabolite whose production by brain is inhibited by apomorphine, emetine, ergotamine, epinephrine, and menadione. *J. Pharmacol.* **82**, 292.

Patton, S. 1960. Response of epihydrin aldehyde and glyceraldehyde in the thiobarbituric acid test for fat oxidation. *Food Research* **25**, 554.

Shepherd, R. G. 1948. A specific analytical method for certain pyrimidines. *Anal. Chem.* **20**, 1150.

Sinnhuber, R. O., and T. C. Yu. 1958. 2-Thiobarbituric acid method for the measurement of rancidity in fishery products. II. The quantitative determination of malonaldehyde. *Food Technol.* **12**, 9.

Sinnhuber, R. O., T. C. Yu, and Te Chang Yu. 1958. Characterization of the red pigment formed in the 2-thiobarbituric acid determination of oxidative rancidity. *Food Research* **23**, 626.

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

Tarladgis, B. G., A. M. Pearson and L. R. Dugan, Jr. 1962. Chemistry of the 2-thiobarbituric acid test for the determination of oxidative rancidity in foods. I. Some important side reactions. *J. Am. Oil Chemists' Soc.* **39**, 34.

Tarladgis, B. G., A. M. Pearson and L. R. Dugan, Jr. 1964. Chemistry of the 2-thiobarbituric acid test for determination of oxidative rancidity in foods. II. Formation of the TBA-malonaldehyde complex without acid heat treatment. *J. Sci. Food Agr.* **15**, 602.

Yu, T. C. and R. O. Sinnhuber. 1964. Further observations on the 2-thiobarbituric acid method for the measurement of oxidative rancidity. *J. Am. Oil Chemists' Soc.* **41**, 450.

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Techniques for Collecting Volatile Components from Haddock Flesh for Gas Chromatographic Analysis

SUMMARY

The volatile compounds isolated from haddock (*Melanogrammus aeglefinus*) flesh were analyzed by cryogenic gas chromatography. Four methods of collecting the volatile compounds were studied and evaluated. Chromatograms showed that the best samples were obtained from the equilibrium vapor above the total condensate removed from the haddock flesh by vacuum distillation. Chromatograms illustrate the changes in the relative amounts of volatile compounds collected from samples of both raw and cooked fish after storage at 2°C for 1, 5, and 10 days. The amount and number of volatile compounds increased during storage, and cooking released additional compounds, some in large amount.

INTRODUCTION

Little is known of the volatile compounds which contribute to the flavor and odor of fishery products. Isolation and identification of these compounds are essential to discover their chemical nature and to understand their contribution to the flavor and odor of the product. Basic studies of these compounds are also needed to determine how they are affected by various storage and processing conditions. Although gas chromatography is widely used for separating the volatile components associated with the flavor and odor of foods, analysis is meaningful most generally only when the sample is collected with little or no change from its natural state. Several techniques have been used by various workers for removing the volatile compounds from foods for analysis by gas chromatography. The most widely used methods are solvent extraction (Hornstein and Crowe, 1960; Hunter *et al.*, 1961; Jennings, 1961) and distillation (Slater, 1961; Yueh and Strong, 1960) or a combination of both (Brandenberger and Müller, 1962; Hiu and Scheuer, 1961; Smith and Coffman, 1960; Vorbeck *et al.*, 1961; Weurman, 1963; Wick *et al.*, 1961). The disadvantages of solvent extraction are the inability of solvents to remove all of the odor components quantitatively and the possibility of contaminating the samples with impurities from the solvent. Distillation at elevated temperatures may lead to chemical changes. Other methods have been reported, such as high-vacuum distillation (Merritt *et al.*, 1959), flushing with an inert gas (Kramlich and Pearson, 1960; Rhoades, 1958), and direct sampling of equilibrium vapors (Buttery and Teranishi, 1961; Carrol and O'Brien, 1959; Mackay *et al.*, 1961; Nawar and Fagerson, 1962; Teranishi *et al.*, 1962). In a previous study (Mangan *et al.*, 1959) of the volatile components isolated from fish, high-vacuum distillation was used, but results obtained from further investigations have revealed that samples differ markedly in composition with the method of isolation. Therefore, a systematic study was undertaken of certain techniques for removing volatiles from haddock flesh. In assessing the significance of the analysis, of paramount importance in addition to the method of sampling is the efficiency of the gas chromatographic procedures in both degree of separability and level of sensitivity. Although, ultimately, it is the objective of the current research to determine the effects of storage and cooking on the composition of the volatile components in fish and their relation to flavor, this study was initiated primarily to evaluate certain methods of collecting an odor concentrate. In the course of the investigation considerable insight has been gained, however, concerning the change in the composition of the volatiles under the various conditions of storage for both raw and cooked fish.

This paper deals with 4 techniques for collecting volatile compounds from cold-stored haddock and the gas chromatographic analy-

ses of these mixtures. It also shows the changes in the volatile compounds from both raw and cooked haddock flesh which are due to storage.

EXPERIMENTAL TECHNIQUES

Storage conditions. The samples of haddock fillets and storage conditions have been described (Mendelsohn and Steinberg, 1962). Sensory odor evaluation of the fillet was made by the experimenters prior to storage and after each storage period. The first sample of volatiles was removed from the flesh the day the fish arrived at the laboratory. Since these fish were stored on ice almost 24 hr prior to arrival, they were considered as 1 day old. Subsequent samples, taken after the fourth and ninth days of storage in the laboratory, are referred to as the 5- and 10-day storage samples. Since haddock stored for 10 days at refrigerated temperatures, though edible, is of poor organoleptic quality, no samples were stored beyond this period. The composition of the volatiles isolated from haddock probably vary slightly with the time of the year, landing area, and handling aboard the boat. Therefore, all samples were obtained from the same source, and storage conditions in the laboratory were held constant. At each storage interval, duplicate samples were removed from storage. Various weights of samples were taken depending on the method of collection to be used. One sample was used for analysis of the volatile compounds from the raw fish, while the other fish sample was placed in a sealed glass vessel and was steamed in a pressure cooker at 110°C. This sample was placed in the cooker as soon as the water had started to boil and cooked for 20 min.

Methods of collecting volatile concentrates. Direct sampling of equilibrium vapor. Three hundred g of the raw haddock fillets were placed in a 1-L flask fitted with a sidearm over which was placed a rubber septum. Ten ml of the vapor in equilibrium with the fish in the flask ("head gas") were removed directly by means of a gas syringe. The raw fish was equilibrated to room temperature, whereas the cooked fish volatiles were removed through the septum while the sample was still hot. Vapors were injected directly onto the chromatographic column.

Flushing with an inert gas. One hundred g of minced raw haddock flesh were placed in the collection trap shown in Fig. 1, and equilibrated for at least 1 hr at room temperature. The volatiles were flushed for 1 min with argon carrier gas onto the head of a gas chromatographic column held at −65°C (see chromatographic procedure below). For analysis of the cooked fish volatiles, the trap was sealed and was cooked in the pressure cooker

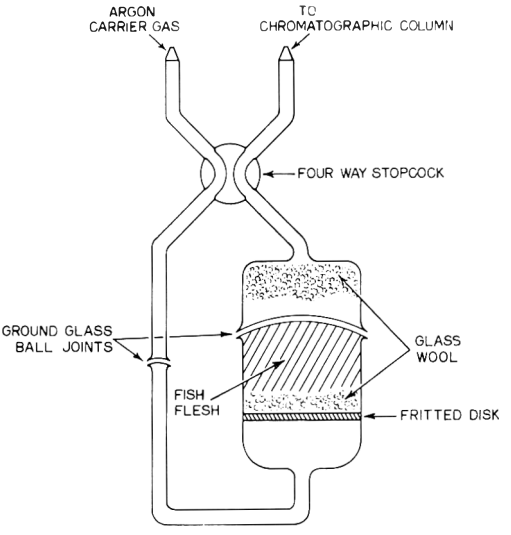

Fig. 1. Sample container used for removal of fish volatiles by flushing.

for 20 min. The hot volatiles were then removed in the same way by flushing for 1 min.

Vacuum collection of equilibrium vapor. At room temperature the equilibrium vapors above 600 g of raw haddock fillets and cooked haddock fillets were collected in gas bottles evacuated to a pressure of about 1 μ Hg. The collection bottles were then immersed in liquid nitrogen and connected to the vacuum manifold, and any uncondensable gases were removed from the system. The collection bottles were brought to room temperature again, and about 1.4×10^{-4} moles of the volatiles (measured by the vapor pressure and calculated by $PV = P'V'$) were transferred to a gas trap, according to the procedure of Bazinet and Walsh (1960). The trap was placed in the stream of carrier gas leading to the chromatographic column.

High-vacuum distillation. The total condensate from 600 g of minced, raw, or cooked haddock flesh was collected with the high-vacuum distillation technique of Merritt *et al.* (1959). The collection was allowed to proceed for 24 hr. The sample was obtained by allowing the equilibrium vapor above the total condensate to expand freely into an evacuated glass collection bottle. By measurement of the vapor pressure of the gases which were allowed to expand into the collection bottle, approximately 1.4×10^{-4} moles of the total condensate vapor were then transferred to a gas trap, and the contents of the trap were flushed by the argon carrier gas onto the gas chromatography column for separation. A comparison was made of the gas chromatograms obtained of the equilibrium vapor above the total condensate with the volatile

material collected in the usual way as "center cut" (Merritt *et al.*, 1959).

Gas chromatography procedure. Preliminary studies showed that isothermal gas chromatography at room temperature or above gave poor resolution. Therefore, all the mixtures of volatile compounds were separated by programmed cryogenic temperature gas chromatography (Merritt and Walsh, 1963) to achieve a high degree of separability. An argon ionization detector (Lovelock, 1958) was used to attain high sensitivity. The gas chromatography column was a 6-ft × 0.25-in.-OD U-shaped glass column with 5% (wt/wt) β,β' oxydipropionitrile (OPN) on 80–100-mesh untreated chromosorb-W. The column, before introduction of the sample, was immersed in a dry-ice-ethanol coolant, and when the temperature had come to −65°C the sample was swept onto the column with carrier gas. Elution of the components from the column was allowed to proceed as the temperature of the column was allowed to rise to room temperature. The column was surrounded by a glass jacket to contain the coolant and was attached at the column exit end to the argon ionization detector of a Barber Colman gas chromatograph, model 10. The argon carrier gas flow was 40 ml per min measured before the start of the chromatogram at the detector outlet by a Fisher Porter Tri-Flat precision-bore flow meter. The ionization detector (20 mc Sr^{90}) was operated at 190°C.

RESULTS AND DISCUSSION

Weurman (1963) investigated various methods of removing the volatile compounds from foods and showed that the compounds detected and identified were directly dependent upon the removal method. The effectiveness of 4 methods of collecting the volatile components was evaluated with the gas chromatograms obtained used as criteria. Chromatograms were obtained of the volatile components from haddock fillets collected by each of the 4 methods.

Direct sampling of equilibrium vapors above the fillets with a syringe provides a gaseous sample of the odor components actually perceived and is simple and rapid. Fig. 2 shows the chromatograms obtained of a 10-ml sample of gas removed by syringe from both raw and cooked haddock fillets that had been stored 10 days at 2°C. Only relatively few peaks are observed. The bulk of the gaseous components of this sample are air and carbon dioxide and the argon ionization

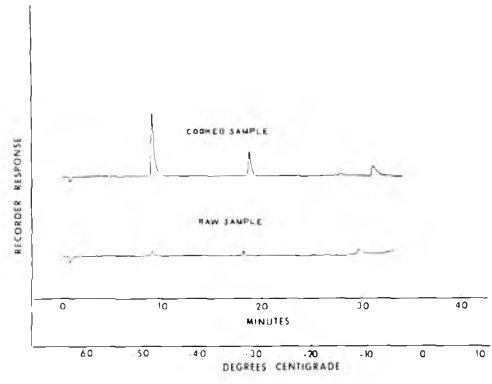

Fig. 2. Programmed cryogenic-temperature gas chromatograms of volatile components collected by syringe from haddock "headspace."

detector does not respond to these components. The peaks observed on the chromatograms are those due to the volatile flavor and odor components in the sample and are shown to be present in extremely low concentration. Since a 10-ml gas sample is considered to be fairly large, and since it was composed almost entirely of air and carbon dioxide, it was concluded that removal of the volatile compounds with a syringe produced a sample which is too dilute to be suitable. Chromatograms for fillets stored 1 and 5 days showed fewer peaks than chromatograms for fillets stored 10 days.

Flushing the equilibrium vapors directly onto the chromatographic column concentrates the volatile odor components on the head of the column. In addition, it may be expected that neither the fish flesh nor the composition of the volatiles is altered by this method of sampling. Another advantage is the fact that components which are normally lost by condensation when liquid nitrogen cold-trapping procedures is employed (see below) are detected on the chromatograms obtained by this method of sampling. Chromatograms obtained by this technique from both raw and cooked fish samples are shown in Fig. 3. Only the sample which was stored for 10 days and then cooked showed large peaks. As with the syringe, the sample flushed onto the column is mainly air and carbon dioxide and contains but a very small proportion of the flavor and odor components. Although the cooked 10-day-old fish shows an abundance of volatiles which is much

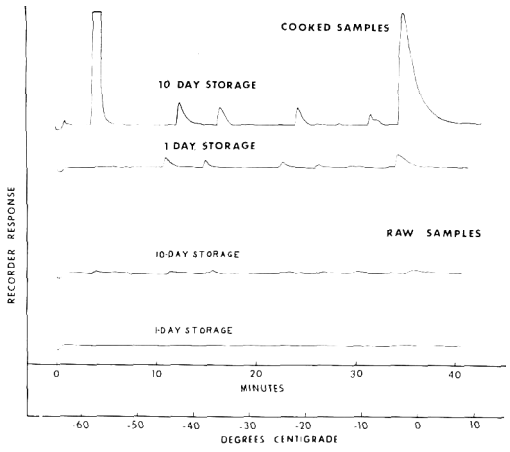

Fig. 3. Programmed cryogenic-temperature gas chromatograms of volatile components in haddock "headspace" flushed onto the column.

greater than that of the other samples, the amounts indicated by the chromatograms are too small for purposes of comparison.

The method of collecting the equilibrium vapor over a sample by free expansion into an evacuated receiver was devised in an attempt to concentrate the volatile components. The chromatograms subsequently obtained from the series of raw fish samples are shown in Fig. 4. As expected, both a larger number as well as a greater quantity of the volatile components are indicated by the chromatograms. This procedure provides the greatest concentration of volatile odor compounds attainable without alteration of the equilibrium composition of the vapor. For comparing the chromatograms from different

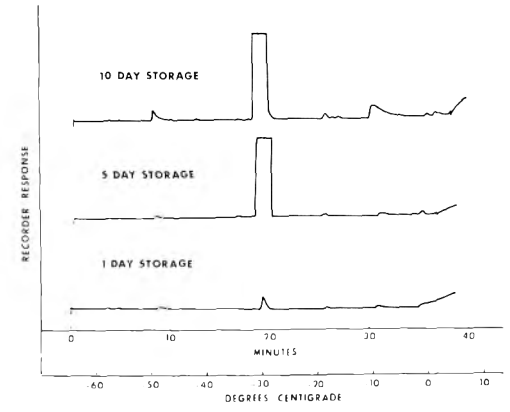

Fig. 4. Programmed cryogenic-temperature gas chromatograms of volatile components collected by vacuum from haddock "headspace."

samples, however, the quantity of volatiles collected is too small to provide much information about composition except for major components of the mixture.

The largest yield of volatile material is provided by the vacuum distillation method (Merritt *et al.*, 1959). In most prior studies using this technique, the total condensate from the distillation was taken for subsequent separation and analysis. In this study the procedure has been simplified by allowing the vapor over the total condensate to expand into an evacuated sample trap. The relative amounts of the compounds in the vapor sample tend to differ, however, from the corresponding amounts in the distillate. Nevertheless, the validity of the qualitative correspondence of the sample composition is maintained since the same peaks are observed on total condensate vapor chromatograms as on chromatograms of liquid total condensate. As with the other methods, the bulk of the material collected consisted mainly of carbon dioxide and water. Since these components are separated from the volatile odor compounds by the gas chromatography procedure subsequently employed, they do not interfere with the evaluation of the sampling procedures. Many more components are found in total condensate vapor samples than in samples which retain the unaltered "headspace" composition. For comparing the change in composition of the volatile components in the samples of fish flesh itself, the greater number of components and larger yield provided by total condensate vapor are superior to the other methods of collection. Chromatograms of samples collected by this procedure (Fig. 5) clearly demonstrate the larger number and higher concentration of compounds collected.

This investigation shows that a vacuum distillation method for collection of volatile compounds can provide a sample of sufficient concentration for further study. This technique has been used in studying the effect of temperature, storage time, cooking, and any other variables on the composition of volatile odor and flavor components in fish. The data provided by this study show that both the number and amount of components increase with storage time and with cooking. Apparent absence of volatile compounds in the

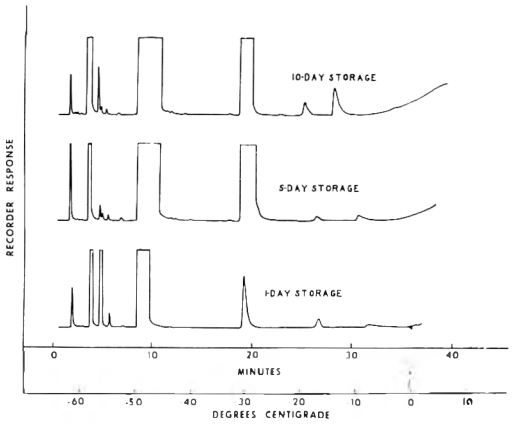

Fig. 5. Programmed cryogenic-temperature gas chromatograms of volatile components in cooked haddock total condensate vapor.

chromatogram of a 1-day raw sample confirms analytically the sensory observation that fresh raw fish is practically odorless. The significance of these results is difficult to evaluate, however, without a knowledge of the identity of the volatile components. The method used to accomplish such identifications by rapid-scanning mass spectrometry of the gas chromatographic eluate has been described (Merritt *et al.*, 1964). A subsequent paper will describe further studies of the composition of the flavor and odor components of fishery products.

REFERENCES

Bazinet, M. L., and J. T. Walsh. 1960. Combination gas sampler and fraction collector for gas chromatography and mass spectrometer application. *Rev. Sci. Instr.* **31**, 346.

Brandenberger, H., and S. Müller. 1962. A gas chromatographic separation of the volatile fatty acids of black tea. *J. Chromatog.* **7**, 137.

Buttery, R. G., and R. Teranishi. 1961. Gas-liquid chromatography of aroma of vegetables and fruit. *Anal. Chem.* **33**, 1439.

Carrol, R. B., and L. C. O'Brien. 1959. Gas-liquid chromatography of whiskies. Paper presented at the 135th Natl. Meeting Am. Chem. Soc., Boston, Mass.

Hiu, D. N., and P. J. Scheuer. 1961. The volatile constituents of passion fruit juice. *J. Food Sci.* **26**, 557.

Hornstein, I., and P. F. Crowe. 1960. Flavor studies on beef and pork. *J. Agr. Food Chem.* **8**, 494.

Hunter, I. R., H. Ng, and J. W. Pence. 1961. Volatile organic acids in pre-ferments for bread. *J. Food Sci.* **26**, 578.

Jennings, W. G. 1961. Volatile esters of bartlett pears. *J. Food Sci.* **26**, 564.

Kramlich, W. E., and A. M. Pearson. 1960. Separation and identification of cooked beef flavor components. *J. Food Research* **25**, 712.

Lovelock, J. E. 1958. A sensitive detector for gas chromatography. *J. Chromatog.* **1**, 35.

Mackay, D. A. M., D. A. Lang, and M. Berdick. 1961. Objective measurement of odor. Ionization detection of food volatiles. *Anal. Chem.* **33**, 1369.

Mangan, G. F., C. Merritt, Jr., and J. T. Walsh. 1959. Chemical components of the odor of fish. Paper presented at the 135th Natl. Meeting Am. Chem. Soc., Boston, Mass.

Mendelsohn, J. M., and M. A. Steinberg. 1962. Development of volatile carbonyls in haddock (*Melanogrammus aeglefinus*) flesh during storage at 2°C. *Food Technol.* **16**, 113.

Merritt, C., Jr., and J. T. Walsh. 1963. Programmed cryogenic temperature gas chromatography applied to the separation of complex mixtures. *Anal. Chem.* **35**, 110.

Merritt, C., Jr., S. R. Bresnick, M. L. Bazinet, J. T. Walsh, and P. Angelini. 1959. Determination of volatile components of foodstuffs. Techniques and their application to studies of irradiated beef. *J. Agr. Food Chem.* **7**, 784.

Merritt, C., Jr., J. T. Walsh, D. A. Forss, P. Angelini, and S. M. Swift. 1964. Wide-range programmed temperature gas chromatography in the separation of very complex mixtures. *Anal. Chem.* **36**, 1502.

Nawar, W. W., and I. S. Fagerson. 1962. Direct gas chromatographic analysis as an objective method of flavor measurement. *Food Technol.* **16**, 107.

Rhoades, J. W. 1958. Sampling method for analysis of coffee volatiles by gas chromatography. *J. Food Research* **23**, 254.

Slater, C. A. 1961. Citrus essential oils. II. Composition of distilled oil of limes. *J. Sci. Food Agr.* **12**, 732.

Smith, D. E., and J. R. Coffman. 1960. Analysis of food flavors by gas-liquid chromatography. Separation and identification of the neutral components from bread pre-ferment liquid. *Anal. Chem.* **32**, 1733.

Teranishi, R., R. G. Buttery, and R. E. Lundin. 1962. Gas chromatography. Direct vapor analyses of food products with programmed temperature control of dual columns with dual flame ionization detectors. *Anal. Chem.* **34**, 1033.

Vorbeck, M. L., L. R. Mattick, F. A. Lee, and C. S. Pederson. 1961. Volatile flavor of sauer-

kraut. Gas chromatographic identification of a volatile acidic off odor. *J. Food Sci.* **26**, 569.

Weurman, C. 1963. Some tentative reasons for the failure in reconstituting natural odors. First International Colloquium on the Chemistry of Green and Roasted Coffee and Their Derivatives. Paris, France.

Wick, E. L., T. Yamanishi, L. C. Wertheimer, J. E. Hoff, B. E. Proctor, and S. A. Goldblith. 1961. An investigation of some volatile components of irradiated beef. *J. Agr. Food Chem.* **9**, 289.

Yueh, M. H., and F. M. Strong. 1960. Some volatile constituents of cooked beef. *J. Agr. Food Chem.* **8**, 491.

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Mention of trade names does not imply endorsement.

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The Relation of Structure to Antioxidant Activity of Quercetin and Some of Its Derivatives
II. Secondary (Metal-Complexing) Activity

SUMMARY

The secondary (metal-complexing) activity of quercetin and some of its derivatives was studied with the conventional Warburg technique in an ascorbic acid system catalyzed with cupric ions.

The 3-hydroxy-4-keto group was the most powerful metal-complexing group in the quercetin molecule; the 5-hydroxy-4-keto group had some activity, but was considerably weaker. The 3′,4′-o-diphenolic group, important for the primary antioxidant activity, possessed virtually no metal-deactivating properties.

Methylation of the 4′- or 7-hydroxyl (or, to a lesser degree, of the 3′-hydroxyl) increased the complexing capacity of the O-methylated derivative of quercetin, while hydrogenation of the 2,3 double bond yielded an almost completely inactive compound (dihydroquercetin).

These experiments were made to study the relationship between the molecular structure of polyhydroxyflavones and their secondary (metal-complexing) properties.

Simpson and Uri (1956), Mehta and Seshardi (1958), and others, have suggested that, although the antioxidant activity of the polyhydroxyflavones is primarily a function of their ability to act as free radical acceptors, the metal-complexing properties of these molecules may make some contribution to their total activity.

Lea (1956, 1958) described the flavonols as multifunctional antioxidants combining in their molecule both chain-breaking and metal-deactivating properties, each of which can operate at several points, and Kelley and Watts (1957) compared the antioxidant effect of various flavonoids with their copper-complexing activity in an aqueous fat system to which ascorbic acid had been added. In their work a simple colorimetric method was used to indicate roughly the rate of oxidation of the ascorbic acid.

In a fat-containing system, however, it is difficult to evaluate the exact contribution of each mode of action to the total antioxidant capacity (Crawford *et al.*, 1961). The present study of the metal-deactivating capacity was therefore carried out on an ascorbic acid system catalyzed with cupric ions. The technique employed was based on that used by Frieden and Alles (1958) in their work with nucleic acid components. The polyphenolic inhibitor quercetin (3,5,7,3′,4′-pentahydroxyflavone) was used again, for the reasons outlined in Part I, and its metal-deactivating capacity was compared with those of its O-methylated, hydroxylated, dehydroxylated, and hydrogenated derivatives and with 6-tert-butyl, 3,7,8,2′,5′-pentahydroxyflavone, an antioxidant synthesized by N. Uri.

EXPERIMENTAL

Apparatus. A conventional-type Warburg apparatus was used, with 20-ml sidearm flasks, and a shaking rate of 100 strokes/min (6-cm stroke).

Cleaning of glassware. Glassware used for the preparation, storage, and delivery of reagent, and the reaction flasks themselves, were cleaned by leaving overnight in concentrated nitric acid and then rinsing with water, distilled and redistilled from glass. This treatment was found both necessary and effective in keeping at an acceptably low level the catalysis of ascorbate oxidation by extraneous ions.

Methods. The rate of the copper-catalyzed oxidation of ascorbic acid was measured at 25.0±0.1°C by the conventional Warburg technique, with 0.01*M* sodium phosphate buffer (pH 7.0±0.1) in a 15% (v/v) aqueous solution of 2-ethoxy-ethanol and air as the gas phase. The concentration of cupric ions in the solution was $0.5\times10^{-5}M$, that of the phenolic inhibitors $0.5\times10^{-6}M$–$20\times10^{-5}M$, and that of the ascorbic acid $0.013M$.

A 40% (v/v) aqueous solution of 2-ethoxy-ethanol was used as solvent for the flavonoids, many of which are sparingly soluble in water. 2-Ethoxy-ethanol was also added to the sidearm of the flasks, so as to equalize the concentration there with that in the main vessel (to avoid heat and/or volume changes on mixing of the contents).

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Table 1. Reagents delivered to Warburg flasks and their volume (ml).

Reagent[a]	Flask						
	Uninhibited O_2 uptake		Inhibited O_2 uptake		Blank		Thermobarometer
	Main vessel	Side arm	Main vessel	Side arm	Main vessel	Side arm	
1. $CuCl_2$, aq., $1.5\times10^{-5}M$	1.0	—	1.0	—	—	—	—
2. 0.26M ascorbic acid, aq., neutralized to pH 6.2 shortly before use	—	0.15	—	0.15	—	0.15	—
3. 2-ethoxy ethanol aq., 30% v/v	—	0.15	—	0.15	—	0.15	0.15
4. ditto, 40% v/v.	1.0	—	—	—	1.0	—	1.0
5. Solution of flavonoid inhibitor in (4), $1.5\times10^{-5}M-60\times10^{-5}M$	—	—	1.0	—	—	—	—
6. pH 7.0 sodium phosphate buffer, aq., 0.043M	0.7	—	0.7	—	0.7	—	0.7
7. Water	—	—	—	—	1.0	—	1.15

[a] Water and 2-ethoxy ethanol used were purified by distillation and redistillation from glass.

Blanks, with ascorbate but without cupric ion, were included in all experiments, and the data were rejected whenever more than 10 μl of oxygen were consumed during the run, indicating contamination.

Table 1 shows the solutions used and their volume, as delivered to different Warburg flasks. Measurements were confined to the period of approximately linear oxygen absorption, and oxidation rates were calculated for reaction times of 20 and 40 min.

The oxygen uptake (U) was expressed as percent of the volume taken up during the uninhibited reaction, and calculated from

$U = 100\,(V_i - V_b)/(V_c - V_b)$, where

V_c = μl oxygen consumed in the copper-catalyzed reaction in the absence of inhibitor;

V_i = ditto, in the presence of inhibitor; and

V_b = μl oxygen consumed in blank experiment (no inhibitor and no added cupric ions).

RESULTS

Detailed results for quercetin over the inhibitor/Cu^{++} range 1:1 to 20:1 (molecular ratio) are given for a reaction time of 20 min in Fig. 1a, and for a reaction time of 40 min in Fig. 1b. Data for the other 15 substances studied cannot be presented with the same completeness, but all results are summarized in Table 2 and Figs. 2a and 2b.

It can be seen that the semilogarithmic plot of oxygen uptake vs. inhibitor/Cu^{++} molecular ratio is normally approximately linear over a considerable stretch.

In Table 3, all 16 substances investigated are arranged in descending order of copper-deactivating ability, 5 being superior and 10 inferior to quercetin.

DISCUSSION

Figs. 2a and 2b and Table 2 indicate that methylation, or replacement with hydrogen, of the 3- or 5-hydroxyl group in the quercetin molecule resulted in derivatives of much lower metal-deactivating capacity (the effect was stronger in the 3-hydroxyl group). It therefore seems that the most powerful copper-complexing part of the quercetin molecule is the 3-hydroxy-4-keto grouping, followed by the 5-hydroxy-4-keto grouping.

The 3′,4′-o-diphenolic group in the B ring (which has strong primary antioxidant prop-

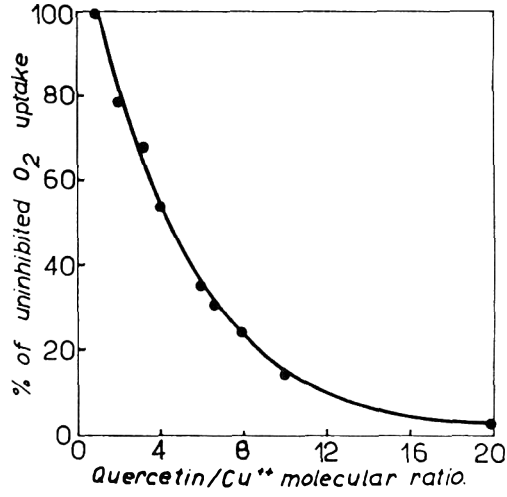

Fig. 1a. Inhibition, by quercetin, of cupric-ion catalysis of ascorbate oxidation. Reaction time, 20 min.

Table 2. Oxygen uptake as function of the inhibitor and its concentration.

Inhibitor	Reaction time (min)	Inhibitor/Cu^{++} molecular ratio					
		1	2	5	10	20	40
Quercetin	20	100	79	—	14.5	2.5	—
	40	98	87.5	—	20.5	4.5	—
	av.	99	83	—	17.5	3.5	—
3-OMeQ	20	—	—	—	98	84	77
	40	—	—	—	100	85.5	76.5
	av.	—	—	—	99	85	77
5-OMeQ	20	—	100	100	68	32	9.5
	40	—	100	100	77	38	12
	av.	—	100	100	72.5	35	11
7-OMeQ	20	100	60	22	4	7	14
	40	98	67	25	6	10	22
	av.	99	63.5	23.5	5	8.5	18
3′-OMeQ	20	100	64	17	11	3	—
	40	98	76.5	24	17	5	—
	av.	99	70	20.5	14	4	—
4′-OMeQ	20	80	24	4	—	0.5	—
	40	82	30.5	6	—	0.9	—
	av.	81	27	5	—	0.7	—
3,5-diOMeQ	20	—	—	—	100	97	83
	40	—	—	—	100	97	85.5
	av.	—	—	—	100	97	85
3,3′-diOMeQ	20	—	100	100	59	34	19
	40	—	100	100	74	48	28
	av.	—	100	100	67.5	41	23.5
3,7,4′-tri OMeQ	20	—	—	—	100	100	100
	40	—	—	—	95	94	94
	av.	—	—	—	97.5	97	97
7,3′,4′-tri OMeQ	20	94.5	69	19	—	11	4
	40	97	66	27	—	16	11.5
	av.	96	67.5	23	—	13.5	7
3,7,3′,4′-tetraOMeQ	20	—	—	—	—	100	100
	40	—	—	—	—	100	100
	av.	—	—	—	—	100	100
Dihydroquercetin	20	—	100	100	100	89	82
	40	—	—	—	100	92	85
	av.	—	100	100	100	90.5	83.5
5-HQ (fisetin)	20	—	—	95	84.5	39	17
	40	—	—	94.5	87	44.5	20.5
	av.	—	—	95	85	41	19
3-HQ (luteolin)	20	—	—	—	88	67.5	32.5
	40	—	—	—	92	71	42
	av.	—	—	—	90	69	37
8-OHQ (gossypetin)	20	100	97	74	55	—	—
	40	98.5	96	81	69	—	—
	av.	99	96	77	62	—	—
"Flavone"[a]	20	94	67.5	25	7	1	—
	40	89	79.5	33.5	9	1	—
	av.	92	73.5	29.5	8	1	—

[a] 6-tert-butyl, 3,7,8,2′,5′-pentahydroxyflavone.

Table 3. Inhibitor/Cu^{++} molecular ratio required for partial deactivation of the cupric ion.

Inhibitor	% of unhibited O_2 uptake 80	50	20	Activity of complexing agent
4′-OMeQ	1.0	1.4	2.5	Considerably stronger than Q
7-OMeQ (rhamnetin)	1.4	2.75	5.5	
7,3′,4′-triOMeQ	1.45	2.75	6.5	Slightly stronger
3′-OMeQ	1.6	3.0	5.5	than Q
"Flavone"[a]	1.6	3.25	6.5	
Quercetin (Q)	2.25	5.0	9.5	
8-OHQ (gossypetin)	4.5	—	—	Weaker than Q
3,3′-diOMeQ	8.0	16.5	—	
5-OMeQ	9.0	15.5	30.0	
5-HQ (fisetin)	11.5	18.0	36.0	Much weaker than Q
3-HQ (luteolin)	15.0	30.0	—	
3-OMeQ	30.0	—	—	Very weak
2,3-dihydroquercetin	45–50	—	—	
3,5-diOMeQ	—	—	—	
3,7,4′-triOMeQ	—	—	—	Inactive
3,7,3′,4′-tetraOMeQ	—	—	—	

[a] 6-tert-but., 3,7,8,2′,5′ pentahydroxyflavone.

erties) seems to have only a weak copper-complexing capacity (see results for 3,5-dimethoxy quercetin).

3,7,4′-tri-OMeQ and 3,7,3′,4′-tetra-OMeQ were both inactive, although still containing the 5-hydroxy-4-keto group intact, and hydrogenation of the 2,3 double bond of quercetin resulted in a derivative (dihydroquercetin) with practically no complexing capacity.

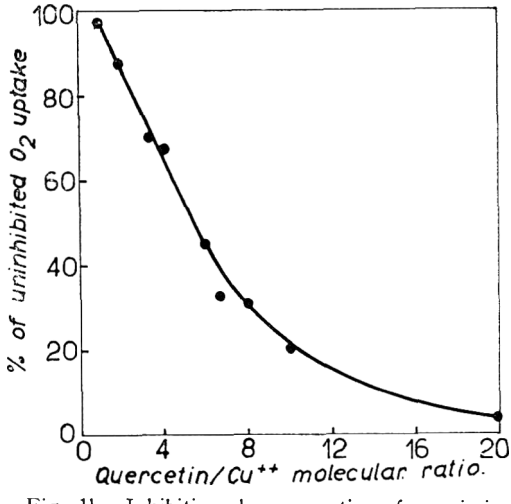

Fig. 1b. Inhibition, by quercetin, of cupric-ion catalysis of ascorbate oxidation. Reaction time, 40 min.

While it is thus possible to evaluate the relative importance of the individual complexing centers to some degree, it nevertheless seems that the metal-deactivating properties of quercetin must be considered as a function of the molecule as a whole. There is obviously interaction of the several active centers along the connecting chain of conjugated unsaturation. In this respect the 2,3 double bond of quercetin is of great importance and it is unfortunate that no O-methylated derivatives of dihydroquercetin were available to test the influence of blocking individual complexing centers in the absence of the connecting link of conjugated double bonds.

With regard to the increased complexing capacity of the 4′-OMe, 7-OMe, or 3′-OMe derivatives it seems that the 7- and 4′-OH's, being conjugated with the 4-carbonyl, are quite strongly acidic. Methylation of these hydroxyls supresses their ionization and increases the electron density of the carbonyl group, thereby its complexing capacity. An adjacent 3′-OH also increases the acidity of

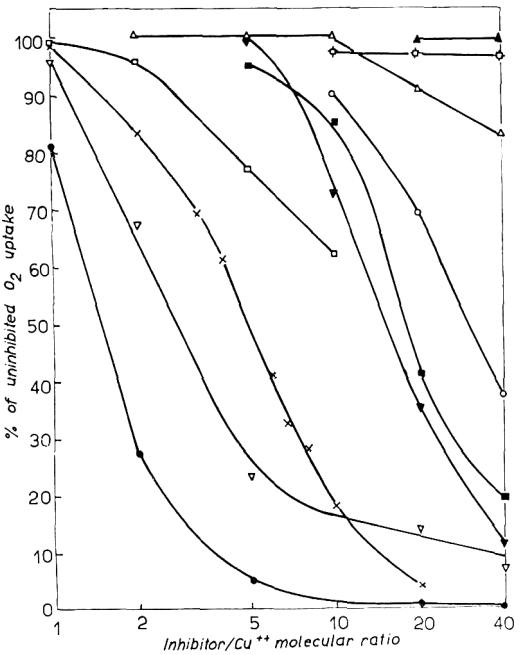

Fig. 2a. Inhibition, by quercetin and its derivatives, of cupric-ion catalysis of ascorbate oxidation. The percent of uninhibited O_2 uptake is the average of the 20- and 40-min reaction times.
X, quercetin (Q); △, dihydroQ;
□, gossypetin (8-OHQ); o, luteolin (3-HQ);
■, fisetin (5-HQ); ▼, 5-OMeQ;
●, 4'-OMeQ; ⊡, 3,7,4'-triOMeQ;
▽, 7,3',4'-triOMeQ; ▲, 3,7,3',4'-tetraOMeQ.

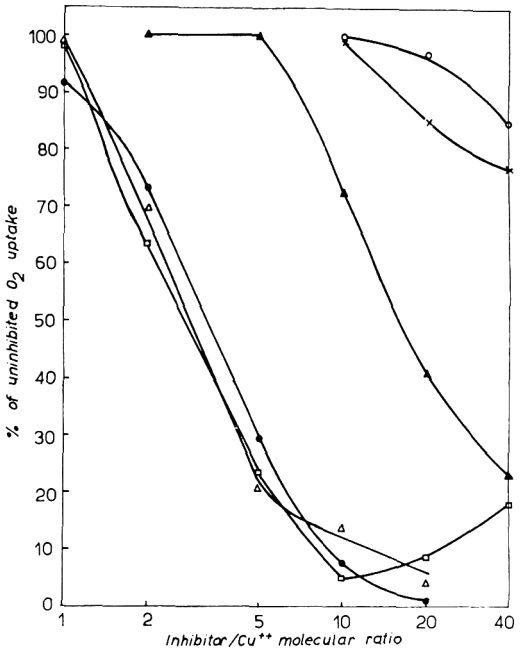

Fig. 2b. Inhibition by quercetin derivatives, of cupric-ion catalysis of ascorbate oxidation. The percent of uninhibited O_2 uptake is the average of the 20- and 40-min reaction times. X, 3-OMeQ;
□, 7-OMeQ; △, 3'-OMeQ;
o, 3,5-diOMeQ; ▲, 3,3'-diOMeQ;
●, 6-tert. but., 3,7,8,2',5' pentahydroxyflavone.

the 4'-OH group. Methylation of the 3'-OH may therefore be expected, in the same way, to reduce the acidity of the 4'-OH and thereby increase the complexing capacity of the 4-carbonyl group.

Surprisingly, the metal-deactivating capacity of 7-OMeQ was less at inhibitor/Cu^{++} ratios 40:1 and 20:1 than at 10:1. No explanation can be suggested for this unexpected behavior, which was confirmed in repeated experiments.

REFERENCES

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

Frieden, E., and J. Alles. 1958. Subtle interactions of cupric ion with nucleic acid and components. *J. Biol. Chem.* **230**, 797.

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

Lea, C. H. 1956. Antioxidants in the food industry. *Research* **9**, 472.

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

Mehta, A. C., and T. R. Seshardi. 1958. Flavonoids as antioxidants. *J. Sci. Ind. Research (India)* **18**B, 24.

Simpson, T. H., and N. Uri. 1956. Hydroxyflavones as inhibitors of the anaerobic oxidation of unsaturated fatty acids. *Chem & Ind.* **1956**, 956.

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Causes of Can Swelling and Blackening of Canned Baby Clams
I. Chemical Factors Involved in Blackening

SUMMARY

Hydrogen sulfide is formed from the meat of baby clams during canning. The amounts of hydrogen sulfide are usually below 50 mg%, which may be the minimum amount necessary to cause blackening. When hydrogen sulfide is produced in amounts more than 50 mg% (e.g. when the raw material is not fresh), blackening may be accelerated, or the canned baby clams may decompose and hydrogen sulfide concentration increase slightly during storage. The blackening is caused by the formation of copper sulfide and iron sulfide. Iron transferred to the intestine from the mud where the clams live, is involved in blackening. Also implicated is copper freed from hemocyanin in baby clam by boiling. Some phosphate compounds (0.2%) are effective in decreasing the blackening.

INTRODUCTION

Among technical problems in the canning industry, blackening of contents and swelling of tin containers have been the subject of research for many years. Investigated besides the blackening of metal surfaces in contact with the headspace of food containers has been blackening of contents of canned shrimp (Thompson, 1963), canned tuna (Pigott and Stanby, 1956), canned salmon (Tanikawa, 1958), and canned crab (Machida, 1911; Oshima, 1927; Arakawa, 1928). Sekine (1926) investigated the blackening of canned baby clams. He concluded that the canned product is blackened by a reaction between iron and hydrogen sulfide.

Blackening of the product can develop during processing at high temperature (Kaneko, 1951). This was also reported by Pigott and Dollar (1963), who also considered that cysteine was the major source of the sulfur and that oxidizing conditions prevent sulfide blackening by oxidizing cysteine to cystine, which does not yield free reduced sulfur to react with ferrous iron. Ferrous iron is formed by the electrochemical reaction of the tin-iron couple in the headspace at temperatures greater than 150°F. From these reports, the mechanisms of the blackening of canned products are considered to be: 1) chemical reactions which involve a large number of interrelated variables; and 2) bacterial action.

Preventive methods for the former are: 1) to keep the raw material fresh (Tanikawa, 1958); 2) to add tartaric or citric acids (Fellers and Parks, 1926; Legendre, 1928; Thompson, 1963; Obata *et al.*, 1957); and 3) to use C-enameled cans. Bacterial action is prevented by appropriate processing temperature and time, and/or added preservatives.

To clarify the causes of blackening and swelling of canned baby clams, we have carried out chemical and bacteriological experiments; 1) on the mechanisms of blackening; and 2) on the bacterial flora in the sea bottoms where baby clams live. This paper reports experiments on the chemical factors involved in the blackening of canned baby clams.

EXPERIMENTS AND RESULTS

Nature of the black substance. *Chemical composition of normal, blackened, and swelled clams.* Chemical composition of the clams was determined as follows: Water contents, crude fat, and crude protein were estimated by the usual methods. Reducing sugar was estimated by an iodometric method (Hagedorn and Jensen, 1923). Iron and copper contents were estimated by a colorimetric method (Breuer and Militzer, 1938; Tompsett, 1934). Hydrogen sulfide was determined by a titration method recommended by Tomiyama and Kanzaki (1951). The results are summarized in Table 1.

There were few differences in water contents, ash, crude fat, iron, copper, and reducing sugar. The amount of hydrogen sulfide was greater in blackened (sometimes blackened and swelled) cans than in normal samples.

Constituents of the black substance. The black substance from canned baby clams has been thought to be a metallic sulfide. It was therefore examined by paper chromatography and chemical analysis.

The contents of two cans of blackened canned baby clams were separated into solid and liquid portions. The liquid portion was filtered through gauze to remove small particles of meat. The filtered liquid (about 250 ml) was centrifuged for

Table 1. Chemical composition of normal, blackened and swollen baby clam cans.

Compositions	Normal	Blackened	Markedly blackened	Blackened and swollen
Water content (%)	68.03	69.80	72.02	70.37
Crude protein (%)	18.74	17.19	17.34	17.35
Crude fat (%)	4.82	2.00	3.56	3.72
Reducing sugar (mg%)	146.34	100.2	127.4	128.6
Iron (mg%)	63.9	78.2	56.9	64.1
Copper (mg%)	0.72	0.83	0.92	0.8
Hydrogen sulfide (mg%)	35.2	73.08	138.6	264.6
Volatile basic-N (mg%)	12.10	14.48	53.1	69.3
Reflectance (%T)	16.5	7.8	6.5	6.7

10 min at 4000 rpm, and the deposit in the tube was washed with water. This procedure was repeated 3 times. About 1 g of a pure black substance was obtained. The metallic components in the black substance were examined by paper chromatography. One-dimensional ascending method was used with n-butanol saturated with water as the developing solvent. The paper was exposed to hydrogen sulfide gas to reveal the metallic components. Results are shown in Fig. 1. The results indicate that the black substance from markedly blackened canned baby clams contains copper and iron. In slightly blackened baby clams, however, the black substance contains copper only. The chemical constituents of the black substance are shown in Table 2.

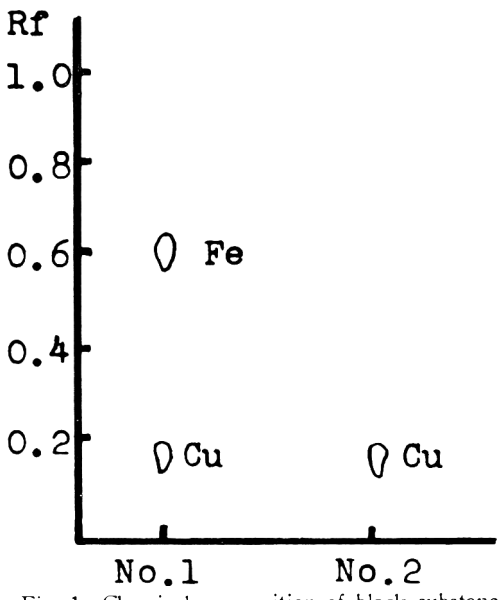

Fig. 1. Chemical composition of black substance detected by paper chromatography.
No. 1) Black substance isolated from markedly blackened canned baby clams.
No. 2) Black substance isolated from slightly blackened canned baby clams.

The extent of formation of metallic sulfides in canned products is dependent on a reaction between the metals and hydrogen sulfide. In general, in combining metallic salts and hydrogen sulfide, the metallic sulfide precipitates when the product of metallic ion and sulfur ion is larger than the solubility product of the sulfide. As the acidity of the solution increases and $[S^{--}]$ gradually decreases, the formation of sulfide is retarded. On the other hand, if the solution is close to neutrality, the formation of sulfide is promoted as $[S^{--}]$ increases.

If $[M^{++}]$ is a divalent metallic ion, and $[S^{--}]$ is formed from hydrogen sulfide, MS, which is a metallic sulfide, is precipitated by combining the two components.

$$M^{++} + S^{--} = MS \qquad [1]$$

$$\frac{[M^{++}][S^{--}]}{(MS)} = K_{MS} \qquad [2]$$

Therefore,

$$[S^{--}] = \frac{K_{H_2S}[H_2S]}{[H^+]^2} \qquad [3]$$

If MS is a precipitate, and [MS] is a coefficient,

$$\frac{[M^{++}][H_2S]}{[H^+]^2} = \frac{K_{MS}}{K_{H_2S}}[MS] = K \qquad [4]$$

From Eq. 4, $[M^{++}]$, which has not been precipitated as the sulfide but exists in the solution as the metallic ion, is in proportion to the square of $[H^+]$. In other words, increasing the acidity of the solution makes it difficult to form the sulfide.

The extent of the formation of sulfide is related to the solubility product (Lp). If Lp is small, $[S^{--}]$ becomes small. Thus, a sulfide whose Lp is sufficiently small is precipitated under comparatively strong acidic solution. The Lp's of copper sulfide and of iron sulfide are respectively 8.5×10^{-45} and 1.5×10^{-10}. This means that copper sulfide is precipitated in 7.5*M* hydrochloric acid solution, and iron sulfide is precipitated in 0.0001*M* hydrochloric acid solution.

Table 2. Chemical properties of black substance isolated from blackened baby clam cans.

	Water (%)	Ash (%)	Total-N (%)	Iron (mg%)	Copper (mg%)	Reflectance (%T)
Black substance	81.3	5.5	0.63	27.5	1.08	4.2
Turbid material (normal can)	77.6	6.2	0.15	16.6	0.69	15.5

Therefore, if both iron and copper coexist in an acidic solution, copper sulfide precipitates to a larger extent than iron sulfide.

From the above consideration it may be assumed that the blackening of canned baby clams would be caused by the formation of copper sulfide if the pH value of the contents were lowered by the addition of acid. Then, as the pH value increases toward the alkaline side, iron sulfide would be gradually formed.

Chemical constituents of headspace gas. The gas of blackened baby clams contains a large quantity of hydrogen sulfide compared to that of the normal canned product. Components other than hydrogen sulfide might also be present in the headspace gas of canned products. Hence, attempts were made to determine the constituents in the headspace.

Gas-chromatographic analysis of the gas-phase from normal and blackened clams was done as follows: the can was heated 10 min at 10 lb (115.2°C) in a pressure cooker. Immediately after heating, the can was placed in a vessel containing water. The cover of the can was opened, and the headspace gas was introduced into a test tube which had been filled with water. The collected gas was used for gas-chromatographic analysis. DNP (dinonyl phthalate) and DMF (dimethyl formamide) were used as fixed phases. The flow speed of the carrier gas was 60 ml/min, the pressure was 0.15 kg/cm^2, and the bridge current was 210 mamp. The temperature of the column was 130°C. Results are shown in Fig. 2.

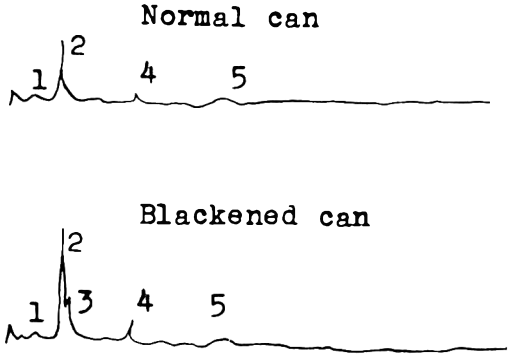

Fig. 2. Composition of headspace gas in canned baby clams detected by gas chromatography
1) air; 2) hydrogen sulfide; 3) methyl mercaptan; 4) ammonia; 5) water.

Hydrogen sulfide and ammonia in the gas were greater for blackened clams than for normal clams. It can be presumed that the blackening in canned baby clams reflects sulfide spoilage. Methyl mercaptan was also present in the blackened samples, but only in small or almost negligible amounts. It is notable, however, that this component reacts with metals, especially iron and copper, to form black precipitates.

Chemical factors in blackening. *Reaction of hydrogen sulfide with iron or copper.* To relate various amounts of the metallic ions and hydrogen sulfide to degrees of blackening, ferrous, ferric, cuprous, and cupric chlorides were reacted with hydrogen sulfide. The concentrations of ferrous and ferric chloride solutions were varied from 0 to 1.0 mg%. Solutions of sodium sulfide had concentrations varying from 0 to 100 mg% as hydrogen sulfide. Aliquots of the solutions were poured into the sodium sulfide solutions, and the development of visible blackening was observed. The results are summarized in Table 3. These data show that more black substance is formed by ferric ion than by ferrous ion. The reason is that the trivalent compound has a lower solubility in water than does the divalent compound. Visible blackening was produced at much lower concentrations with the divalent copper compound than with the divalent iron compound. Thus, copper salts reacting with hydrogen sulfide should cause more blackening than iron salts reacting with hydrogen sulfide.

When the hydrogen sulfide level was above the lowest limit for blackening by iron and copper, the degree of blackening did not increase with an increase in concentration of metal ions. When the amounts of iron or copper were above the lowest limit, however, an increase in hydrogen sulfide increased blackening.

Iron and copper in the meats. The iron and copper contents of the meats of raw and canned baby clams were estimated by gravimetric methods after 200 g of raw or canned meats were burned to ash in crucibles. The amounts of iron and copper were calculated as FeO and CuO. The results (Table 4) indicate that the iron or copper contents of raw or canned baby clams were above the lowest limit for blackening formation.

Blackening formation by hydrogen sulfide in meats and hemocyanin from baby clams. Since the

Table 3. Iron, copper, and hydrogen sulfide concentration as related to development of blackening.

		Hydrogen sulfide (mg%)					
		0–30	40	50	60–70	80–90	100
Ferrous	0–15	—	—	—	—	—	—
chloride	20	—	—	—	—	+—	+—
(mg%)	25	—	—	—	—	+	+
	30	—	—	+—	+—	+	+
Ferric	0–10	—	—	—	—	—	—
chloride	15	—	—	+—	+—	+—	+—
(mg%)	20–30	—	—	+	+	+	+
Cuprous	0	—	—	—	—	—	—
chloride	0.2	—	—	—	—	—	+—
(mg%)	0.6–1.0	—	—	—	+	+	+
Cupric	0	—	—	—	—	—	—
chloride	0.2	—	+—	+	+	+	+
(mg%)	0.6–1.0	—	+—	+	+	+	+

—: no blackening; +—; slight blackening; +: blackening.

Table 4. Copper and iron contents in raw and canned baby clams.

	Raw baby clams	Canned baby clams (normal)
Iron as FeO (mg%)	71.5	63.9
Copper as CuO (mg%)	0.59	0.72

iron or copper contents in raw baby clams are above the lowest limit for formation of visible blackening, hydrogen sulfide added to boiled meats of baby clams might be expected to cause blackening. The experiment was carried out to show whether blackening occurs when boiled meats and hemocyanin from baby clams are treated with hydrogen sulfide.

Ten ml of solutions of sodium sulfide with final concentrations varying from 0 to 120 mg% as hydrogen sulfide, were mixed with about 2 g of raw or boiled meats of baby clams. Also 2 ml of 0.1% solutions of unheated and boiled (7 min at 100°C) hemocyanin were poured separately into solutions of sodium sulfide. Hemocyanin isolated from body fluid was gathered with an injection-syringe after the shells of baby clams were carefully opened. The hemocyanin was prepared by the method of Allison and Cole (1940). A 0.1% solution of hemocyanin was employed for the experiment.

Results are shown in Table 5. Hydrogen sulfide added in amounts of less than 50 mg% to boiled baby clams did not cause visible blackening. Above this concentration, blackening developed with increasing amounts of hydrogen sulfide. The findings show almost the same tendency as those found in Table 3.

According to Tables 1 and 4, there is no significant difference between normal and blackened canned baby clams in the amounts of iron or copper. This suggests that the amounts of copper or iron in the raw meat of baby clams are always sufficient to develop the black discoloration. It is therefore thought that the development of blackening in baby clams is affected more by the

Table 5. Formation of blackening in baby clam meat and hemocyanin with hydrogen sulfide.

	Hydrogen sulfide added (mg%)					
	0–35	40	50–60	70–80	85	90–120
Baby clam meat						
Raw	—	—	—	—	—	+
Boiled	—	+—	+	++	++	++
Hemocyanin						
Unheated	—	—	—	—	+—	+
Boiled	—	+—	+	+	+	++

—: no blackening; +—: slight blackening; +: blackening; ++: marked blackening.

concentration of hydrogen sulfide than by the copper or iron contained. When hydrogen sulfide was added in the form of sodium sulfide to the raw clams, 50 mg% of hydrogen sulfide was insufficient to cause the black discoloration, but more than 100 mg% of hydrogen sulfide did produce this reaction.

In Table 5, it is obvious that various concentrations of hydrogen sulfide added in the form of sodium sulfide to hemocyanin did not cause blackening. When hemocyanin isolated from baby clams was boiled 7 min at 100°C and more than 50 mg% of hydrogen sulfide was added, blackening resulted.

Blackening of heat-treated hemocyanin. We next observed whether blackening occurred when hydrogen sulfide at various levels was added in the form of sodium sulfide to the heat-treated hemocyanin. Two ml of 0.1% hemocyanin solution was poured into 10 ml of the solution of hydrogen sulfide at various concentrations from 0 to 70 mg% and the mixtures were heated 0–10 min at 70–100°C, and the degree of blackening was observed. As seen in Table 6, hemocyanin from baby clams is quite stable when heated. Black discoloration did not develop on heating below 80°C for 10 min, but did develop on heating above 90°C for 5 min or 100°C for 2–5 min. In practical canning procedures, raw baby clams are first boiled 4 min at 100°C so as to remove the meat from the shell. Therefore, hemocyanin in raw baby clams would be decomposed to liberate copper by the heating, and become a factor of blackening.

Table 6. Blackening of hemocyanin from baby clams by hydrogen sulfide after heat processing at various temperatures and times.

Temp. (°C)	Time (min)	Hydrogen sulfide added (mg%) 0–30	40	50–60	70
70	0–10	—	—	—	—
80	0– 8	—	—	—	—
	10	—	+—	+	+
90	0– 2	—	—	—	—
	5– 8	—	—	+	+
	10	—	+—	+	+
100	0	—	—	—	—
	2	—	—	+—	+—
	5– 8	—	—	+	+
	10	—	+—	+	+

—: no blackening; +—: slight blackening; +: blackening.

Formation of hydrogen sulfide and blackening. *Amount of hydrogen sulfide formed by heating.* Blackening in canned baby clams is affected not so much by the amounts of copper or iron as by the concentration of hydrogen sulfide. In general, heating fish and shell fish produces hydrogen sulfide. To show the amount of hydrogen sulfide produced from baby clams by heat processing, 20 g of the meat was put into large test tubes (4.0 × 25.0 cm) and heated 30–100 min at 80–120.9°C. The amount of hydrogen sulfide produced was estimated by Tomiyama and Kanzaki's method (1951).

The results (Table 7) indicate that hydrogen sulfide was produced with heating at above 80°C for 40 min. The amount of hydrogen sulfide produced varied with the heating temperature. Thus, if fresh raw material is used, sulfide spoilage by heat should not occur since the smallest amount of hydrogen sulfide to cause blackening is 50 mg%. Blackening will not develop even if the canned baby clams are processed for 100 min at 15 lb (120.9°C).

Volatile basic nitrogen (VBN), hydrogen sulfide, and pH values. The relation between the amounts of hydrogen sulfide, volatile basic nitrogen (VBN), and pH values was investigated. Baby clams were packed into No. 7 cans and processed 60–100 min at 10–15 lb (115.2–120.9°C). After processing, each can was opened and only the meat portion was used for the experiment. The amounts of hydrogen sulfide, VBN, and pH value were determined for each sample. The results (Table 8) show that, as processing temperature and time are increased, the amounts of hydrogen sulfide and VBN are increased.

Table 7. The amount of hydrogen sulfide (mg%) produced from baby clams by heat processing.

Heating temp. (°C)	Heating time (min) 30	40	60	80	100
80	0	1.6	2.3	5.5	7.2
100	5.7	5.8	6.4	7.8	10.2
105	10.3	10.3	12.6	13.8	14.5
111.5[a]	16.3	19.1	20.6	19.6	28.8
117.6[a]	29.6	23.7	28.1	30.4	38.2
120.9[a]	30.1	30.1	40.0	41.9	45.2

[a] Temperatures equivalent to 10, 12, and 15 lb steam pressure.

Table 8. The volatile basic nitrogen (VBN) and hydrogen sulfide contents and pH values after heat processing of canned baby clams.

Temp. (°C)	Time (min)	H_2S (mg%)	VBN (mg%)	pH
115.2 (10 lb)	60	18.9	10.5	6.6
	80	20.4	12.0	6.6
	100	31.5	14.4	6.6
117.6. (12 lb)	60	29.3	11.4	6.6
	80	31.6	12.1	6.6
	100	36.5	18.8	6.6
120.9 (15 lb)	60	35.6	15.3	6.4
	80	41.5	20.5	6.6
	100	43.2	32.3	6.6

Hydrogen sulfide and volatile basic nitrogen with aged clams. The amount of VBN, hydrogen sulfide, and pH values after processing of canned baby clams from fresh and aged materials were studied. The control was raw fresh meats immediately after shucking. The other meats had been held 48 hr in the shell at 30°C. Two hundred g of each of these materials was boiled 7 min at 100°C, packed into No. 7 cans, and processing for 100 min at 15 lb (120.9°C). The cans were then opened and the amounts of VBN and hydrogen sulfide content, percent reflectance, and pH values determined.

The results (Table 9) indicate that the amounts of VBN and hydrogen sulfide were higher in aged meats than in fresh meats. Aged meats were higher in pH values. Slight blackening occurred in the aged meats. From these results, only fresh baby clams should be canned. The blackening seen several times in boiled meat of baby clams is thought to be due to the use of dead shell or unfresh raw materials, which easily produce large quantities of hydrogen sulfide and volatile bases during canning.

Hydrogen sulfide in sea-bottom mud and clam meats. In general, baby clams inhabit the sea bottom, where many organic substances are deposited. Hydrogen sulfide in the mud of the sea bottom could be introduced into the digestive tracts of baby clams. To know the amounts of hydrogen sulfide in the sea bottom where baby clams occur, samples of mud were obtained from 11 places in Ariake-Sea in Kyushu, Japan, and their hydrogen sulfide contents were estimated. The baby clams used were prepared by the usual method and packed into No. 7 cans.

The amounts of hydrogen sulfide in the muds varied from 2.75 to 21.1 mg%. This variation may depend upon the mud characteristics, e.g. the organic substance present. Nevertheless, no matter where the baby clams were caught, hydrogen sulfide in the canned meats was 30–48 mg%, not directly related to the hydrogen sulfide in the sea-bottom mud.

Chemical changes in stored clam meats. Canned baby clams were stored for two months at 55 and 37°C. At 1 and 2 months the cans were opened and the solid portion was used to estimate volatile basic nitrogen and hydrogen sulfide, percent reflectance, and pH value.

As seen in Table 10, the amount of hydrogen sulfide and volatile basic nitrogen increased gradually during storage. The change was greater at 55°C than at 37°C. At the higher storage temperature, a slight, though not significant, blackening occurred. Canned baby clams should not be stored at high temperature.

Table 9. The volatile basic nitrogen (VBN) and hydrogen sulfide and pH after heat processing of canned baby clams from aged raw material.

	After boiling (100°C, 7 min)		After processing (120.9°C, 100 min)	
	Fresh meat	Aged meat	Fresh meat	Aged meat
VBN (mg%)	15.1–19.6	17.4–25.3	24.9–32.3	37.6–42.3
H_2S (mg%)	30.2–37.7	35.7–51.6	40.5–43.2	40.7–56.7
Reflectance (%T)	19.6	10.4	14.5	7.5
pH	6.2–6.4	6.6–6.8	6.4–6.6	7.0–7.2

Table 10. Change in chemical composition of canned baby clams during storage.

		After 1 month		After 2 months	
	Before storage	37°C	55°C	37°C	55°C
VBN (mg%)	12.1	15.9	20.0	14.8	24.2
H_2S (mg%)	35.2	36.6	46.3	45.8	49.7
pH	6.4	6.6	6.6	6.6	6.6
Reflectance (%T)	16.5	14.7	9.7	13.3	8.5

Effect of phosphates. *Behavior of phosphates to copper.* As a method of preventing blackening, complete removal or deactivation of free copper might be effective. Studied first was the effectiveness of adding phosphates to cupric chloride solution, and then to heated hemocyanin. Each 5 ml of 2% cupric chloride solution was mixed with, first 5 ml, then 10 ml, of 0.2% phosphate solution, and the amount of precipitate was observed. The kinds of phosphate were sodium phosphate (monobasic), sodium phosphate (tribasic), sodium pyrophosphate, sodium tripolyphosphate, sodium hexametaphosphate (pH 4.4), and sodium polymetaphosphate (pH 5.6). For the hemocyanin, about 5 ml of 0.1% solution was heated 7 min at 100°C and then mixed, first with 5 ml, and then 10 ml, of 0.2% phosphates solution, and the amount of precipitate was observed. It was found that sodium phosphate (tribasic) and sodium pyrophosphate were the most effective in forming copper phosphate compounds from cupric chloride or hemocyanin in baby clams. Sodium phosphate (monobasic) and sodium tripolyphosphate were less effective. These results suggest that added phosphates might decrease blackening.

Effect of phosphates on the reaction of hydrogen sulfide and copper. With respect to discoloration, the relation between phosphate, copper, and hydrogen sulfide was studied. To each 5 ml of 1% $CuCl_2$ solution or 0.1% hemocyanin solution from baby clams was added 2 ml of sodium sulfide solution (0–100 mg%) as hydrogen sulfide and then 5 ml of 0.2% sodium phosphate (tribasic) or sodium pyrophosphate. Precipitation was then observed.

The results (Table 11) show that the sodium phosphates are effective in preventing the development of blackening. However, even though the phosphates were added directly into the container, black discoloration was not prevented when a large quantity of hydrogen sulfide was present. Thus, it would be difficult to prevent blackening of canned baby clams by the simple addition of phosphates.

Iron in sea water and sea-bottom mud. Results of Sekine (1926) and the present researchers indicate that baby clams have a higher iron content than other fish. Iron in the meat of baby clams is biologically important in shell formation and also involved in oxygen transport in the respiration system. The absorbed iron of baby clams comes mainly from the mud or sea water in which they are found. Thus, the iron content of baby clams and of mud or sea water where the clams were obtained was determined quantitatively. The method of estimation was the same colorimetric method as above. The samples were collected from six places where baby clams live along Ariake-Sea in Kyushu, Japan.

Considerable differences were found in the distribution of iron in the mud (2.6–8.7%) or sea water (0.1–0.4%) where baby clams live. The iron content of the meat of baby clams, however, was relatively constant (58.9–78.4 mg%), regardless of where they were harvested. In all cases it was greater than 20 mg%, the lowest limit of iron for blackening to occur. The lack of difference in iron content of the meat of baby clams harvested from a wide area is due to the fact that almost all of the iron absorbed into baby clams is utilized to meet physiological requirements.

Reaction of phosphates with iron. As described above, tribasic sodium phosphate and sodium pyrophosphate are effective in forming metallic compounds with copper. Experiments were carried out to determine whether the phosphates are also effective in forming metallic compounds with iron. Each 5 ml of 2% $FeSO_4$ solution was mixed with, first 5 ml, and then 10 ml, of 0.2% phosphates solution, and the amount of precipitate was observed. It was found that sodium and potassium metaphosphates are effective in forming metallic compounds with iron, but tribasic sodium phosphate and sodium pyrophosphate were not effective.

Blackening in clams containing phosphates. In order to find out whether the phosphates which were effective in preventing blackening of copper

Table 11. Effect of added phosphates on blackening of copper and hydrogen sulfide.

H_2S (mg%)	1% $CuCl_2$ soln			Cu in hemocyanin		
	Control	Na phosphate tribasic	Na pyrophosphate	Control	Na phosphate tribasic	Na pyrophosphate
0–30	—	—	—	—	—	—
40	+—	—	—	+—	—	—
50	+	—	—	+	—	+—
60	+	—	+—	+	+—	+—
70	++	+—	+	+	+	+
80	++	+	+	++	+	+
90	++	+	+	++	+	+
100	+++	++	++	++	+	+

—: no blackening; +—: slight blackening; +: blackening; ++, +++: marked blackening.

and hydrogen sulfide were also effective in commercially prepared canned baby clams, the following experiment was carried out. Sodium pyrophosphate or sodium phosphate (tribasic) was added to each container in the amount of 0.2% of the total weight of the contents; then the cans were packed with boiled meat of baby clams and processed 110 min at 15 lb (120.9°). The product was stored for about a month at room temperature (20±2°C) and then opened. The solid portion was used for the determination of copper, iron, hydrogen sulfide, and volatile basic nitrogen by the methods given above. Reflectance of the meat was estimated as follows: About 10 g of the meat was blended to a slurry in a mortar, then put on a disk of reflectometer attached to a photoelectric colorimeter. Reflectance was expressed as %T of the reading of the colorimeter. The standard for the estimation of reflectance was CaO. The crushed meat of baby clams was extracted with ten times its volume of water, and the pH value was estimated with a glass-electrode pH meter.

The results (Table 12) indicate that sodium pyrophosphate and tribasic sodium phosphate were effective to some extent in preventing blackening in canned baby clams. It is obvious that copper in canned baby clams is liberated from blood hemocyanin by denaturation or decomposition. Iron, however, is also a component that causes blackening. Although it could be dissolved from the tin container rather than from the contents, this should not be an important factor, since the container was coated with C-enamel or other paints. It should be recalled that there were only slight differences between the iron contents of canned and raw meat of baby clams. Hence dissolution of iron from the tin container is thought to have little or no relation to blackening.

The copper associated with blackening is, at least in part, liberated from the hemocyanin of baby clams by processing. Then, the copper and iron react with hydrogen sulfide to produce the blackening. Some phosphate compounds (0.2%) appear to be effective in preventing the development of black discoloration by forming the metallic phosphate compounds.

Table 12. Effect of added phosphates on blackening of canned baby clams.

	Control (without phosphate)	Na phosphate tribasic	Na pyrophosphate
Cu as CuO (mg%)	0.75	0.82	0.78
Fe as FeO (mg%)	67.3	55.7	59.1
H_2S (mg%)	38.9	37.2	36.5
Reflectance (%T)	17.3	20.3	19.6
VBN (mg%)	15.4	14.6	12.8
pH	6.4	6.5	6.2

DISCUSSION

The data indicate that blackening of canned baby clams is a phenomenon of precipitation of metallic sulfides. The metallic constituent of the black substance is copper and iron. The copper content of hemocyanin from *Gastropod* is usually about 0.25% higher than that from *Arthropoda*. The relatively high copper content of hemocyanins from *Gastropod* represents a remarkable concentration of copper from sea water, which contains only 0.01 μg per 100 ml (Goodwin, 1960). Thus, the development of blackening of canned baby clams may easily occur.

Without any definite evidence, the copper in various hemocyanins has for many years been assumed to be in the cuprous form in both the reduced and oxygenated pigments (Redfield, 1952). More recently, considerable evidence has been obtained that the copper in *Busycon canaliculatum* hemocyanin is almost entirely in the cuprous state in the non-oxygenated pigment, and that only about one-half is in the cupric state in the oxygenated pigment (Klotz and Klotz, 1954). Also, strong oxidizing agents, e.g. permanganate, are necessary to convert the copper in *Homarus* hemocyanin from the cuprous to the cupric state. From these findings, the valency state of the copper in hemocyanin from baby clams may be mainly in the cuprous state. Whether the valency state of the copper in the hemocyanin in canned baby clam remains in the cuprous state or is converted to the cupric state by heating, is yet to be determined.

Some phosphate compounds are effective in forming metallic phosphate compounds with iron and copper. These phosphate compounds may be effective in preventing blackening in canned baby clams. The reversion of polyphosphate compounds to orthophosphate is affected by pH and heat treatments (Green, 1950). Therefore information regarding the final pH of canned baby clams after addition of various phosphates and heat "stability" of the added polyphosphate compounds in the canned clams is

necessary before a recommendation can be made on the type of phosphate compounds to be used in preventing blackening in canned baby clams.

REFERENCES

Allison, J. B., and W. H. Cole. 1940. The nitrogen, copper content of the serums of several arthropods. *J. Biol. Chem.* **135**, 259.

Arakawa, G. 1928. Studies on blackening and corrosion of tin container of canned crab. *Canners J.* **7**(7), 45.

Breuer, R., and M. W. Militzer. 1938. Determination of iron in blood. *J. Biol. Chem.* **126**, 561.

Fellers, C. R., and C. T. Parks. 1926. Biochemical study and proximate composition of Pacific coast crabs. *Univ. Washington Publ. Fisheries.* **1**(7), 139.

Goodwin, T. W. 1960. Biochemistry and pigments. The physiology of Crustacea, Vol. 1, pp. 101–140. Academic Press, N. Y.

Green, J. 1950. Reversion of molecularly dehydrated sodium phosphates. *Ind. Eng. Chem.* **42**(18), 1542.

Hagedorn, H. C., and B. N. Jensen. 1923. Micro determination of blood sugar by means of ferricyanide. *Biochem. Z.* **135**, 47.

Kaneko, I. 1951. On manufacture of canned crab. Nichiro Fisheries Company, Japan.

Klotz, T. A., and I. M. Klotz. 1954. The nature of oxydation reaction in haemocyanin. *Biol. Bull.* **107**, 300.

Legendre, R. 1928. Industrial application of pH. Cure for the blackening of canned crustaceae. *Chem. Ind. Spec.* No. **842**.

Machida, S. 1911. Cause of blackening of canned crab meat. *J. Agr.* **108**, 1.

Obata, Y., S. Sakamura, Y. Ishikawa, and J. Mizutani. 1957. The factor responsible for blackening of the wall of cans containing cooked seasoned beef. *Nippon Nogei Kagaku Kaishi* **31**, 585.

Oshima, K. 1927. Preservative methods of blackening of canned crab, shrimp, shellfish, corn, etc. Japan Pat. No. 4889.

Pigott, G. M., and A. M. Dollar. 1963. Iron sulfide blackening in canned protein foods; Oxidation and reduction mechanisms in relation to sulfur and iron. *Food Technol.* **17**(4), 115.

Pigott, G. M., and M. E. Stanby. 1956. Iron sulfide discoloration of tuna cans. 2. Analytical methods. *Comm. Fisheries Rev.* **18**(2), 5.

Redfield, A. C. 1952. Haemocyanin. Symposium of copper metabolism. 1950. (W. D. McElroy and H. B. Glass, eds.), pp. 174–190, Johns-Hopkins, Baltimore.

Sekine, S. 1926. Blackening of canned baby clam and chemical composition of black substance. *Canners J.* **5**(5), 71.

Tanikawa, E. 1958. Studies on the technical problems in the processing of canned salmon. *Mem. Fac. Fisheries. Hokkaido Univ.*, **6**(2), 67.

Thompson, M. H. 1963. The mechanism of iron sulfide discoloration in cans of shrimp. *Food Technol.* **17**(5), 665.

Tomiyama, T., and K. Kanzaki. 1951. A semimicro method for determination of sulfide contained in muddy deposits. *Bull. Japan. Soc. Sci. Fisheries* **17**(5), 115.

Tompsett, S. L. 1934. The copper content of blood. *Biochem. J.* **28**, 1544.

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Effect of Temperature and pH on the Soluble Proteins of Ham

SUMMARY

A sensitive heat gelling test based upon the least protein concentration as the endpoint (LCE) was developed for salt-soluble protein evaluation in extracted muscle. Although this is not readily applicable to quality-control procedures, it appears to be an excellent research tool to study subtle protein changes occurring in muscle proteins during the post-mortem transformation of muscle into meat. Using the LCE to study the effect of simulated physiological temperature and pH on muscle protein extracts it was found that pH has a profound and partially, but not complete, reversible effect on these proteins. A definite relation was found between post-rigor muscle pH and the LCE of the extracted salt-soluble proteins, which suggests that the rigor mortis transformation of muscle to meat determines the subsequent characteristics of these proteins. The most desirable post-rigor muscle pH was 5.8–6.1. No mechanism is proposed to explain the effect of pH on the salt-soluble protein LCE. The effect of decreasing pH was linear on the solubility of both water- and salt-soluble proteins, and on the increase of free heme with concomitant decrease in oxymyoglobin visual color. Mixing salt-soluble proteins from extreme-pH post-rigor muscle resulted in an improved LCE while averaging the solution pH and ATP(ase) activity. This mixing effect may account for the lack of relation between sausage batter variation and product quality.

INTRODUCTION

In the production of processed sausage, products of marginal quality should be significantly reduced by the application of computor-derived formula, single 10–15,000-lb batch mixing of trimmings representing several thousand animals (Kielsmeier and Gara, 1962), and continuous processing including chopping (Eberman, 1961), stuffing (Sloan *et al.*, 1965), smoking, heating, and relative-humidity conditions (Sloan and Ahern, 1965). While this has been true, occasional product problems such as fat separation, soft texture, poor color development, and stability are still found. Studies in this laboratory of samples taken from large lots of the individual beef and pork meat components and large mixed batches, and examined by various water-binding, fat emulsification, and heat gel strength tests suggested that these problems are related to variations in the meat quality. Although meat quality was indicated as a major variable, good statistical relationships could not be established, because of the poor sensitivity of these methods. To understand these meat quality variations, attention was directed toward characterizing some of the muscle post-mortem glycolytic-associated changes, particularly regarding the soluble muscle proteins. In most processed sausage products it is essential that a portion of the contractive (salt-soluble) muscle proteins be solubilized with sodium chloride before heat processing. Although post-mortem changes in muscle have recently received a great deal of attention (Bendall, Briskey, Hamm, Wismer-Pedersen), it is unfortunate that these studies have not included sodium chloride solubility properties which would be similar to those necessary for sausage manufacture.

Variations in many post-rigor carcass properties and characteristics have been reported to be associated with the biochemical transformation of muscle into meat. Specifically, Bendall and Wismer-Pedersen, (1962), Wismer-Pedersen (1964), and Briskey and Wismer-Pedersen (1961) have reported that rapid glycolysis at body temperatures result in pale, soft, exudative pork tissue. Bendall and Wismer-Pedersen (1962) concluded that solubility, swelling, and water retention of the muscle fibrils are inhibited by an insoluble surface layer of sarcoplasmic protein which is altered by post-mortem temperature and pH changes. They also concluded that the myofibrillar proteins are relatively unaffected by these post-mortem changes. Scopes (1964) reported the denaturation of sarcoplasmic protein to be both temperature- and pH-dependent and associated with decreased myofibrillar solubility. The loss in solubility of both sarcoplasmic and myofibrillar proteins due to post-mortem changes has been reported by Sayre and Briskey (1963). Cassens *et al.* (1963-a,b) reported evidence conflicting with Ben-

dall and Wismer-Pedersen's (1962) interpretation of the dense irregularly banded material. They suggest that the disorganization of the myofilaments may be due to changes in the myofibrillar proteins and that the precipitated sarcoplasmic proteins may be an artifact of myofibrillar solubility.

Although there is general agreement that the kinetics and extent of post-mortem glycolysis are responsible for the undesirable properties of the pale, soft, watery muscle, the causative factors and components involved have not been fully elucidated. Considerable disagreement exists about which reactions of glycolysis are most responsible for these properties and which meat components are most susceptible to the changes.

While both temperature and pH contribute to ultimate carcass properties, no attempt has been made to distinguish between temperature effects on the rate of glycolysis and the kinetics of chemical reactions between the products of glycolysis and meat components. Hence, it is desirable to determine which is most important to manufacturing meat quality: a) the ultimate carcass pH resulting from various rates of glycolysis; or b) the nonglycolytic reactions, whose rates are temperature-dependent. Of equal importance is determination of which meat components are involved.

A model system of soluble meat components was selected so that undefinable variables could be minimized and the experimental parameters closely controlled. Such a model system has a very practical basis since: a) except for the dilution effects of water and the presence of fat and connective tissue, finely comminuted sausage batters are quite similar; and b) the heat treatments and pH range studied are those encountered in rigor mortis conditions and during cooked sausage manufacturing. Although some of the properties of the soluble proteins studied here in model systems may be analogous to their reactions in muscle and meat, these systems must be considered different in at least two respects:

First, the model system contained only those proteins which were soluble after limited or extensive post-mortem reactions took place. Because the extraction may be viewed as a fractionation step of soluble from insoluble protein of the same or mixtures of proteins, the extracts may not be truly representative of meat, especially with respect to molar ratios of myoglobin, actin, myosin and actomyosin.

Second, the myofibrillar proteins were studied in their NaCl-soluble states versus their potentially soluble or superprecipitated condition in the muscle. The subject of this report is the observed influence of simulated carcass temperatures and pH conditions on the soluble protein components from ham muscle.

MATERIALS AND METHODS

Muscle samples. *Pre-rigor.* Semimembranosus muscles were obtained from 5½–6½-month pigs ranging in weight from 195–210 lb within five min of slaughter at the University of Wisconsin Meats Laboratory and immediately ground and frozen in thin layers on dry ice. The muscle from each animal was identified and treated as individual samples. The frozen samples were placed directly in the salt extraction solution to avoid thaw rigor, which is similar to certain sausage practices (Turner and Olson, 1959).

Post-rigor. Semimembranosus muscles were obtained from swine carcasses 18–24 hr after carbon dioxide anesthesia and slaughter in a commercial meat processing plant. Average 24-hr carcass temperature was 38–40°F. Numerous individual muscles were selected on their color basis, their distilled-water pH was determined, and those representing the desired pH range were taken for individual extraction.

Extraction. Samples to be extracted were cooled to 32–33°F, extracted 1 hr at 32°F on a constant-speed stirring rack at a ratio of 100 g sample to 275 ml of 0.05*M*, 0.67*M*, or 1.2*M* NaCl (depending upon which major component was desired), and centrifuged for 10 min at approximately 9,000 × G. The use of 0.67*M* NaCl and 1.2*M* NaCl extraction solutions was justified on the basis that many sausage products have 0.67*M* NaCl in their aqueous phase and, during certain periods of the blending of sausage ingredients, the NaCl concentration in the aqueous phase may be as high as 1.4–1.5*M*. Subsequent mixing and chopping melts the remaining ice and lowers the NaCl concentration. The protein components are referred to herein as water- or salt-soluble instead of sarcoplasmic or myofibrillar because their manner of preparation was based upon their solubility. Electrophoretic examinations of similarly prepared extracts have confirmed the validity of this terminology with respect to the

presence of actin and myosin and actomyosin in pre- and post-rigor purified extracts, respectively.

Water-soluble protein (sarcoplasmic proteins). The 0.05*M* NaCl supernatants contained only water-soluble protein and NPN. The latter was removed by dialysis for two days at 32°F against three changes (10× supernatant volume) of 0.05*M* NaCl.

Water- and salt-soluble protein. The 0.67*M* NaCl supernatant contained approximately equal amounts of water- and salt-soluble proteins plus NPN. The latter was removed by dialysis against 0.67*M* NaCl.

Salt-soluble protein (myofibrillar proteins). The 1.2*M* NaCl supernatants contained significantly more salt-soluble protein than water-soluble protein and were utilized as sources of purified salt-soluble protein. These supernatants were dialyzed for three days at 32°F against 0.05*M* NaCl with four changes, and then centrifuged for 10 min at approximately 9,000 × G. The insoluble salt-soluble protein was recovered and washed twice with 0.05*M* NaCl. Finally it was solubilized in 1.2*M* NaCl and dialyzed for two days with 0.67*M* NaCl. At 1½% concentration these soluble purified salt-soluble proteins were clear, whitish solutions with no acid-acetone-soluble heme.

Heat-gelling test. A test called "least concentration," developed to determine the quality differences of soluble proteins, was based upon the rigidity and elasticity of a heat-induced gel. It consists of the following steps:

1) Calculated portions of a soluble protein solution and 0.67*M* NaCl or buffer are pipetted into a series of 15-ml conical centrifuge glass tubes so that 10 ml of these solutions should cover the anticipated concentration endpoint range. Generally, pre-rigor salt-soluble protein will have a range of 0.3–0.5%; post-rigor salt-soluble protein will have a range of 0.6–1.0%, depending on its original muscle pH; and water-plus-salt-soluble protein mixtures will have a range of 1.2–2.0%. Increments of 0.1% are satisfactory.

2) The 10-ml contents of each tube are thoroughly mixed by repeated gentle inversions.

3) The series of tubes are transferred to a 176°F water bath, held quiescently for 10 min, and then transferred to an ice-water bath for 1 hr.

4) The air-gel-tube interphase of each tube was loosened with a micro-spatula to avoid anomalous endpoints. Each tube was then gently inverted three times. The endpoint was the tube in which all the content of the least protein concentration remained in the tube when inverted.

5) Once the endpoint range is established, an endpoint accuracy of ±0.05% protein may be obtained by repeating the test with the protein solution diluted to a concentration 0.1–0.2% greater than the endpoint previously determined. With experience, this endpoint is quite definite and reproducible.

Pure water-soluble protein solutions will form a flocculated coagulum when heated to 176°F at a concentration of 2% or greater. However, slight physical distortions caused a complete breakdown of the coagulum over a wide range (2.0–3.0%) of protein concentration, so that this test is not applicable to pure water-soluble proteins.

Buffers. The pH changes were accomplished by dialysis for at least 2 days against one of the following buffers at 32°F with four changes:

1) pH 7.0–4.5 were prepared containing 0.67*M* NaCl and 0.05*M* phosphate and adjusted to the desired pH with NaOH or HCl.

2) Similar NaCl-PO_4 buffers were prepared containing 0.01*M* sodium ascorbate.

Heme determinations. Total acid-acetone-soluble heme was determined by Hornsey's (1956) method. Acetone-soluble free heme was determined on 10-ml protein solutions plus sufficient acetone and 0.01*M* ascorbate to give a ratio of 80% acetone to 20% water at 50 ml. The absorption spectra of the free heme was similar to that of acid hematin. Thus, in a 1-cm cell at 640 mμ, a factor of 680 was used to obtain ppm of free heme (Hornsey, 1959).

ATP(ase) activity. The magnesium-activated ATP(ase) activity of salt-soluble protein was determined by the method of Perry and Kielley (1955) at pH 7.5, and inorganic phosphate by Allen's method (1940).

Heating protein solutions. After dialysis against buffer for 3 days, each protein solution in its dialysis bag was transferred to its corresponding buffer, which had been prewarmed to the desired temperature. Each sample was held for 30 min and then returned to its 32°F buffer, where dialysis was continued for two days.

RESULTS AND DISCUSSION

Pre-rigor proteins. The frozen pre-rigor ham muscles from only one animal were extracted for each experiment under conditions which avoided thaw rigor. All the results reported here were confirmed by at least three similar experiments. Pre-rigor muscles were selected so that the condition of the extracted proteins would be as near the premortem state as possible before the cellular organization was disrupted. The amount of labile phosphate was also determined in each muscle and expressed as the percent of total

phosphate which was acid-hydrolyzed. A range of 57–70% labile phosphate was found.

Effect of temperature and pH on water- and salt-soluble proteins. A 0.67*M* NaCl extract of the ⅛-in. reground frozen pre-rigor muscle was prepared. Portions were immediately dialyzed against phosphate buffer at pH 7.0, 6.5, 6.0, 5.5, and 5.0. After three days of dialysis, fractions of each pH were heated to 65, 75, 85, 95, and 105°F to determine if there was a critical temperature effect which might be similar to that reported by Briskey (1963). After two additional days of buffer dialysis, the protein concentration, least-concentration endpoint (LCE), and pH of each sample were determined. Below pH 5.5, the samples were in a gel condition, which, when centrifuged, yielded a dense residue and a pink-gray supernatant. The bright-pink oxymyoglobin changed to gray with decreasing pH from 6.5. This observation was in agreement with Goldspink and McLoughlin (1964). The effects of pH and heat on the LCE are illustrated in Fig. 1.

The results in Fig. 1 gave a family of curves representing the five heat treatments from 65 to 105°F. Very little significant deleterious effect was observed between the 65 and 105°F heat treatments. The most significant effect was in pH. The best LCE (0.7-0.8%) was between 6.0 and 5.7. Below this pH range, a very sharp positive slope in the curves continued until the sample gelled into an unworkable condition, at pH 5.3 and at approximately twice the LCE. Above pH 6.0 the slope of the curves increased in a positive manner to approximately pH 6.75 and, again, about twice the LCE. The LCE results suggested that there was an optimum pH range in which the heat-denatured soluble proteins gave a more rigid gel. The pink-gray supernatants containing 10–20% of the protein below pH 5.7 indicated that not all the sarcoplasmic proteins had precipitated. Since the LCE reflects the effects of pH before and during heating of the NaCl solubilized protein system, these results cannot be compared with work of Hamm (1959) on the pH and water-binding capacity of raw meat systems. However, because sausage batters are heat-processed at pH values representing the composite of many muscle pH's or individual hams representing pH's from 5.7 to 6.6, the LCE test at various pH's is considered to have a significant practical relationship.

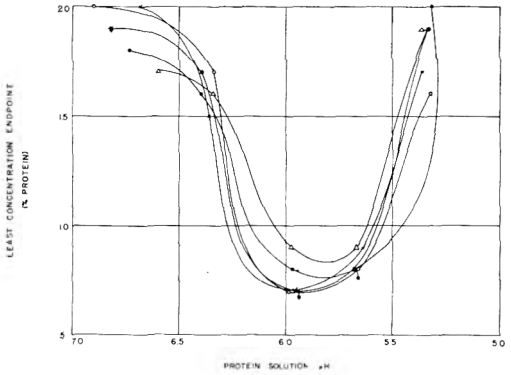

Fig. 1. The effects of pH and temperature on the least-concentration endpoint of a pre-rigor extract containing water- and salt-soluble proteins. Heat treatments for 30 min.: x——x 65°; o——o 75°; *——* 85°; ●——● 95°; and Δ——Δ 105°F.

Effect of temperature and pH on salt-soluble protein and pH reversal. Since the solubility of myofibrillar proteins has been reported by Sayre and Briskey (1963) to be influenced by physiological conditions, the effects of pH on purified salt-soluble proteins were examined. Pre-rigor salt-soluble proteins were prepared from a single semimembranosus muscle. Portions of the resolubilized protein solution were dialyzed against phosphate buffers. Since no significant effect of temperature was previously found, half of each sample was warmed only at 65°F for 30 min. Following further dialysis, the least-concentration endpoint and pH were determined. Afterwards, aliquots of each sample were dialyzed individually for three days against phosphate buffer, pH 6.4, to study the reversible nature of the pH effect. The LCE and solution pH were again determined. The results are presented in Fig. 2.

The results in Fig. 2 were on purified salt-soluble proteins. The effect of changing the protein pH is represented by the solid lines, while the effect of returning the protein solution pH to 6.4–6.5 is represented by broken lines and arrows. Again, an LCE minimum was found in the pH range of 5.8–6.1. Similar sharp positive slope changes were found at pH 5.4 and 6.75. No significant deleterious effects of the 65°F heat treatment were

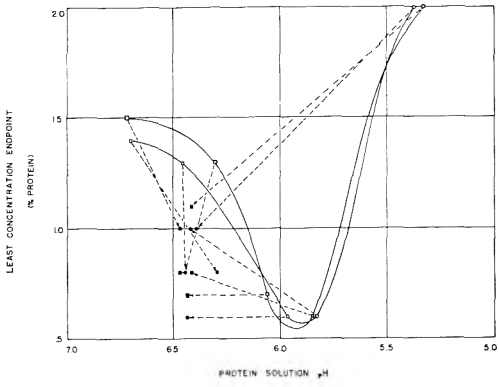

Fig. 2. The effect of pH, temperature, and pH reversal on pre-rigor salt-soluble protein.
Control o——o
Heated to 65°F for 30 min □——□.
Filled symbols with dotted line connecting arrows indicate pH-reversal effect.

found. Returning the pH to approximately 6.4 tended to increase slightly the LCE of the samples at pH 5.8–6.1. A decrease (improvement) was found in samples either above or below this range. Changing the pH appeared to restore partially, though not completely, the LCE of the samples initially at the pH extremes. In other words, the extreme pH of 6.75 and 5.4 tended to imprint certain properties which were irreversible during subsequent pH changes. Hamm (1959) has stated that pH changes of muscle from 4.5 to 7.0 are completely reversible for water-holding capacity of uncooked meat systems. The pH reversal is not complete with this heated system. The partial reversal of the pH effect observed here illustrates the desirability of studying isolated proteins and the sensitivity of the least-concentration test in determining subtle protein changes. Because the LCE for pure salt-soluble protein was obtained at significantly lower protein concentrations than those for water-and-salt-soluble protein mixtures (0.5–0.6, versus approximately 1.5–2.0% protein), it was concluded from an intact cooked-meat water-binding standpoint that pH may have a greater influence on the salt-soluble protein than on the water-soluble protein.

Post-rigor proteins. The indication that initial pre-rigor pH may determine subsequent characteristics of salt-soluble proteins suggested that post-rigor muscle pH may also determine muscle or salt-soluble protein properties.

Effect of temperature and pH on salt-soluble proteins. Salt-soluble proteins were prepared individually from post-rigor ham muscles having 24-hr pH's of 5.25, 5.55, 6.27, 6.68, and 6.80. Portions of each preparation were divided into the following lots: three control samples which were exhaustively dialyzed against 0.67*M* NaCl followed by one not being heated; a second heated to 65°; and a third heated to 105°F; and three series which were dialyzed against the range of pH buffers before similar heat treatments. Because specific ions have been reported (Hamm, 1960; Yasui *et al.*, 1964a,b) to influence the water binding of raw and cooked meats, phosphate, malonate, and EDTA buffers were also studied. The LCE and pH were determined and plotted. The results of the pH 6.68 ham-muscle salt-soluble proteins in EDTA buffer are presented in Fig. 3. Again, the pH caused an increase in LCE at the extremes of pH studied, and a minimum was found in the pH range 5.7–6.0. A small deleterious effect of heating was noted in the

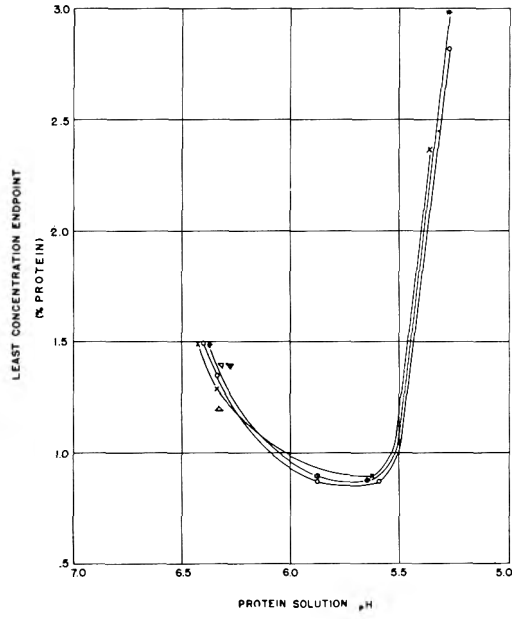

Fig. 3. The effect of pH and temperature on the least-concentration endpoint of post-rigor salt-soluble protein.
x——x pH control, o——o heated to 65°F for 30 min.
+——+ heated 30 min at 105°F.

control unbuffered samples. No heating effect was found in the buffered samples until the pH was below 5.5. Similar families of curves were obtained with salt-soluble proteins from semimembranosus muscles of various pH's. Portions of the same protein sample dialyzed against these three buffers and at the same pH's result in very similar curves. The buffer ion studied did not exhibit a significant specific effect. While the shapes of the curves from each sample were similar, the LCE minimum varied from pH 5.6 to 6.2. When all of the results from this pH range of muscles were plotted, it became apparent that, at a given buffer pH, the pH effect was not of the same intensity.

Relationship between post-rigor ham muscle pH and its salt-soluble protein least-concentration endpoint. Since post-rigor salt-soluble proteins from different pH muscles did not respond equally to the same pH, the previously noted effect of initial pH on pre-rigor proteins suggested that these differences may have been related to the post-rigor muscle pH. To determine whether a relationship existed, the results of the unheated control samples from the previous experiments were plotted versus their respective muscle pH as presented in Fig. 4. A minimum LCE was found in the salt-soluble protein extracted from ham muscles in the pH range of 5.8–6.25. The endpoint then increased in a similar manner on both sides of this minimum when the muscle was

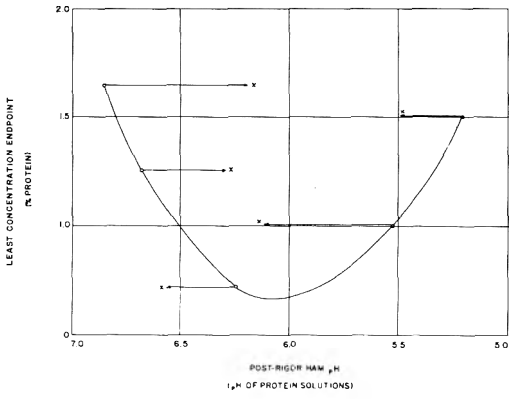

Fig. 4. Relationship between post-rigor ham pH and its salt-soluble protein least-concentration endpoint. o———o ham muscle pH versus LCE; o———→ x direction and magnitude of shift from muscle pH to SSP solution pH at equilibrium dialysis.

of either higher or lower pH. There appears to be a very definite relationship between LCE and the post-rigor muscle pH. This indicates that the properties of the salt-soluble proteins are determined by and are characteristic of the glycolytic events during rigor-mortis which determine ultimate muscle pH.

The pH of each protein solution was also plotted in Fig. 4, with arrows connecting each respective muscle pH. The direction and length of each arrow indicate the pH shift from muscle to salt-soluble protein solutions. At muscle pH of 6.5 and higher after exhaustive NaCl dialysis, the salt-soluble proteins had a lower solution pH, while at muscle pH of 6.25 and lower the solutions had an increase in pH. This shift in pH would appear to explain the differences in response at a given pH of salt-soluble proteins from different muscle pH. It may also be interpreted as differences in sodium and chloride ion-binding capacity as determined by post-rigor pH. During an 18-month period following these experiments, 14 similar salt-soluble protein solutions from known muscle pH were found to have LCE's which fit on this curve. Hamm (1960) has explained various muscle-pH water-hydration curves in terms of inter- and intramolecular bonds. His curve differences may have been due to changes brought about by bond changes caused by differences in muscle pH which may be related to the pH shift from muscle to protein solution observed here.

Although additional studies will be necessary to explain the protein structural differences responsible for this pH 5.8–6.25 LCE minimum, a practical relationship has been found. The amount of free moisture in a vacuumized impervious film package of comminuted sausage after 14 days at 45°F was found to be related directly to the LCE of the salt-soluble proteins extracted from the sausage batter (Trautman, 1963). Thus it is concluded that LCE is related to water binding in cooked meat systems which contain sufficient NaCl to solubilize the salt-soluble proteins.

For several years it has been observed in this laboratory that the addition of NaCl to post-rigor ham muscles consistently lowers

the pH. This has been reported by Hamm (1957), and Van Logtestijn (1964). The increase in pH upon exhaustive NaCl dialysis of salt-soluble proteins from low-pH muscles suggests that the water-soluble and insoluble muscle components mask these subtle changes in the salt-soluble proteins.

Effect of pH on protein solubility and heme distribution. *Salt-soluble protein.* One percent salt-soluble protein solutions containing no acid-acetone-soluble heme were prepared from post-rigor ham muscles of pH 6.51 and 5.46. Portions were dialyzed individually for three days against the range of NaCl-phosphate buffers. The protein content of each sample was determined before and after centrifuging, and the effect of pH on protein solubility was calculated. The results are presented in Fig. 5. No difference in solubility was found between these two samples at the same buffer pH. Both samples were completely soluble above pH 5.9 and 95% insoluble at pH 4.9. Additional studies of salt-soluble proteins from muscles of various pH's found very similar curves, with no apparent relationship between muscle pH and loss in protein solubility, as found with the LCE in Fig. 4.

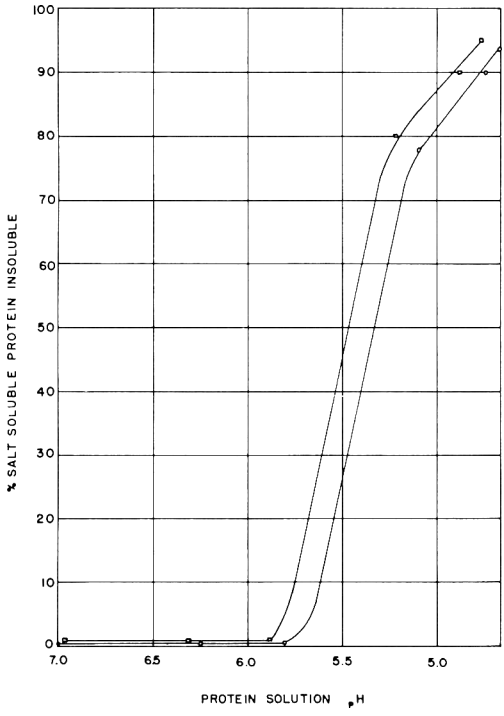

Fig. 5. The effect of pH on the solubility of post-rigor salt-soluble protein. Original ham muscle pH: 0——0, 5.46; □——□ 6.51.

These results indicate that solubility criteria are not as sensitive a test for protein alterations as the least-concentration test. Although protein solubility may be a poor criterion for subtle protein changes, it has been used by Sayre and Briskey (1963) to indicate differences of physiological conditions in the muscle. These effects of pH on salt-soluble protein solubility support the position of Sayre and Briskey that rigor-mortis pH changes do alter the solubility of myofibrillar proteins and are not confined to the sarcoplasmic proteins as reported by Bendall and Wismer-Pederson (1962).

The disagreement about the pH effects on muscle protein solubility (Bendall and Wismer-Pederson, 1962; Sayre and Briskey, 1963; and Scopes, 1964) may be due to the use of buffers during extraction which have pH's different from the muscle. Thus anomalies may be introduced by these buffers before experimental observations are made. As shown in Fig. 2 and confirmed in Fig. 4, certain salt-soluble protein properties are influenced by their first exposure to pH decrease and respond differently following subsequent pH changes.

Water-soluble protein. A decrease in the color intensity of myoglobin with decreasing pH was noted similar to that shown in Fig. 1. This loss in color may have been due to a change from a covalent to an ionic bonding (Haurowitz and Hardin, 1954) between the iron of the ferriheme and the F-8 histidyl residue of Kendrew *et al.* (1960) 2A model; or splitting of the approximately 9 salt linkages between the heme group and the protein moiety. Snyder (1963) found 40% of the heme in beef muscle myoglobin to be soluble in acetone at pH 6.6. Although Fronticelli and Bucci (1963) were unable to find this amount of soluble heme from myoglobin derivates at pH 6.6, they did find a sharp increase at pH 5.0 and below. To determine whether acetone-soluble heme increased concomitantly with visual color loss and decreasing pH, the following study was made.

Water-soluble proteins were extracted from single post-rigor muscles. The protein

content of the extracts was standardized at 1% and the free and total heme determined. Portions of each extract were then dialyzed against the NaCl-phosphate buffers containing 0.01*M* ascorbate. Without ascorbate in the buffers, the lower-pH samples were found to analyze lower heme contents than the higher-pH samples and original unbuffered extract. This loss in heme suggested that free heme is less stable at lower pH even at 32°F. After three days of buffer dialysis, portions of each sample were removed for determinations of free and total heme, pH, and protein. The remaining portion of each sample was centrifuged for 10 min at 9,000 × G and the supernatant protein determined. The effects of pH on heme distribution and water-soluble protein solubility of an extract from pH 6.28 post-rigor ham muscle are presented in Fig. 6. The ratio of ppm heme to % protein is plotted against protein solution pH to correct for small changes in bag volume during dialysis. In the presence of ascorbate the total heme concentration remained constant while the free heme increased linearly with pH decrease. The visual intensity decreased at a rate apparently similar to the increase in free heme. Protein insolubilization with decreasing pH was also linear and prevented a more objective measurement of the color intensity.

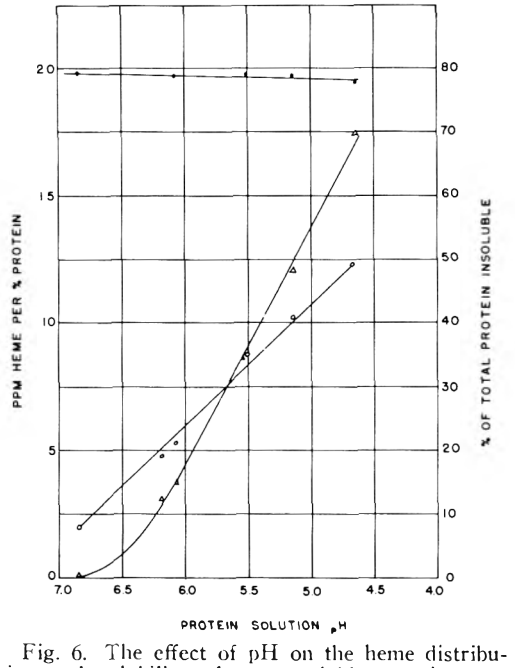

Fig. 6. The effect of pH on the heme distribution and solubility of water-soluble proteins. ●——● total heme; 0——0 free heme; Δ——Δ percent of total protein insoluble.

Although the increase in free heme and protein insolubilization was linear with pH in a series of similar experiments, the slope of the lines was not constant. This may have been due to the difference in extract composition. For example, the ratio of ppm heme to % protein ranged from 19 to 29 extracted from muscles of pH 5.9–6.5. This narrow range of muscle pH may account for the lack of a correlation between heme content and muscle pH as reported by Hornsey (1959).

These results on water-soluble protein extracts suggested that the paleness of pork muscle studied by Briskey and others may have been initiated by simple pH effects of the muscle during rigor mortis. If this were the case then analysis of free heme, total heme, and pH of muscle pairs, one pre-rigor and one post-rigor, should reveal a relationship between free heme and decrease of visual color with decreasing post-rigor muscle pH. Approximately two dozen pairs of muscles were examined, and significant differences found in pH, color, and total heme. However, no free heme was found in any of the pre- or post-rigor muscles. The failure to find free heme in low-pH muscles, while readily detected in water-soluble protein extracts of similar pH, prompted further studies on the nature of the protein-moiety heme binding in myoglobin, which will be the subject of a future paper.

Mixing salt-soluble proteins. The relationship shown in Fig. 4 explains the LCE range of 0.7–1.2 found in studies of several years ago in which muscle pH was not determined. However, it did not explain the LCE of 0.8–0.9 of salt-soluble proteins extracted from large sausage batters containing muscles ranging from pH 6.6–5.4 (Moss, 1963). No significant statistical correlation was found between 38 sausage batter pH's and least-concentration endpoints.

To study the effect of mixing salt-soluble proteins from muscles of known pH, three post-rigor muscles were selected having pH's of 6.85, 6.15, and 5.60. The salt-soluble proteins were individually extracted and puri-

fied, and exhaustively dialyzed against 0.67*M* NaCl. The LCE, pH, and ATP(ase) activity were determined on the individual samples and 50:50 mixtures of each. The results are presented in Table 1.

The LCE of the individual samples closely followed the relationship shown in Fig. 4 with respect to muscle pH. The mixture of pH 6.85 + 6.15 (sample D) and pH 6.15 + 5.60 (sample E) resulted in an averaging effect on the LCE. However, when the pH 6.85 sample (A) was mixed with the pH 5.60 sample (C) to obtain sample F, a lower endpoint was obtained than either of these individually. Since the pH of the latter mixture approximated that of the two former mixtures, pH alone did not appear to account for this effect. This improvement effect may be similar to that noted by Shaw (1958) that mixing pre-rigor and post-rigor muscles together resulted in a pre-rigor electrophoretic pattern of the extract. His analysis suggested that this effect was not merely an ATP effect but was probably protein complexing. Although the improvement by mixing may involve protein complexing, a thorough study including ultracentrifugal molecular-weight distributions should be made before this phenomenon is suggested to be due to different rigor mortis conditions causing different protein molecular stoichiometric combinations or alterations.

The ATP(ase) activity of the individual and mixed samples was on the order expected if this activity was primarily a function of ultimate muscle pH and a secondary function of protein solution pH, i.e. greater activity with higher post-rigor muscle pH. The ATP(ase) activity of the mixed samples supported this position in that their activity was a numerical average of the activity of the two components. The variability of muscle ATP(ase) activity reported in literature may be the result of pH differences in the sources of myofibrillar proteins.

The improvement in least-concentration endpoint by mixing and averaging ATP(ase) activities was confirmed in two similar experiments with three muscles having post-rigor pH in the range 6.5–5.5.

Table 1. The effect of mixing post-rigor salt-soluble proteins on their LCE, solution pH, and ATP(ase) activity.

Sample	Muscle pH	Protein solution pH	LCE (% protein)	ATP(ase) activity[a]
A	6.85	6.01	1.4	2.50
B	6.15	5.80	0.7	1.85
C	5.60	5.61	1.0	0
D	½ A + ½ B mixture	5.76	1.1	2.20
E	½ B + ½ C mixture	5.70	0.9	0.90
F	½ A + ½ C mixture	5.80	0.9	1.20

[a] $\mu M PO_4$ released in 5 min per mg enzyme N.

REFERENCES

Allen, R. J. L. 1940. The estimation of phosphorus. *Biochem. J.* **34**, 858.

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.

Briskey, E. J., and J. Wismer-Pedersen. 1961. Biochemistry of pork muscle structure. I. Rate of anaerobic glycolysis and temperature change versus the apparent structure of muscle tissue. *J. Food Sci.* **26**, 297.

Briskey, E. J. 1963. Influence of ante- and post-mortem handling practices on properties of muscle which are related to tenderness. *Proc. Campbell Soup Co. Meat Tenderness Symp.*

Cassens, R. G., E. J. Briskey, and W. G. Hoekstra. 1963. Similarity in the contracture bands occurring in thaw-rigor and in other violent treatments of muscle. *Biodynamics* **9**, 165.

Cassens, R. G., E. J. Briskey, and W. G. Hoekstra. 1963b. Electron microscopy of post-mortem changes in porcine muscle. *J. Food Sci.* **28**, 680.

Eberman, A. H. 1961. Continuous chopper. U.S. Patent 2,974,701.

Fronticelli, C., and E. Bucci. 1963. Acetone extraction of heme from myoglobin and hemoglobin at acid pH. *Biochim. et Biophys. Acta* **79**, 530.

Goldspink, G., and J. V. McLoughlin. 1964. Studies on pig muscle. 3. The effect of temperature on the solubility of the sarcoplasmic proteins in relation to colour changes in post-rigor muscle. *Irish J. of Agr. Research* **3**, 9.

Hamm, R. 1957. Water-binding capacity of mammalian muscle. III. The action of neutral salts. *Z. Lebensm. Utersuch. u.-Forsch* **106**, 281.

Hamm, R. 1959. Biochemistry of meat hydration. *Proc. 11th Research Conf. A.M.I.F.* **50**, 17.

Hamm, R. 1960. Biochemistry of meat hydration. *Food Research* **10**, 355.

Haurowitz, F., and R. L. Hardin. 1954. Respiratory proteins. *Proteins* **2A**, 279.

Hornsey, H. C. 1956. The colour of cooked pork. I. Estimation of the nitric oxide-haem pigments. *J. Sci. Food Agr.* **7**, 534.

Hornsey, H. C. 1959. The colour of cooked pork. III. Distribution and relationship of pigments, pH and cysteine. *J. Sci. Food Agr.* **10**, 114.

Kendrew, J. C., H. C. Watson, B. E. Strandberg, R. G. Hart, D. R. Davies, D. C. Phillips, and V. C. Shore. 1960. Structure of myoglobin. Three-dimensional Fourier synthesis at 2A resolution. *Nature* **185**, 422.

Kielsmeier, E. W., and W. B. Gara. 1962. Preparation and composition control of sausage materials. U.S. Patent 3,050,399.

Moss, V. G. 1963. Unpublished data. Oscar Mayer & Co., Madison, Wisconsin.

Perry, S. V., and W. W. Kielley. 1955. Mg-activated muscle ATP(ase). *Methods of Enzymol.* **2**, 582, Academic Press, N.Y.

Sayre, R. N., and E. J. Briskey. 1963. Protein solubility as influenced by physiological conditions in the muscle. *J. Food Sci.* **28**, 675.

Scopes, R. K. 1964. The influence of post-mortem conditions on the solubilities of muscle proteins. *Biochem. J.* **91**, 201.

Shaw, J. L. 1958. Unpublished data. Oscar Mayer & Co., Madison, Wisconsin.

Sloan, E. C., E. Schmook, Jr., W. D. Paynter, and E. W. Kielsmeier. 1965. Method and apparatus for forming a sausage meat product. U.S. Patent 3,203,807.

Sloan, E. C., and W. B. Ahern. 1965. Continuous manufacture of small smoked sausages. U.S. Patent 3,170,797.

Snyder, H. E. 1963. Heme dissociation and autoxidation of myoglobin. *Biochim. et Biophys. Acta* **79**, 200.

Trautman, J. C. 1963. Unpublished data. Oscar Mayer & Co., Madison, Wisconsin.

Turner, E. W., and F. C. Olson. 1959. Manufacture of sausage and ground meat products. U.S. Patent 2,874,060.

Van Logtestijn, J. G. 1964. Some data about the influence of heating and salt on the pH value of meat. *10th European Meeting of Meat Research Workers,* Roskilde, Denmark.

Wismer-Pedersen, J. 1964. Some physico-chemical properties of raw meat fibers in relation to myofibrillar water-binding capacity. *17th An. Recip. Meat Conf.,* Univ. of Wisconsin.

Yasui, T., M. Sakanishi, and Y. Hashimoto. 1964a. Effect of inorganic polyphosphate on the solubility and extractability of myosin B. *J. Agr. Food Chem.* **12**, 392.

Yasui, T., T. Fukazawa, K. Takahaski, M. Sakanishi, and Y. Hashimoto. 1964b. Specific interaction of inorganic polyphosphate with myosin B. *J. Agr. Food Chem.* **12**, 399.

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Microbial Growth Patterns of Rehydrated Freeze-Dried Foods. I. Shrimp

SUMMARY

Microbial growth patterns of rehydrated shrimp were investigated at 4, 20, and 37°C. Storage temperature greatly influenced the growth rates. The lag period was longest at 4°C and shortest at 37°C. The maximum population, almost a million times that of the initial population, was reached twice as fast at 37°C as at 20°C. Samples stored at 4°C increased 10,000-fold in total aerobic counts but required two weeks to do so.

Growth patterns indicate a shift in microbial spectrum in response to temperature. This was pronounced when rehydrated shrimp was stored at 4°C, the essentially mesophilic population becoming a minority.

It is apparent that, in common with other types of perishable food products, rehydrated shrimp can have a storage life significantly extended by low temperatures. The shrimp storage life was 7 times as long at 4°C as at 20°C, and 20 times as long as at 37°C.

INTRODUCTION

The production of commercial freeze-dried foods for consumer acceptance has shown a steady increase after the initial developmental stages. In 1962, the volume of freeze-dried foods in the U.S. was 5.6 million pounds, and now is five times that of 1962. It is predicted that by 1970, there will be further a 12-fold increase in the production of freeze-dried foods (Bird, 1965). It is therefore important that these foods be evaluated microbially.

A previous study (Sinskey *et al.*, 1964) demonstrated that certain organisms of public-health significance and other contaminants in model systems and foods can survive freeze-drying to various extents, and that survival is influenced by the composition of food to be freeze-dried and by the processing temperature. Saleh *et al.* (in press) surveyed eight randomly selected commercial freeze-dried foods and detected organisms of public-health significance. As stated elsewhere (Silverman and Goldblith, in press), the microbial flora of a freeze-dried food is related to but not necessarily identical to that of the original raw material. The differences may be greater after storage and rehydration. Rehydration studies are important because, once a freeze-dried food is rehydrated, it becomes a perishable product which requires immediate consumption or refrigeration. Therefore, both for product quality and for anticipating specific situations of potential public-health significance, it is important to predict growth patterns of various groups of organisms subsequent to rehydration at specific temperatures.

In this study three temperatures, 4, 20, and 37°C, were selected to estimate psychotrophs (Eddy, 1960; Mossel and Zwart, 1960) and mesophiles. The selection of these temperatures is somewhat arbitrary since, for example, certain psychrophilic organisms can grow over an appreciable temperature range (Ingraham and Stokes, 1959; Witter, 1961).

Sea foods such as shrimp, which are susceptible to deterioration at refrigerated temperatures and which undergo protein denaturation during frozen storage, are being freeze-dried commercially for institutional use. For this reason, it was felt desirable to evaluate the microbial growth patterns of rehydrated freeze-dried shrimp.

MATERIALS AND METHODS

Two samples of commercial peeled, deveined, and cooked freeze-dried shrimp of H (higher bacterial plate count) and L (lower bacterial plate count) were obtained from commercial sources and stored at 20°C until analyzed. The shrimp had been packaged under nitrogen in 13¼-oz cans which were of the same lot number.

Portions of freeze-dried shrimp (225 g) were rehydrated with 1,000 ml of either sterile distilled water or trypticase diluent. The rehydration medium was equilibrated at either 4, 20, or 37°C prior to use. After 15 min of rehydration the samples were drained to remove excess liquid, and each portion was stored at a temperature corresponding to that of the rehydration liquid.

For plate counts, a 50-g portion of rehydrated

shrimp was blended for 3 min with 75 ml trypticase diluent, resulting in a 1:10 dilution (dry basis). The trypticase diluent consisted of 0.003*M* KH_2PO_4 containing 0.1% trypticase (BBL, pH 7.2). Blenders and diluents were always cooled to 5°C before use. Unless otherwise indicated, trypticase diluent was used for rehydration.

Total aerobic pour-plate counts were made in trypticase soy agar (BBL) supplemented with 0.5% yeast extract (Difco). Serial dilutions were made in trypticase diluent (Sinskey *et al.*, 1964). For each rehydration temperature, three sets of plates were prepared and incubated at 4, 20, and 37°C, respectively. Plates incubated at 37°C were counted after 3 days, those incubated at 20°C after 5 days, and those incubated at 4°C after 7 days.

RESULTS

Enumeration of the aerobic microbial flora in shrimp is, to a certain extent, dependent upon the composition of the rehydration medium (Fig. 1). Rehydration with trypticase diluent gave a higher recovery than rehydration with distilled water. The difference in recoverable organisms was minimal initially, and increased with incubation. Growth rates in the logarithmic phase were much higher in shrimp rehydrated and stored at 20°C than in shrimp stored at 4°C. At 20°C, a population of 10^9 cells was obtained in 50–55 hr, while at 4°C, a population of 10^8 cells was obtained in 55 hr. As noted below, growth was even faster at 37°C.

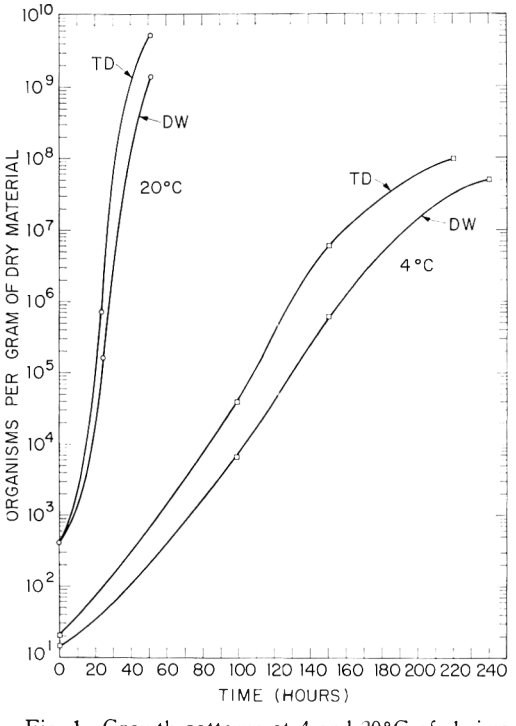

Fig. 1. Growth patterns at 4 and 20°C of shrimp rehydrated in distilled water (DW) or in trypticase diluent (TD).

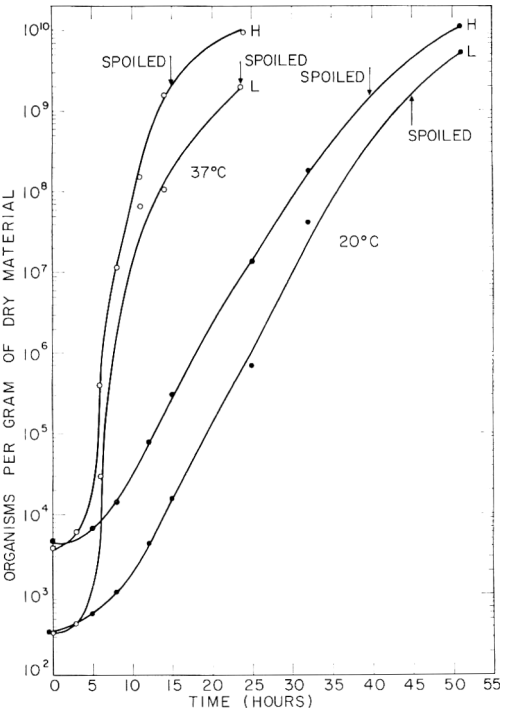

Fig. 2. A comparison at 20 and 37°C of the growth patterns of two samples of shrimp: higher count (H) and lower count (L).

The initial number of organisms present in the two shrimp samples, one of higher plate count (H) and the other of lower plate count (L), did not materially affect the growth rates but did influence the total numbers detected at any given interval of storage. As seen in Fig. 2, the growth rates of the bacteria in H and L samples stored at 20 or at 37°C were comparable. At any given interval, those stored at 37°C reached a higher total population than those at 20°C. After 25 hr, sample H reached a population of 10^{10} organisms per gram of dry material, compared to 10^7 organisms per gram at 20°C. Sample L at 37°C had a population of 10^9 cells in 25 hr, and at 20°C a population of 10^6 cells. A comparison of Figs. 1 and 2 indicates that plate counts for shrimp rehydrated and incubated at 4°C was much lower initially than replicates incubated at 20 or 37°C. This is, however, an oversimplification of the problem, as is discussed below (Fig. 3).

Spoilage was detected organoleptically at 37°C after 15 hr in sample H and 24 hr in sample L. At 20°C this period was extended to 40 hr in sample H and 45 hr in L. Both H and L samples at 37, 20, and 4°C spoiled when the total numbers of aero-

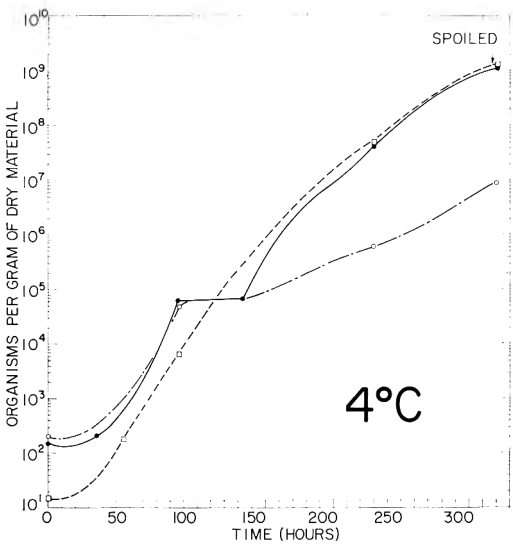

Fig. 3. The aerobic plate counts at three incubation temperatures of shrimp rehydrated and stored at 4°C. The incubation temperatures were 4°C, □- - - -□; 20°C, ●——●; 37°C, ○— - — -○.

bic organisms were in the neighborhood of 15 to 20 × 10^9 organisms per gram of dry material (40 × 10^7 per gram of wet material).

Storage life of the rehydrated shrimp is extended appreciably by low-temperature storage. At 4°C it took about two weeks for sample L to show obvious organoleptic deterioration. At this time, and in agreement with the studies conducted at 20 and 37°C, the microbial population was about 15 × 10^8 organisms per gram of dry material (Fig. 3). For samples stored at 4°C, three sets of plates were prepared at each sampling period and a set incubated at 4, 20, and 37°C. Approximately a 10-fold average higher initial plate count was obtained from plates incubated at 20 and 37°C than from plates incubated at 4°C.

Using growth temperature as a criterion, it is seen that a shift occurs in the microbial spectrum. In this study, psychrotrophs (Eddy, 1960) are defined as those bacteria capable of relatively rapid growth at or below 4°C. Mesophilic bacteria are those bacteria which grow at 37°C. Recovery at 20°C would therefore not differentiate between psychrotrophs and mesophiles.

The majority of the microbial population found in freeze-dried shrimp upon rehydration was incapable of growing at 4°C but did grow at 20 and 37°C; recovery was highest at 37°C. During storage at 4°C a significant change occurred in the nature of the microbial flora as shown by their growth response.

After 2 weeks, the counts from plates incubated at 4 and 20°C were about 100 times as high as those incubated at 37°C. This indicates that the psychrotrophs capable of growth at 20 and 4°C became the predominant flora in the shrimp stored at low temperatures and the mesophiles capable of growth at 37°C decreased proportionately after 140 hours of storage. The counts of plates incubated at 4°C increased rapidly until they were equal to those at 37°C after 5 days of storage. Essentially the same result (not presented) was obtained with shrimp sample H.

The microbial growth pattern for sample L rehydrated and stored at 37°C is shown in Fig. 4 and analyzed in the same manner as shown in Fig. 3. In agreement with the data of Fig. 3 the numbers of organisms capable of growth at 4°C were 10% of the organisms capable of growth at 37°C. After 24 hr of storage, the total counts from plates incubated at 37 and 20°C were about a hundred times as great as counts from plates incubated at 4°C.

Table 1 indicates that when shrimp sample L was rehydrated and stored at 20°C, the initial counts from plates incubated at 20°C were 10 times as great as the counts from plates at 4°C. After 51 hr of storage, the counts from plates at 20°C were still 10 times as great as the plate counts at 4°C. In fact, once the dominant populations were established by storage at a given incu-

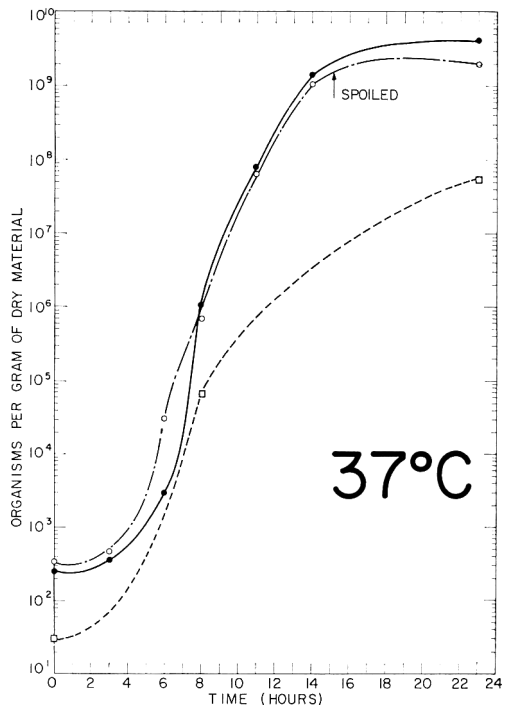

Fig. 4. The aerobic plate counts at three incubation temperatures of shrimp rehydrated and stored at 37°C. The incubation temperatures were: 4°C, □- - - -□; 20°C, ●——●; 37°C, ○— - — -○.

Table 1. Total aerobic plate counts of shrimp L rehydrated and stored at 20°C.

Incubation time (hr)	Organisms/g dry sample		
	37°C	20°C	4°C
0	35×10^1	35×10^1	3×10^1
5	.....	60×10^1	.....
8	.....	11×10^2	.....
12	55×10^2	45×10^2	55×10^1
15	.....	16×10^3	.....
24	.....	70×10^4	.....
32	.....	40×10^6	.....
51	14×10^8	50×10^8	50×10^7

bation temperature, plate counts were always higher at 20°C than at 37°C. For storage at 4°C, a somewhat different pattern emerged (Fig. 3). The plate counts obtained at 20°C were higher than the 4°C plates only in the initial stage of incubation. In the latter stages the plates incubated at 4°C had counts higher or comparable to counts of the 20°C plates.

DISCUSSION

The temperature, time of storage, initial number of microorganisms present, composition of rehydration media, and nature of the food influenced the microbial growth patterns of rehydrated freeze-dried shrimp. The influence of the composition of rehydration medium on recovery of total plate count from shrimp indicates that shrimp does not appear to support optimal microbial growth, even though it is conceivable that rehydration can cause the liberation of food constituents which may be present due to the destruction of cellular integrity as a result of processing. In this study only two rehydration media were compared, and quantitation may be increased by other media, but from a public-health viewpoint, rehydration in water is one of the more important considerations.

Interestingly enough, rehydrated shrimp was organoleptically spoiled, as determined by odor, when a population of 15 to 20 $\times 10^8$ organisms/g at 4, 20, and 37°C was reached for both H and L shrimp samples even though the growth patterns differed. The type of spoilage could not be distinguished by the organoleptic examination. Maximum populations were not determined at 4, 20, and 37°C, because the shrimp became organoleptically unacceptable before the maximum population was attained.

The samples from regular commercially produced batches which were used in this study were of relatively low initial counts. Silverman *et al.* (1961) found aerobic plate counts of commercial frozen, raw, peeled, and deveined shrimp that were higher than those found in this study. Plate counts for frozen shrimp varied from a low of 70,000 organisms to a high of 9,420,000 organisms per gram of wet material. Studies are being conducted by the senior author on the effect of processing conditions on microbial survival in freeze-dried shrimp. Preliminary data show that the decrease in plate counts due to freeze-drying can be only 50–70%, because moderate drying temperatures must be used in order to obtain an acceptable product. Therefore, should there be shrimp with much higher initial plate counts, the spoilage time can be shorter than that shown in this study, particularly at the higher incubation temperatures.

When rehydrated shrimp is stored at 4°C, higher initial aerobic total counts are obtained at incubation temperatures of 20 and and 37°C. Therefore, it may be said that the initial flora of the rehydrated shrimp was mesophilic in character. However, after five days of storage, that is, when the microflora have undergone a change to favor the multiplication of psychrotrophs, higher plate counts were obtained from plates incubated at 4 and 20°C. When rehydrated shrimp is stored at 37°C, the growth of mesophiles is favored, as was shown by highest counts being obtained from plates incubated at 37°C during the entire rehydration period.

It is evident from these experiments that the incubation temperature of choice for the enumeration of the microflora of rehydrated shrimp depends upon the temperature at which the product is stored and the interval of storage. Intial counts will reflect the survivors of processing, whereas subsequent counts will reflect their growth patterns after incubation at a given temperature.

In spite of the large increase in microbial populations at the temperatures studied, the pH of the homogenate varied only over the narrow range of 7.1–7.4.

In this study, only those organisms intimately associated with shrimp were included.

For rehydration the manufacturer's recommendations were followed. This resulted in a certain amount of excess liquid (about 300 ml), which was found to contain a small number of microorganisms. Aerobic plate counts of the excess liquid varied between between 30 and 200 organisms per ml, which, based on the total number of organisms, shows that approximately 1–6% of the microbial flora appeared in the rehydration water. The extent of this removal of organisms in the excess rehydration fluid will, of course, depend upon the location of an organism on a shrimp portion. Comparable experiments at 20 and 37°C (results not presented) where the excess water used for rehydration was not drained, resulted in growth rates very similar to those for the samples from which the water was removed.

Since standards should reflect the degree of potential public-health hazards (Goldblith, 1963), it is important to include organisms of public-health significance in the study of growth patterns in rehydrated freeze-dried food. The commercial shrimp samples H and L used in this study did not contain *Staphylococcus aureus* (Baird-Parker Medium, 1962), fecal enterococci (KF Medium, Kenner *et al.*, 1961), or coliforms as part of the natural flora. Present studies in this laboratory on other sources of frozen shrimp in which these organisms are found to be present will determine the survival of these organisms as affected by processing variables and storage. A subsequent paper will discuss the growth patterns of *Staphylococcus aureus* and fecal enterococci as part of the natural flora of chicken.

REFERENCES

Baird-Parker, A. C. 1962. An improved diagnostic and selective medium for isolating coagulase-positive staphylococci. *J. Appl. Bacteriol.* **25**, 12.

Bird, K. 1965. Freeze-drying—12-fold increase by 1970. *Food Processing* **26**, 75.

Eddy, B. P. 1960. The use and meaning of the term "psychrophilic." *J. Appl. Bacteriol.* **23**, 189.

Goldblith, S. A. 1963. *In*: Exploration in Future Food Processing Techniques. S. A. Goldblith, ed. M.I.T. Press, Cambridge, Mass., p. 70.

Ingraham, S. L., and J. L. Stokes. 1959. Psychrophilic bacteria. *Bacteriol. Revs.* **23**, 97.

Kenner, B. A., H. F. Clark, and P. W. Kabler. 1961. Fecal streptococci. I. Cultivation and enumeration of streptococci in surface waters. *Appl. Microbiol.* **9**, 15.

Mossel, D. A. A., and H. Zwart. 1960. The rapid tentative recognition of psychrotrophic types among Enterobacteriaceae isolated from foods. *J. Appl. Bacteriol.* **23**, 185.

Saleh, B., G. J. Silverman, and S. A. Goldblith. Microbial evaluation of commercial freeze-dried foods. *Food Technol.* **20**(5), 103.

Silverman, G. J., and S. A. Goldblith. 1965. The microbiology of freeze-dried foods. *In*: Advances in Applied Microbiology. Vol. 7. W. W. Umbreit, ed. Academic Press, New York. In press.

Silverman, G. J., N. S. Davis, J. T. R. Nickerson, D. W. Duncan, I. Tezcan, and M. Johnson. 1961. Microbial analysis of frozen raw and cooked shrimp. I. General results. *Food Technol.* **15**, 455.

Sinskey, T. J., A. H. McIntosh, I. S. Pablo, G. J. Silverman, and S. A. Goldblith. 1964. Considerations in the recovery of microorganisms from freeze-dried foods. *Health Lab. Sci.* **1**, 297.

Witter, L. D. 1961. Psychrophilic bacteria—A review. *J. Dairy Sci.* **46**, 983.

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Development of Yeast on Irradiated Pacific Crab Meat

SUMMARY

Yeasts increased rapidly on air-packed irradiated (0.2 and 0.4 megarad) samples of crab meat stored at 0.5 and 5.6°C. The growth of the yeasts on irradiated crab meat was inhibited by vacuum-packing the sample. No appreciable increase in yeast counts occurred on the unirradiated controls. A total of 569 yeast cultures were isolated from unirradiated and irradiated King crab and Dungeness crab meat. The genera represented included *Rhodotorula*, *Cryptococcus*, *Torulopsis*, *Candida*, *Trichosporon*, and a yeastlike organism resembling *Aureobasidium pullulans*. Members of the *Cryptococcus*, *Candida*, and *Aureobasidium pullulans* were gelatinolytic and lipolytic. *Trichosporon* isolates were very active in attacking gelatin, casein, and triglycerides.

INTRODUCTION

Of the factors affecting the spoilage of unfrozen fishery products, by far the most important is that of microbial activity. Most of the earlier investigations concerning the spoilage of fishery products have demonstrated that the predominating microorganisms on spoiling fish are psychrophilic gram-negative bacteria belonging to the *Pseudomonas* and *Achromobacter* genera (Shewan, 1960; Castell and Anderson, 1948; Liston, 1960).

Because of the extreme perishability of unfrozen seafoods, the fisheries industry has continually searched for a satisfactory method of retaining the quality of the freshly harvested product. Current research on ionizing radiation at pasteurization levels shows that marine products can be preserved for extended periods with only minor alterations from the fresh untreated product (Nickerson and Goldblith, 1950; Shewan and Liston, 1958; Scholz *et al.*, 1962; Mosurovsky *et al.*, 1963; Miyauchi *et al.*, 1964; Lerke and Farber, 1961).

The effect of irradiation at pasteurization levels is to inactivate a large portion of the microorganisms indigenous to the fishery product. The increased storage life of the irradiated product therefore depends on the longer period for the survivors to reach sufficient numbers to cause spoilage. Factors that may influence this increase in the storage life include: 1) a qualitative change in the microbial flora after radiation; 2) possible radiation damage to the surviving microorganisms, with a resulting longer lag period; and 3) alteration of the substrate by radiation with a resulting inhibition of growth in the surviving microorganisms.

Yeasts are not of major importance on untreated fresh fishery products, because they normally represent only a small percentage of the initial microbial population and are rapidly overgrown by the bacteria at refrigerated temperatures. However, it has been observed in this laboratory that yeast and mold counts often exceed 1×10^5/g on crab meat that has been irradiated and stored at 0.5 and 5.6°C. In contrast, yeast and mold counts seldom exceed 1×10^3/g on untreated crab meat.

It is generally agreed that yeasts are more resistant to radiation than are bacteria (Ingram and Thornley, 1959; Gunter and Kohn, 1956). In fact, the initial microbial population after irradiation can be composed predominantly of yeasts (Ingram and Thornley, 1959). The predominance of yeasts was however only temporary and they were overwhelmed by bacteria in the final spoilage flora.

Because of the radiation resistance of yeasts and their ability to grow on crab meat during refrigerated storage, they have assumed greater importance than before as potential contributors to spoilage of the product. Furthermore, some yeasts may be of public-health significance.

This paper reports: 1) the factors affecting growth of yeasts in irradiated crab meat; 2) the genera of yeasts present on the unirradiated product and those surviving the irradiation treatment and growing on the refrigerated product; and 3) the biochemical activities of the different genera that might be potentially deleterious to the product.

FACTORS AFFECTING YEAST GROWTH IN IRRADIATED CRAB MEAT

Results were very much the same for irradiated Dungeness and King crab meat. Accordingly, only the results for King crab meat are discussed here except where differences were significant. Considered in this phase of study are: 1) the effect of air and vacuum packing on yeast count; and 2) the effect of bacterial populations.

Effect of air and vacuum packing. *Materials and methods.* King crab and Dungeness crab meat were purchased from local processors and then packed into 307 × 200.25 cans leaving a headspace of approximately ½ inch. The cans were sealed either under atmospheric pressure or under vacuum (26 inches Hg). Irradiation procedures used by Miyauchi *et al.* (1964) were followed. After being irradiated, the crab meat was stored at either 0.5 or 5.6°C until sampled.

In the preparation for total yeast and mold counts, 100 g of sample was blended with 100 ml of sterile diluent (0.1% peptone water). Forty g of this sample was again blended with 160 ml of sterile diluent, and serial dilutions were prepared. Pour plates were made by the use of malt agar acidified to pH 3.8 with sterile 10% lactic acid. Samples on all pour plates were incubated 7 days at 22°C, and yeast and mold were then counted.

Results and discussion. Very few molds were isolated from any of the samples; therefore, these counts will be referred to as yeast counts in the remainder of the report. Figs. 1 and 2 illustrate the changes in yeast counts on air- and vacuum-packed crab meat stored at 0.5 or 5.6°C. The main yeast count prior to irradiation was 2×10^2/g. After the crab meat was irradiated at 0.2 and 0.4 megarad, the yeast count was reduced to less than

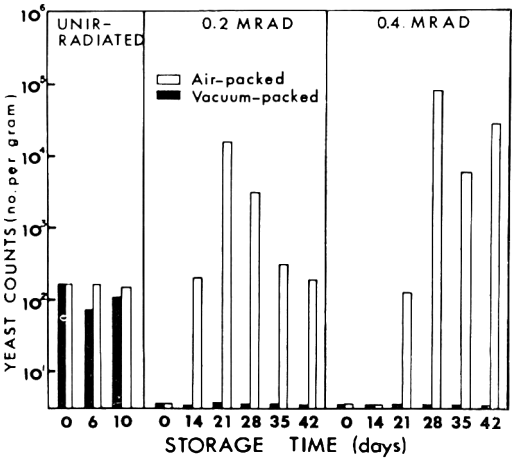

Fig. 1. Comparison of yeast counts from air-packed and vacuum-packed king crab meat irradiated at 0.0, 0.2, and 0.4 megarad and stored at 0.5°C.

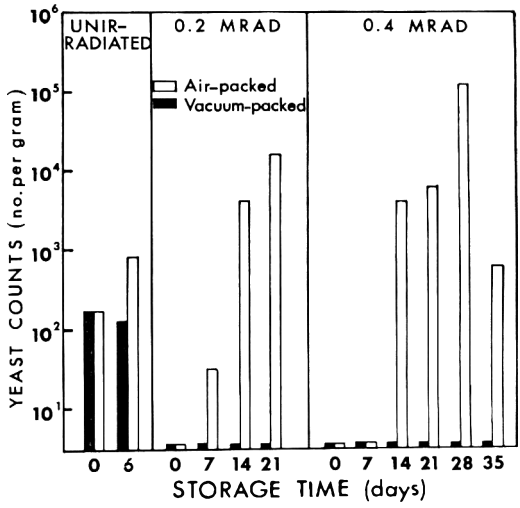

Fig. 2. Comparison of yeast counts from air-packed and vacuum-packed king crab meat irradiated at 0.0, 0.2, and 0.4 megarad and stored at 5.6°C.

10/g—that is, over 95% of the original yeast flora was inactivated.

During subsequent storage, a slight increase in yeast counts was observed on unirradiated crab meat that was air packed and held at 5.6°C, but no increase was observed on unirradiated samples stored at 0.5°C. When the samples were irradiated at 0.2 and 0.4 megarad, yeast grew only on the air-packed samples. Yeast counts on the vacuum-packed irradiated samples remained at less than 10/g even after 6 weeks at either 0.5 or 5.6°C. Therefore, subsequent discussions in this report are confined to the air-packed samples.

In samples stored at 0.5°C, increases in yeast counts were not observed until after 7–14 days on samples irradiated at 0.2 megarad and 14–21 days on samples irradiated at 0.4 megarad. At the higher storage temperature of 5.6°C, yeast counts increased on the 0.2- and the 0.4-megarad samples after 7 days and after 7–14 days, respectively. The greater time required for the yeasts to multiply on the 0.4-megarad samples may be the result of a combination of effects: 1) increased damage to the cell at the higher irradiation level, resulting in inability to synthesize protein (Stapleton *et al.*, 1955); or 2) possible temporary inhibition of the irradiated substrate on microbial growth (Solberg and Nickerson, 1963).

Similar observations on the development of yeasts have been reported in other food products when antibiotics were used as food preservatives to inhibit bacterial growth. Tarr *et al.* (1952) observed a predominance of yeasts on spoiled fish treated with tetracycline antibiotics. Ziegler and Stadelman (1955) and Wells and Stadelman (1958) have observed appreciably larger yeast populations

on antibiotic-treated than on untreated chicken carcasses.

Effect of bacterial population. *Methods and materials.* Total bacterial plate counts were made with the same procedures as for yeast and mold counts except that trypticase, phytone yeast-extract (pH 7.1) was used in place of malt agar. The plates were incubated 5 days at 22°C, and the bacteria then counted. Yeast counts were made with the same procedures discussed in the previous section.

Results and discussion. The yeast population was considerably higher on all the irradiated samples than on unirradiated samples. This increase may be due, in part, to the longer storage time, but it is most likely due to the reduction in types of bacteria that are antagonistic to and compete with the yeasts for the same nutrients. These factors are illustrated in Figs. 1 and 2—higher yeast counts appearing on the 0.4-megarad samples than on the 0.2 megarad or the untreated samples. The yeast counts on the 0.4-megarad samples exceeded 1×10^5/g. In contrast, the unirradiated samples treated with 0.2 megarad of ionizing radiation respectively reached mean counts of 1×10^3 and 1×10^1/g.

Figs. 3 and 4 illustrate the relative changes in total bacterial counts and yeast counts in irradiated air-packed King crab meat. Unirradiated samples of crab meat showed very rapid increases in total bacterial plate counts, and spoilage generally occurred when the bacterial population exceeded 1×10^6/g. In these same instances, the yeast population never exceeded 1×10^3/g. When the crab meat received irradiation doses of 0.2 and 0.4 megarad, spoilage was not detected until the bacterial counts exceeded 1×10^5/g, and spoilage was more of an acidic or sour type, particularly at the 0.4 dose level, than of the normal amine-like type observed on unirradiated samples, indicating a possible difference in the type of surviving microflora. It is noteworthy that yeast counts on the air-packed irradiated samples increased until the bacterial counts were 1×10^5/g; thereafter they decreased. This trend perhaps indicates a depletion of nutrients; however, it might also indicate that a bacterial population of 1×10^6/g is required before a certain segment (genus) of the bacterial population reaches sufficient numbers to cause spoilage. In turn, it may also be that these species are inhibitory. Whitehill (1957) reported that the bacterial flora developing on meat seemed to produce antifungals that inhibited the growth of yeasts. Walker and Ayres (1959) and Eklund (1962) also observed inhibitory action against yeast by *Pseudomonas fluorescens* and *Ps. geniculata.*

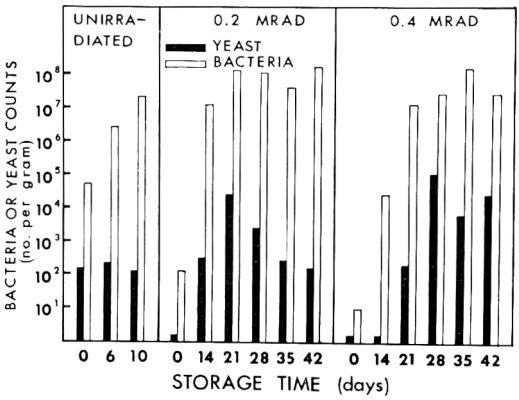

Fig. 3. Comparison of total bacterial and yeast counts from air-packed king crab meat irradiated at 0.0, 0.2, and 0.4 megarad and stored at 0.5°C.

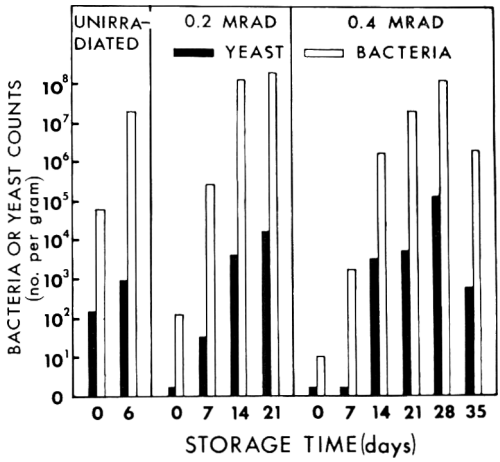

Fig. 4. Comparison of total bacterial and yeast counts from air-packed king crab meat irradiated at 0.0, 0.2, and 0.4 megarad and stored at 5.6°C.

GENERA OF YEASTS ISOLATED

This phase of the study was made to determine the genera of yeast developing on air-packed unirradiated and irradiated King crab and Dungeness crab meat at 0.5 and 5.6°C.

Methods and materials. Procedures for yeast pour plates were as discussed in earlier sections. Twenty colonies of yeast were selected randomly from each of the countable plates. Before the physiological and biochemical characteristics of each isolate were studied, it was streaked twice successively on acidified malt agar (pH 3.8). Isolated colonies were transferred onto malt agar slants and stored at 3°C until characterized.

Physiological and biochemical characteristics of these isolates were determined by procedures of Lodder and Kreger-Van Rij (1952). Malt agar, V-8 agar (Wickerham *et al.*, 1946), and Gorodokowa's agar were used to induce spore formation at incubation temperatures of 20, 25, and 30°C.

Table 1. Genera of yeast and frequency of occurrence on unirradiated and irradiated King crab meat stored at 0.5 and 5.6°C.

Irradiation level (Mrad)	Storage temp. (C°)	Storage time (days)	Genera and number of times isolated					
			Rhodotorula	*Cryptococcus*	*Torulopsis*	Black yeast	*Candida*	*Trichosporon*
0.0	0.5	0–5	9	....	1	1	8	15
	5	0–5	17	9	2	....	10	16
0.2	0.5	13	....	6	5	....	....	8
		21	....	10	9	....	....	....
		28	2	1	14	3	....	3
	5	13	6	5	7	....	....	....
		21	1	4	11	1	....	2
0.4	0.5	21	....	12	8	....	....	....
		28	2	4	12	....	2	....
	5	13	7	13	....	....	....	1
		21	6	9	4	1	....	1
		28	15	1	....	6	....	3

Results and discussion. During the study, 293 cultures were isolated from unirradiated and irradiated King crab meat, and 276 cultures from unirradiated and irradiated Dungeness crab meat. These isolates were taken from air-packed samples. All of the isolates failed to produce ascospores under the conditions of the experiment. These isolates represent both unpigmented and pigmented yeasts.

Isolates from King crab. Table 1 lists the genera of yeasts and frequency of occurrence on unirradiated and irradiated King crab meat stored at 0.5 and 5.6°C.

Six different genera are represented by the isolates—*Trichosporon, Rhodotorula, Cryptococcus, Torulopsis, Candida,* and a "black yeast" resembling *Aureobasidium pullulans.* Five of these genera were isolated from samples irradiated at 0.2 and 0.4 megarad; *Candida* was not isolated, however, from 0.2-megarad samples, and was isolated only infrequently from 0.4-megarad samples.

The largest number of isolates from the unirradiated samples of King crab meat was of the *Trichosporon* and *Rhodotorula* genera. After irradiation and storage of the samples, the largest number of isolates occurred in the *Cryptococcus* and *Torulopsis* genera. *Rhodotorula* isolates were observed more frequently on samples stored at 5.6°C than on samples stored at 0.5°C.

Forty-nine cultures belonged to the *Trichosporon* genus. It was isolated more frequently from the irradiated crab meat than was the other mycelium-forming genus, *Candida.* Lodder and Kreger-Van Rij (1952) reported that the production of a starchlike material is characteristic of the genus *Cryptococcus.* We observed that the *Trichosporon* isolates also produced starchlike material. This characteristic was not observed in the isolates of the genus *Candida.* Similar observations have been made by Walker and Ayres (1959) and Aschner and Cury (1951), though the latter authors found starchlike material also to be produced by *Candida humicola.*

"Black yeasts," which resemble *Aureobasidium pullulans,* were isolated only one time from the unirradiated crab meat but 11 times during the later storage period of the irradiated crab meat. This difference is not surprising, considering that this organism has an exceptionally high resistance to gamma radiation. Saravacos *et al.* (1962) reported a lethal dose for this organism of 2.0 megarad using a 500-curie cobalt-60 source of radiation, five times the highest irradiation dose used in these experiments.

Isolates from Dungeness crab. Table 2 lists genera of yeasts and frequency of occurrence on unirradiated and irradiated Dungeness crab meat stored at 0.5 and 5.6°C.

The six genera isolated from King crab meat were also represented on both unirradiated and irradiated Dungeness crab meat. *Rhodotorula* and *Trichosporon* genera constituted the larger portion of the initial yeast flora prior to irradiation; whereas, after irradiation, *Cryptococcus* and *Trichosporon* were represented most often in the irradiated product. Neither the *Rhodotorula* nor the *Torulopsis* genus was isolated from the crab meat receiving 0.4 megarad of ionizing radiation. "Black yeasts" were isolated much more frequently from Dungeness crab meat than from King crab meat.

Table 2. Genera of yeast and frequency of occurrence on unirradiated and irradiated Dungeness crab meat stored at 0.5 and 5.6°C.

Irradiation level (Mrad)	Storage temp. (C°)	Storage time (days)	Genera and number of times isolated						
			Unidentified	*Rhodotorula*	*Cryptococcus*	*Torulopsis*	Black yeast	*Candida*	*Trichosporon*
0.0	0.5	0–6	1	17	10	6	12	6	20
	5	0–6	.....	13	3	3	3	8	12
0.2	0.5	1	.....	3	3	5	.....	.....	.....
		14	.....	3	8	2	6	.....	9
		21	.....	.....	5	.....	.....	5	14
		28	.....	.....	2	3	.....	4	11
	5	14	.....	2	3	1	1	3	15
0.4	0.5	21	.....	.....	17	.....	1	.....	.....
		28	.....	.....	13	.....	.....	4	3
	5	14	.....	.....	2	.....	1	6	9

BIOCHEMICAL ACTIVITIES OF YEAST ISOLATES

Yeasts undoubtedly play a very minor role in the spoilage of untreated crab meat. However, "yeasty" odors have often been detected on irradiated (0.2 and 0.4 megarad) samples that are spoiled.

The biochemical activities of representatives of each of the genera of yeasts were determined so that the deleterious changes that the various genera of yeasts might have on crab meat could be evaluated.

Methods and materials. Lipolytic activity was determined using cottonseed oil in nile-blue sulfate agar (Turner, 1929) and spirit-blue agar (Starr, 1941).

Gelatinolytic activity was determined using gelatin tubes and the plate procedure of Smith *et al.* (1946). Caseinolytic activity was determined using tryptone, yeast-extract agar, plus 3% skim milk. Clearing around the colonies indicated a positive caseinolytic culture. The ability of the isolates to hydrolyze casein was confirmed, using a modified procedure of Anson (1938), with casein as the substrate.

Results and discussion. Tables 3, 4, and 5 summarized the gelatinolytic, caseinolytic,

Table 3. Gelatinolytic activity of yeast isolates.

Irradiation level (Mrad)	Storage temp. (C°)	Storage time (days)	Gelatinolytic [a] activity among yeast genera					
			Rhodotorula	*Cryptococcus*	*Torulopsis*	Black yeast	*Candida*	*Trichosporon*
0.0	0.5	0–6	0/9	.....	0/1	1/1	6/8	10/15
	5	0–6	0/17	3/9	0/2	.....	4/10	12/16
0.2	0.5	13	.....	0/6	0/5	.....	.....	8/8
		21	.....	8/10	0/9	.....	.....	.....
		28	0/2	0/1	0/14	3/3	.....	3/3
	5	13	0/6	0/5	0/7	.....	.....	.....
		21	0/1	0/4	0/11	1/1	.....	2/2
0.4	0.5	21	.....	0/12	0/8	.....	.....	.....
		28	0/2	0/4	0/12	.....	2/2	.....
	5	13	0/7	0/13	.....	.....	.....	1/1
		21	0/6	0/9	0/4	1/1	.....	1/1
		28	0/15	1/1	.....	6/6	.....	3/3

[a] Number of isolates yielding positive test/number of isolates tested.

Table 4. Caseinolytic activity of yeast isolates.

Irradiation level (Mrad)	Storage temp. (C°)	Storage time (days)	Caseinolytic [a] activity among yeast genera					
			Rhodotorula	*Cryptococcus*	*Torulopsis*	Black yeast	*Candida*	*Trichosporon*
0.0	0.5	0-6	0/9	.....	0/1	0/1	0/8	6/15
	5	0-6	0/17	0/9	0/2	.....	0/10	10/16
0.2	0.5	13	.....	0/6	0/5	.....	.....	8/8
		21	.....	0/10	0/9	.....	.....	.....
		28	0/2	0/1	0/14	0/3	.....	0/3
	5	13	0/6	0/5	0/7	.....	.....	0/3
		21	0/1	0/4	0/11	0/1	.....	2/2
0.4	0.5	21	.....	0/12	0/8	.....	.....	.....
		28	0/2	0/4	0/12	.....	0/2	.....
	5	13	0/7	0/13	.....	.....	.....	0/1
		21	0/6	0/9	0/4	0/1	.....	1/1
		28	0/15	0/1	.....	0/6	.....	0/3

[a] Number of isolates yielding positive test/number of isolates tested.

and lipolytic activities of representatives of the different yeast genera.

None of the *Torulopsis* or *Rhodotorula* isolates from either the unirradiated or the irradiated samples displayed lipolytic or proteolytic activity under the conditions of these experiments. Certain of the isolates representing the *Cryptococcus, Candida,* and "black yeasts" were gelatinolytic and lipolytic but failed to attack the casein substrate.

Members of the *Trichosporon* genus isolated from both the irradiated and the unirradiated samples were very active biochemically in attacking casein, gelatin, and triglyceride substrate.

All of the genera isolated from Dungeness and King crab meat have at one time or another caused the spoilage of other food products, such as: 1) refrigerated beef (Jensen, 1942); 2) fats [spoilage indicated by

Table 5. Lipolytic activity of yeast isolates [a]

Irradiation level (Mrad)	Storage temp. (C°)	Storage time (days)	Lipolytic [b] activity among yeast genera					
			Rhodotorula	*Cryptococcus*	*Torulopsis*	Black yeast	*Candida*	*Trichosporon*
0.0	0.5	0-6	0/9	.....	0/1	1/1	1/8	8/15
	5	0-6	0/17	7/9	0/2	.....	2/10	10/16
0.2	0.5	13	.....	2/6	0/5	.....	.....	8/8
		21	.....	5/10	0/9	.....	.....	.....
		28	0/2	1/1	0/14	3/3	.....	3/3
	5	13	0/6	5/5	0/7	.....	.....	.....
		21	0/1	1/4	0/11	1/1	.....	2/2
0.4	0.5	21	.....	4/12	0/8	.....	.....	.....
		28	0/2	3/4	0/12	.....	1/2	.....
	5	13	0/7	4/13	.....	.....	.....	1/1
		21	0/6	2/9	0/4	1/1	.....	1/1
		28	0/15	1/1	.....	6/6	.....	3/3

[a] Nile blue sulfate and spirit blue indicators used.
[b] Number of isolates yielding positive test/number of isolates tested.

discoloration (Jensen, 1942)]; 3) oysters [spoilage indicated by discoloration (McCormack, 1950)]; 4) poultry treated with tetracycline antibiotics (Wells and Stadelman, 1955); and 5) fruit juices (Lawrence *et al.*, 1959).

In a separate report, Eklund *et al.* (1965) reported on an organism tentatively identified as *Trichosporon* sp. that would attack casein and soluble crab protein. This organism was also observed to grow very well at 0.5°C.

From the public-health standpoint, the *Candida* and *Cryptococcus* genera have been implicated in the past in mycotic infections in man (Ainsworth, 1958; Skinner *et al.*, 1961). However, none of the species described by Eklund *et al.* (1965) from crab meat had the characteritsics of the pathogenic species.

REFERENCES

Ainsworth, G. C. 1958. Pathogenic yeasts. *In*: Chemistry and Biology of Yeasts. Academic Press, New York.

Anson, M. L. 1938. Estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.* **22**, 79.

Aschner, M., and A. Cury. 1951. Starch production in the genus Trichosporon. *J. Bacteriol.* **67**, 350.

Castell, C. H., and G. M. Anderson. 1948. Bacteria associated with spoilage of cod fillets. *J. Fisheries Research Board Can.* **7**, 370.

Eklund, M. W. 1962. Biosynthetic responses of poultry meat organisms under stress. Ph.D. thesis. Purdue University.

Eklund, M. W., J. Spinelli, D. Miyauchi, and H. Groninger. 1965. Characteristics of yeasts isolated from Pacific crab meat. *Appl. Microbiol.* **13**, 985.

Gunter, S. E., and H. I. Kohn. 1956. The effect of X-rays on the survival of bacteria and yeasts. 1. A comparative study of dose-survival curves of Azotobacter agile, Escherichia coli, Pseudomonas fluorescens, Rhodopseudomonas spheroides, and Saccharomyces cereviscae irradiated in the resting state. *J. Bacteriol.* **71**, 571.

Ingram, M., and M. J. Thornley. 1959. Changes on spoilage pattern of chicken meat as a result of irradiation. *Intern. J. Appl. Radiation and Isotopes.* **6**, 122.

Jensen, L. B. 1942. Microbiology of meats. Garrard Press, Champaign, Illinois.

Lawrence, N. L., D. C. Wilson, and C. S. Pederson. 1959. The growth of yeast in grape juice stored at low temperature. II. The types of yeasts and their growth in pure culture. *Appl. Microbiol.* **7**, 7.

Lerke, P. A., and L. Farber. 1961. Preservation of fish and shellfish by relatively low doses of beta radiation and antibiotics. *Food Technol.* **15**, 145.

Liston, J. 1960. The bacterial flora of fish caught in the Pacific. *J. Appl. Bacteriol.* **23**, 469.

Lodder, J., and N. J. W. Kreger-Van Rij. 1952. The Yeasts, a Taxonomic Study. Interscience Publishers, New York.

McCormack, G. 1950. "Pink yeast" isolated from oysters grows at temperatures below freezing. *Comm. Fisheries Rev.* **12**(11a), 28.

Miyauchi, D., M. Eklund, J. Spinelli, and N. Stoll. 1964. Irradiation preservation of Pacific Coast shellfish. 1. Storage life of king crab meats at 33° and 42°F. *Food Technol.* **18**, 138.

Mosurovsky, E. B., S. A. Goldblith, and J. T. R. Nickerson. 1963. Effects of substerilization doses of Co^{60} gamma radiation on the cold-storage life extension of shucked soft-shelled clams and haddock fillets. *Appl. Microbiol.* **11**, 220.

Nickerson, J. T. R., and S. A. Goldblith. 1950. A comparison of chemical changes in mackerel tissues treated by ionizing radiation. *Food Technol.* **4**, 84.

Saravacos, G. D., L. P. Hatzipetrou, and E. Geogiadou. 1962. Lethal doses of gamma radiation of some fruit spoilage microorganisms. *Food Irradiation* **3**, A6.

Scholz, D. J., R. O. Sinnhuber, D. M. East, and A. W. Anderson. 1962. Radiation-pasteurized shrimp and crab meat. *Food Technol.* **16**, 118.

Shewan, J. M. 1960. Fish as food. Vol. 1. G. Borgstrom, ed. Academic Press, New York.

Shewan, F. M., and J. Liston. 1958. Experiments on the irradiation of fish with 4 mev. cathode rays and Co^{60} gamma rays. *Proc. Intern. Conf. Peaceful Uses Atomic Energy*, Geneva **27**.

Skinner, C. E., C. W. Emmons, and H. M. Tsuchiya. 1961. Henrici's molds, yeasts, and actinomycetes. John Wiley and Sons, New York.

Smith, N. R., R. E. Gordon, and F. E. Clark. 1946. Aerobic mesophilic sporeforming bacteria. *U. S. Dept. Agr. Misc. Publ.* **559**.

Solberg, M., and J. T. R. Nickerson. 1963. A biological after effect in radiation processed chicken muscle. *J. Food Sci.* **28**, 243.

Stapleton, G. E., A. J. Sbarra, and A. Hollander. 1955. Some nutritional aspects of bacterial recovery from ionizing radiation. *J. Bacteriol.* **70**, 7.

Starr, M. P. Spirit-blue agar. A medium for the detection of lipolytic microorganisms. *Science* **93**, 333.

Tarr, H. L. A., B. A. Southcott, and H. M. Bissett. 1952. Experimental preservation of flesh foods with antibiotics. *Food Technol.* **6**, 363.

Turner, R. H. The action of bacteria on fat. *J. Infectious Diseases* **44**, 126.

Walker, H. W., and J. C. Ayres. 1959. Characteristics of yeasts isolated from processed poultry and the influence of tetracyclines on their growth. *Appl. Microbiol.* **7**, 251.

Wells, F. G., and W. J. Stadelman. 1958. Development of yeast on antibiotic treated poultry. *Appl. Microbiol.* **6**, 420.

Whitehill, A. R. 1957. Inhibition of yeasts by food spoilage organism. *Bacteriol. Proc.* **1957**, 20.

Wickerham, L. J., M. H. Flickinger, and K. A. Burton. 1946. A modification of Henrici's vegetable juice sporulation medium of yeasts. *J. Bacteriol.* **52**, 611.

Ziegler, F., and W. J. Stadelman. 1955. The effect of aureomycin treatment on the shelf-life of fresh poultry. *Food Technol.* **9**, 107.

Ms. rec'd 10/5/65.

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Effect of Bivalent Metals on the Production of Aflatoxins in Submerged Cultures

SUMMARY

Optimum growth and maximum aflatoxin production by *Aspergillus flavus* were obtained on a basal medium containing 0.8 mg of zinc per liter. The effect of several other trace metals on the toxin production was evaluated. Aflatoxin production was inhibited by the presence of barium ions in the medium. Cadmium ions, in contrast, stimulated aflatoxin production.

INTRODUCTION

Aflatoxins, a mixture of toxic metabolites produced by certain strains of *Aspergillus flavus* and related organisms, are found to consist of structurally related coumarin-like derivatives which are designated as B_1, B_2, G_1, and G_2 in the order of decreasing R_f value on thin-layer chromatography. According to Hartley *et al.* (1963), the infrared and ultraviolet absorption spectra of the four aflatoxins are very similar. Several investigators, including Asplin and Carnaghan (1961), Sargeant *et al.* (1961), and Allcroft and Carnaghan (1963), have reported that these toxins, isolated from moldy animal feeds, are responsible for "Turkey X" disease and outbreaks of poisoning among livestock in England and elsewhere.

In tissue cultures, Gabliks *et al.* (1965) reported that the toxic effect of aflatoxin B_1 on several cell cultures including HeLa cells (human cervical carcinoma) was shown by an inhibition of growth followed by progressive granulation, rounding and finally sloughing of the cells from the glass.

HeLa cells were reported to be less sensitive (50% toxic dose TD_{50} = 5.0 to 7.0 μg/ml) than primary chick-embryo cells (TD_{50} < 5.0 μg/ml), human Chang liver cells (TD_{50} = 1.0 to 1.75 μg/ml), and primary duck-embryo cells (TD_{50} = 1.0 μg/ml). Our unpublished data on cytotoxic effect on HeLa cells and mouse L-strain (NCTC clone 929, connective tissue) cells are in agreement with those observations on metabolic and morphological changes. However, visible signs of toxicity to aflatoxins added in the medium of our newly established stationary serum cultures of HeLa cells appeared at a level as high as 24–25 μg per ml, approximately five times that of the HeLa cell assay of Gabliks *et al.* (1965).

Mateles and Adye (1965) described the effect of different growth and cultural conditions on the production of aflatoxins in submerged culture. Among their data, the effect of trace elements on aflatoxin production is most interesting, especially the zinc requirement. It has been suggested that metal chelates are formed by the trace metals that function in metalloenzymes or as enzymatic activators (Dwyer and Mellor, 1964) and that the stability of the metal complexes may depend on the pH of the medium in which it is formed.

This study was made to determine the role played by divalent metals other than zinc in the generation of aflatoxins in liquid shake cultures of *Aspergillus flavus*.

The studies were conducted in the following sequence:

1) To determine the minimum concentration of zinc required for obtaining optimum growth while maintaining maximum aflatoxin production;

2) To investigate the effect of various trace metals on growth and aflatoxin production when added to a basal medium.

MATERIALS AND METHODS

Organism. The *A. flavus* used was strain No. 91019b received from Culture Collection, Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, England. The original culture was inoculated on moist peanut meals, and incubated at 30°C until good sporulation was obtained. These spores were used to inoculate flasks containing 100 ml of medium.

Media and cultures. The basal medium containing glucose, ammonia, and inorganic salts was as described by Adye and Mateles (1964) except that the concentration of zinc was reduced to 0.8 ppm, for reasons stated in the text.

On hundred ml of medium contained in a 500-ml Erlenmeyer flask was steam-sterilized at a pressure of 15 psi for 15 min, inoculated, and incubated for

5 days at 30°C on a gyratory shaker. Barium carbonate and barium acetate were sterilized separately by filtration and added to the medium immediately prior to inoculation. The yellow pigment produced in the culture was found to parallel aflatoxin production and for this reason served as a fast visual indication of the presence of toxins. According to Mateles and Adye (1965), four days of incubation at 30°C was sufficient to obtain a maximum aflatoxin production. Differences in aflatoxin production between duplicate flasks were generally less than 10%, although, very rarely, they differed as much as 40–50%.

Titration. The conidia inoculated into the basal medium containing 0.5 mg (1.9 μM) of barium acetate per 100 ml were allowed to germinate and to grow for 24 hr prior to titration. The established cultures were then placed in a water-bath shaker maintained at a constant temperature of 30°C and were titrated by an automatic Radiometer titrator to maintain the pH at 4.5 (the initial pH of the medium) either with 0.5N NaOH or with 0.5N $Ba(OH)_2$. The control cultures were treated under the same conditions without pH adjustment.

Assays. The procedure outlined by Mateles and Adye (1965) was used to determine the presence of aflatoxins, particularly B_1 in the unfiltered broth.

Total aflatoxin determination was performed in duplicate 1-ml aliquots of the unfiltered media. Each aliquot was extracted by shaking for 30 sec with 5 ml of chloroform. The optical density of the chloroform extract was measured at 362 mμ. The remaining broth and mycelia were extracted with 100 ml chloroform, evaporated, and redissolved in 5 ml methanol for thin-layer chromatography.

A portion of methanolic extract equivalent to 25 ml of culture media was spotted on a 4 × 8-inch glass plate coated with a 0.3-mm layer of "Kieselgel" previously activated at 90°C for 1 hr and then developed with 5% methanol in chloroform. The plate was examined under ultraviolet light. The relative visual intensities and R_f values were recorded. The aflatoxin bands, detected by fluorescence under ultraviolet light, were scraped off and eluted with methanol. Again, the presence of aflatoxin was determined by measuring the absorbancy of the methanol eluant at 362 mμ.

Where preliminary examination failed to reveal typical absorption at 362 mμ, the following procedure was followed to eliminate the possibility of any loss of aflatoxins through complexing with barium ions. The cultures were saturated overnight with ethylenediaminetetraacetic acid (EDTA) and then extracted with chloroform. The chloroform extracts were examined for the presence of aflatoxins following separation by thin-layer chromatography.

RESULTS AND DISCUSSION

Fig. 1 indicates the effect on growth and aflatoxin production of various concentrations of zinc in the basal medium. In agreement with data of Mateles and Adye (1965), the minimum concentration of zinc required for optimum growth and maximum aflatoxin production was 0.8 ppm. This concentration was used subsequently in our basal medium.

Of the trace metals investigated, Co^{++}, Ba^{++}, $CrO_4^{=}$, Cd^{++}, Fe^{+++}, and Mn^{++}, only barium reduced aflatoxin production (Table 1). Cadmium, on the other hand, increased production. Visual observations of the intensity of the yellow color in the culture agreed with numerical data obtained from absorption spectra of the chloroform extracts. In the flasks in which aflatoxin production was inhibited by added barium acetate, the mycelium remained white until the third day and started to turn yellow on the fourth day. Thus, barium ions appeared to delay the production of aflatoxin.

It was of interest to find that significant amounts of aflatoxins were produced in zinc-deficient cultures (0.2 ppm of zinc) containing 0.5 ppm of cadmium ions, while the same medium without cadmium produced only a trace or no aflatoxins. This implies that the cadmium ions directly stimulated production or lowered the zinc level required for aflatoxins.

Since the presence of barium ions in the medium appeared to indicate an inhibition of the occurrence of aflatoxins, experiments were carried out to further elucidate the effect of this ion. In concentrations of barium ace-

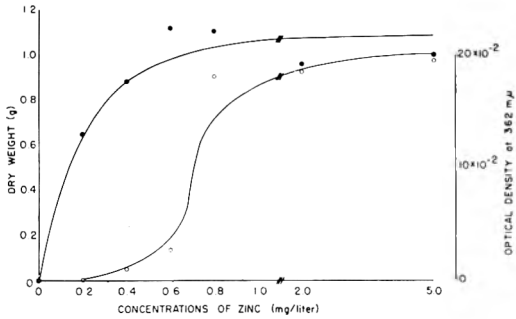

Fig. 1. Effect of zinc on growth and aflatoxin production.

Symbols: ● = growth
○ = aflatoxins

Table 1. Effect of trace elements on aflatoxin production

Compound added to basal medium	Conc. of compound added (μM/L)	Color of mycelium	Total dry weight (g)	Aflatoxin optical density at 362 mμ per ml of unfiltered broth
none		yellow	1.033	0.18
$CoCl_2$	42.0	yellow	1.018	0.23
$Ba(CH_3COO)_2$	3.9	white	1.027	0.02 or none
$CaSO_4$	5.8	yellow	1.026	0.23
K_2CrO_4	4.3	yellow	1.023	0.17
$CdCl_2$	4.3	strongly yellow	1.035	0.34
$Fe(SO_4)_3$	250.0	slightly pink	1.012	0.17
$MnSO_4$	59.0	yellow	1.017	0.20

tate, ranging from 1.0 mg to 500 mg [1.9mM of $Ba(CH_3COO)_2$] per 100 ml of basal medium, aflatoxin production was delayed 24 hr. By the end of 4 days the total yield of aflatoxins was equivalent to that of the control cultures, and the final pH was approximately 2.2. To determine whether the high final acidity developed in the cultures nullified the aflatoxin inhibitory effect of a possible hypothetical barium chelate, the barium acetate cultures were maintained at the constant pH of 4.5 by adding approximately 40 ml (2×10^{-3} mole) of 0.5N NaOH over 5 days. Maintenance of the pH at 4.5 did not alter the final yield of aflatoxins from that of the control cultures. However, when the said cultures were maintained at pH 4.5, using approximately 25 ml (6.2×10^{-3} mole) of 0.5N $Ba(OH)_2$, no aflatoxins were found in cultures incubated for as long as 5 days at 30°C (aflatoxins are usually produced after 2 days incubation). The same effect of barium was observed for the series of cultures grown in the basal media containing 0.5 g to 0.75 g (3.8×10^{-3} mole) of $BaCO_3$ per 100 ml. Any concentration greater than 1.0 g of $BaCO_3$ per 100 ml of medium inhibited the germination of the conidia. The absorption spectra of the chloroform extracts of the medium from the control, the $Ba(OH)_2$-titrated, and $BaCO_3$ cultures are shown in Fig. 2. The control culture was the only culture which contained aflatoxin as indicated by the characteristic absorption peak at 362 mμ.

On examination of the aflatoxins from the above chloroform extracts after thin-layer chromatography (Fig. 3), it was apparent by visual estimation that aflatoxin was either not present or that it occurred in very small amounts in the barium-treated cultures. As indicated by fluorescence, only trace amounts of material occurred at R_f 0.55 in the barium-treated cultures, compared to the large amount found at this R_f value in the control culture. A purified crystallized B_1 aflatoxin sample also had an R_f value of 0.55 in this chromatographic system. However, the absorption spectra of the material at R_f 0.55 from the barium-treated cultures was not typical of aflatoxin, i.e., a strong absorption

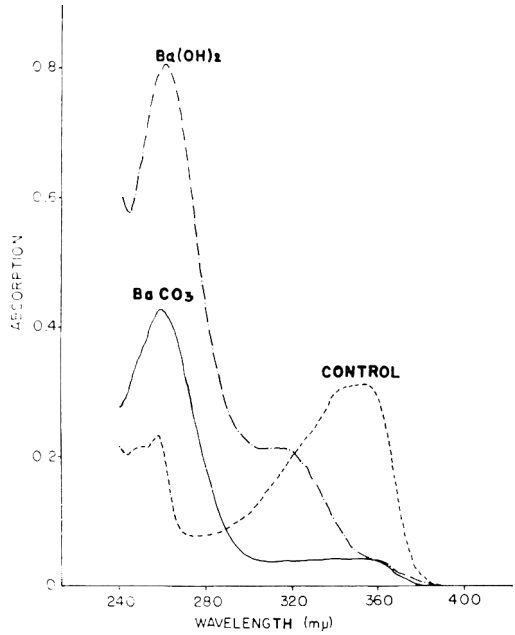

Fig. 2. Absorption spectra of chloroform extracts from culture media.

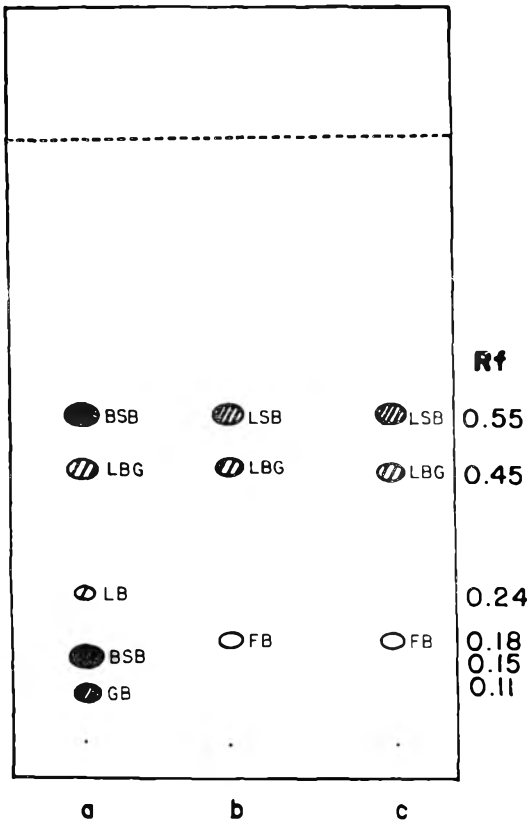

Fig. 3. Thin-layer chromatography of chloroform extracts of culture medium: a) control cultures; b) $Ba(OH)_2$-titrated cultures; c) $BaCO_3$-added cultures.

Fluorescence in ultraviolet:

BSB = bright sky blue
LSB = light sky blue
LBG = light blue green
LB = light blue
GB = green blue
FB = faintly blue

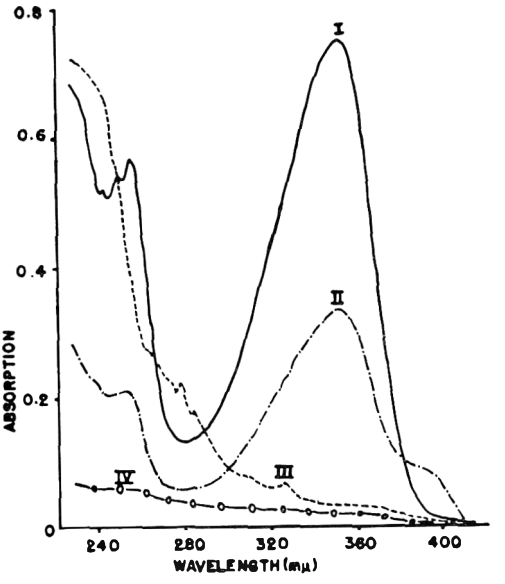

Fig. 4. Absorption spectra of fluorescent materials separated by thin-layer chromatography: I, bright sky-blue spot having R_f of 0.55 from control cultures; II, bright sky-blue spot having R_f 0.15 from control cultures; III, light sky-blue spot having R_f 0.55 from either $Ba(OH)_2$-titrated or $BaCO_3$-added cultures; IV, faintly blue spot having R_f 0.18 from either $Ba(OH)_2$-titrated or $BaCO_3$-added culture.

at wavelength 362 mμ was not obtained (Fig. 4). The fluorescent material at R_f 0.15 found in the control culture, which was not found in the barium-treated culture, was not identified. The substance, R_f = 0.15, had an absorption spectrum similar to that of the aflatoxins (Fig. 4), and may be similar to the fluorescent materials found in cultures grown in synthetic media described by A. Sheridan. It was noted that a new, weakly fluorescent material of R_f 0.18, which did not show any similarity to the aflatoxin absorption spectrum (Fig. 4), occurred in the cultures containing barium. All other fluorescent spots (Fig 3), R_f 0.45, 0.24, and 0.11, did not show any absorption spectrum at wavelength 362 mμ. Thus, the supply of barium ions to the basal medium, according to the changes of pH in the medium or as barium carbonate, inhibited aflatoxin production.

The possibility of failure to extract the aflatoxins in the first chloroform extraction in the barium-treated cultures was checked by the addition of EDTA to the cultures, followed by extraction with chloroform. These extracts were chromatographed and absorption spectra were taken. The very small amount of fluorescent materials isolated from these extracts did not differ from the previous primary extraction in either R_f values or absorption spectra.

REFERENCES

Adye, J., and R. I. Mateles. 1964. Incorporation of labelled compounds into aflatoxins. *Biochim. et Biophys. Acta.* **86**, 418.

Allcroft, R., and R. B. A. Carnaghan. 1963. Biological effects of toxic products in groundnuts. *Chem. Ind.* **2**, 50.

Asplin, F. D., and R. B. A. Carnaghan. 1961. The toxicity of certain groundnut meals for poultry

with special reference to their effect on ducklings and chickens. *Vet. Record*, **73**, 1215.

Dwyer, F. P., and D. P. Mellor. 1964. Chelating agents and metal chelates. p. 383. Academic Press, New York and London.

Gabliks, J., W. Schaeffer, L. Friedman, and G. Wogan. 1965. Effect of aflatoxin B_1 on cell cultures. *J. Bacteriol.* **90**(3), 720.

Hartley, R. D., B. F. Nesbitt, and J. O'Kelley. 1963. Toxic metabolites of Aspergillus flavus. *Nature* **198**, 1056.

Mateles, R. I., and J. C. Adye. 1965. Production of aflatoxins in submerged culture. *Appl. Microbiol.* **13**(2), 208.

Sargeant, J., J. O'Kelley, R. B. A. Carnaghan, and R. Allcroft. 1961. The assay of a toxic principle in certain groundnut meals. *Vet. Record* **73**, 1219.

Sheridan, A., cited in Hartley, R. D., *et al., op. cit.*, p. 1056.

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Structure of Spores of Rough and Smooth Variants of Bacillus Stearothermophilus with Special Reference to Their Heat Resistance

SUMMARY

A single-cell method was used to separate two variant forms of *Bacillus stearothermophilus*. The heat resistance of rough and smooth variant spores was determined. The $D_{250°F}$ values for the smooth and rough variants were respectively 2.32 and 1.42 min. Thin sections of spores of both variants revealed that there were three layers in the spore wall of both variants. In the smooth variant, however, these layers adhere together whereas in the rough they separated. Also, the spore wall was thicker in the smooth variant than in the rough variant. Immediately adjacent to the spore wall of the smooth variant was a "two-layer" area of less electron density than the spore wall. The cortex was thicker in the rough variant than in the smooth. The spore core wall, or a layer surrounding the core of the spore, was present in the rough variant but not in the smooth.

INTRODUCTION

Bacillus stearothermophilus can cause flat-sour spoilage in low-acid foods such as peas, corn, beans, and spinach. The spores of this organism are heat resistant and may survive the heat process and cause flat-sour spoilage. It is important, therefore, to investigate the factors which affect this heat resistance.

Different F values (time to kill all the spores at 250°F) are found in the literature for this organism. Bigelow (1921) found that the F value of *B. stearothermophilus* strain NCA 1518 was 5½ min at 250°F. Reed *et al.* (1951), however, published F values for this organism of 25.3 min in phosphate buffer, pH 7.0, and 44 min in pea purée. Fields and Finley (1962) found the F value at 250°F to be 7.0 min for a suspension of NCA 1518. Some of these differences may have been due to traditionally recognized factors such as: nutritional status of the organism, the suspending menstruum, the age of the spore, and concentration of spores; however, the differences between spore suspensions of the same organism may have been due to the presence of variant forms in the spore suspensions, particularly in Fields and Finley's suspension.

In work on the heat resistance of *B. stearothermophilus* spores reported in the literature, no reference is made to variant forms except in that conducted by Fields.

According to some investigators (Church and Halvorson, 1959; Hashimoto *et al.*, 1960; Levinson *et al.*, 1961) the heat resistance of a spore depends greatly on the amount of dipicolinic acid and calcium. Hachisuka and Kuno (1963) suggest that the cortex is the site of the dipicolinic acid and calcium. Recently, Thomas (1964) showed that the spore core as well as the coat are mineral-rich. He avoided making a clear decision about the cortex, because of the possibility of artifacts. Knaysi (1965) observed a higher concentration of mineral matter in the core than at the periphery of the endospore. He suggested that the bulk of the dipicolinate complex is in the core.

Since earlier work in our laboratory showed that the rough variant of NCA 1518 was less heat-resistant than the smooth, and since thin sections of spores had not been made on variants to relate morphological structure to heat resistance, this research was instituted.

MATERIALS AND METHODS

The organism used was *B. stearothermophilus* NCA 1518. The two variants, rough and smooth, were isolated by using single-cell method. From these isolated single vegetative cells, spore suspensions were prepared by the procedure used by Finley and Fields (1962). These spore suspensions were used in both destruction-rate studies and spore sectioning.

Thermal destruction rates were determined by the classical method of Esty and Williams (1924). Phosphate buffer (M/15, pH 7.0) was used as heating menstruum. The tubes were sealed after 2 ml of suspension, with a concentration of 10^5 spores per ml, were introduced into each tube. Four tubes were run per heating time. The "come-up times" were corrected by the method of Ball

et al. (1937). One ml from each TDT tube was plated in dextrose tryptone agar and incubated 48 hr at 52°C. The "come-up" spore count (count at the time the suspension reached the oil bath temperature) was used as the original spore concentration in determining the number of survivors.

For morphological observations, the spores were fixed for 20 min with $KMnO_4$ (5%) at 0–5°C, since Rode *et al.* (1962) demonstrated that $KMnO_4$ fixation yields spores with uniform and characteristics appearance. The compact fixed spores were dehydrated through an ethanol series 25, 50, 75, and 100% (Mercer and Birbeck, 1961).

The fixed dried spores were embedded in a polymerized monomer which was prepared by mixing 20 ml methylmethacrylate (inhibitor-free) with 80 ml of butylmethacrylate (inhibitor-free). Two g of dried, cooled Luperco CDB (Lucidol Division, Wallace & Tiernan Inc., 1740 Military Road, Buffalo 5, New York) and 0.07 g uranyl acetate $UO_2(C_2H_3O_2)_2$ were added. The embedding procedure used in this study is described in detail by Mercer and Birbeck (1961).

The microtome used was a hand-operated model designed by Porter and Blum for use with glass knives. Sections 600 to 3200 Å thick were placed on electron microscope copper grids coated with an extremely thin film of Formvar (Peas, 1960). The microscope used was Akashi (Bendix Tronscope Model TRS-50 EL.

The double-exposure method (Tronscope Model Instructions) was used to measure the magnitude of the specimens.

RESULTS AND DISCUSSION

The smooth variant was found to be of greater resistance to heat than the rough variant, as shown in Fig. 1. This is in agreement with the work of Fields (1963). The *D* (time required to kill 90% of the spores at 250°F) values were found to be 2.32 min for the smooth and 1.42 min for the rough variant. The break in the curve for the smooth variant indicates that there were two levels of resistance in the spore population, with the major part being less resistant than the minor.

About 125 sections were examined by the electron microscope for each variant. In both variants, three layers were observed in the spore wall (Fig. 2-A, 3-A, 4-A). The outermost layer has the lowest electron density. The spore wall of the smooth variant is thicker than the wall of the rough variant by 175.3 Å (Table 1). This value does not

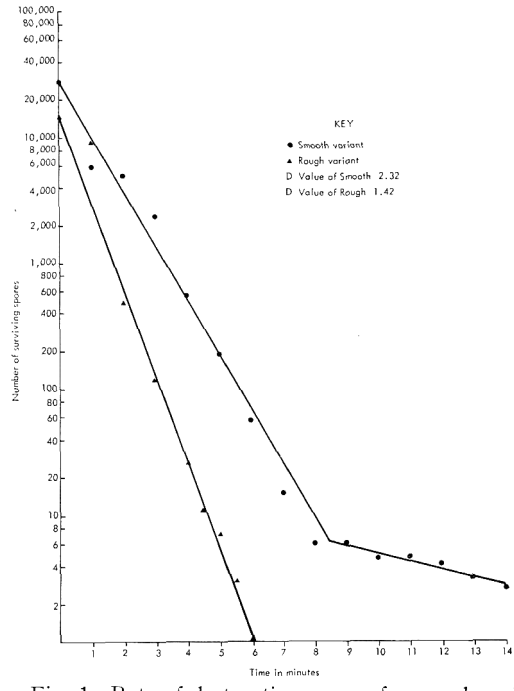

Fig. 1. Rate of destruction curves for rough and smooth variants of *Bacillus stearothermophilus* strain NCA 1518.

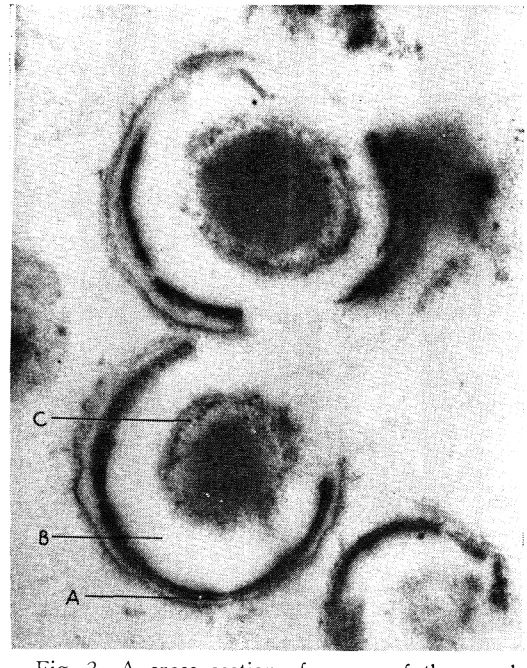

Fig. 2. A cross section of spores of the rough variant (Mult. 70,000). The spore wall (A) consists of three layers. (B) is the cortex. The spore core wall (C) is separated from the cytoplasm of the spore core.

Table 1. Comparison between the rough and the smooth variants (average of 10 sections in Angstroms).

Variant	Spore wall	Cortex	Spore core wall
Rough	1163.0	1437.5	856.8
Smooth	1338.3	1137.8	[a]

[a] Could not be observed.

show the actual differences between the two variants, since the layers of the spore wall of the rough variant were separated to a greater extent than in the smooth variant. The method used to measure the thickness of the spore wall included the gaps between the layers, which artificially increased the thickness of the spore wall of the rough variant. Observed inside the spore wall of the smooth variant spores were two layers with low electron density (Fig. 3B). This structure could not be seen in sections of the rough variant spores. Together with the relatively thick spore wall, this structure may act as a mechanical barrier to heat, in spores of the smooth variant.

Two layers of equal electron density were seen around the core of the rough variant spores (Fig. 2-C) but could not be observed in sections of the smooth variant spores. In the study reported herein, this structure is referred to as the spore core wall.

The area between the spore core wall and the spore wall is an electron-transparent zone (Fig. 2-B, 3-C, 4-B) referred to as the cortex (Holbert, 1960). Spores of the rough variant had thicker cortices than spores of the smooth variant (Table 1). The literature on the composition of the cortex is full of contradictions and discrepancies. According to Hachisuka and Kuno (1963), the cortex is the site of dipicolinic acid and calcium, which affect the thermo-resistance of spores (Church and Halvorson, 1959; Hashimoto *et al.*, 1960; Levinson *et al.*, 1961). Thomas (1964) and Knaysi (1965) seemed to suggest that the core contains high concentra-

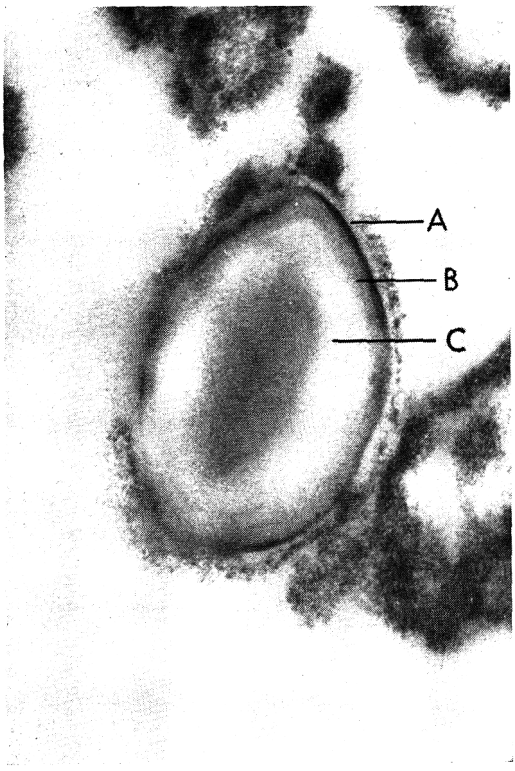

Fig. 3. A longitudinal section of a smooth variant spore (Mult. 70,000). The spore wall (A) has three layers. Inside the spore wall is a "two-layer" structure (B). The cortex surrounds the cytoplasm of the spore core (C).

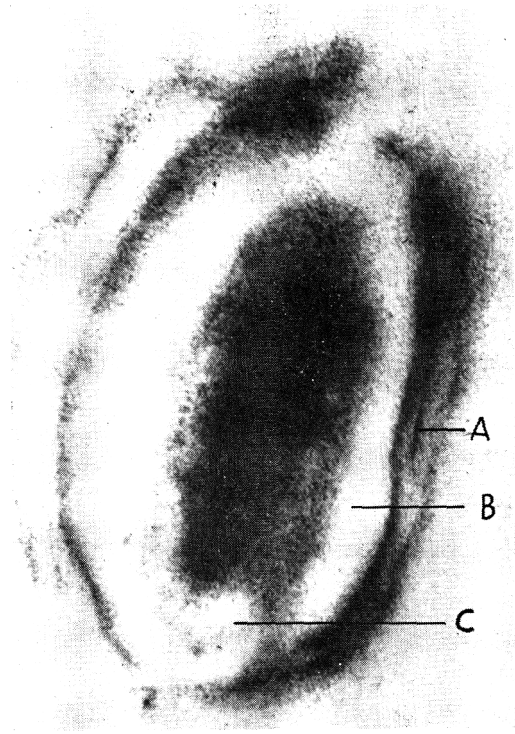

Fig. 4. A longitudinal section of a rough spore (Mult. 70,000). The spore wall (A) has three layers. The cortex (B). The spore core wall (C).

tion of "mineral matter." Knaysi also suggests that the site of the dipicolinate complex is in the core. The role of the cortex in regard to heat resistance has not been established.

Since the data in this study indicate that there was more cortex in the rough variant, which was the least heat resistant, the amount of cortex does not seem to be related to heat resistance. The presence of core wall in the rough variant and its absence in the smooth variant suggests that this structure does not act as a mechanical barrier to heat.

The amount and the location of dipicolinic acid and calcium for both variants, is currently under investigation in our laboratory.

REFERENCES

Ball, C. O., C. M. Merril, C. C. Williams, and D. J. Wessel. 1937. Unpublished data. As cited in Sognefest, P., and H. A. Benjamin. 1944. Heating lag in thermal death time cans and tubes. *Food Research* **9**, 234.

Bigelow, W. D. 1921. The logarithmic nature of thermal death curves. *J. Infectious Diseases* **29**, 528.

Church, B. D., and H. Halvorson. 1959. Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content. *Nature* **183**, 124.

Esty, J. R., and C. C. Williams. 1924. Heat resistance studies. I. A new method for the determination of heat resistance of bacterial spores. *J. Infectious Diseases* **34**, 516.

Fields, M. L. 1963. Effect of heat on spores of rough and smooth variants of Bacillus stearothermophilus. *Appl. Microbiol.* **11**, 100.

Fields, M. L., and N. Finley. 1962. Studies on heat responses of bacterial spores causing flat sour spoilage in canned foods. III. Effect of storage time on activation and thermal destruction responses. *Missouri Univ. Agr. Expt. Sta. Research Bull.* No. **807**.

Finley, N., and M. L. Fields. 1962. Heat activation and heat-induced dormancy of Bacillus stearothermophilus spores. *Appl. Microbiol.* **10**, 231.

Hachisuka, Y., and T. Kuno. 1963. The location of dipicolinic acid in Bacillus subtilis spores and the role as a barrier to stainability. Abstr. Intern. Symp. on Physiology, Ecology, Biochemistry of Germination. Greifswald. In *Spore Newsletter* **7**(1964), W. G. Murrell (ed.) P. O. Box 43, Ryde, N.S.W., Australia.

Hashimoto, T., S. H. Black, and P. Gerhardt. 1960. Development of fine structure, thermostability and dipicolinate during sporogenesis in bacillus. *Can. J. Microbiol.* **6**, 203.

Holbert, P. E. 1960. An effective method of preparing sections of Bacillus polymyxa sporangia and spores for electron microscopy. *J. Biophys. Biochem. Cytol.* **7**, 373.

Knaysi, G. 1965. Further observations on the sporogram of Bacillus cereus endospores. *J. Bacteriol.* **90**, 453.

Levinson, H. S., M. T. Hyatt, and F. E. Moore. 1961. Dependence of the heat resistance of bacterial spores on the calcium:dipicolinic acid ratio. *Biochem. and Biophys. Research Commun.* **5**, 417.

Mercer, E. H., and M. S. C. Birbeck. 1961. Electron microscopy. A handbook for biologists. p. 60. Blackwell Scientific Publications, Oxford.

Peas, D. C. 1960. Histological technique for electron microscopy. p. 67. Academic Press, New York and London.

Reed, J. M., C. W. Bohrer, and J. E. Cameron. 1951. Spore destruction rate studies on organisms of significance in the processing of canned foods. *Food Research* **16**, 383.

Rode, F. J., C. W. Lewis, Jr., and J. W. Foster. 1962. Electron microscopy of Bacillus megaterium with special reference to the effect of fixation and thin sectioning. *J. Cell Biol.* **13**, 423.

Thomas, R. S. 1964. Ultrastructural localization of mineral matter in bacterial spores by microincineration. *J. Cell Biol.* **23**, 113.

Tronscope Model TRS-50 EL. "Electron microscope" Instructions. Akashi Seisakusho, LTD., Tokyo, Japan.

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Immunofluorescent Detection of Staphylococcal Enterotoxin B
I. Detection in Culture Media

SUMMARY

Application of the fluorescent antibody technique (FAT) to detection of staphylococcal enterotoxin *B* and of the cells that produce it in culture media was investigated. Enterotoxin *B* was detected by FAT in culture media both in the presence and in the absence of bacterial cells. Two methods of detection were developed. The first involved staining of fixed smears with fluorescein-isothiocyanate-conjugated anti-enterotoxin *B* serum. The second technique involved the precipitation of enterotoxin by mixing one drop of fluid containing either living cells and toxin or only toxin with two drops of serially diluted conjugate. After incubation, the formed precipitates were caught on Millipore filter membranes and washed, and impression smears were made on slides. Enterotoxin *B* was demonstrated with the first technique only when there was a minimum of 15 μg/ml. The second technique detected as little as 1 μg toxin/ml.

INTRODUCTION

Much progress has been made lately in methodology for detecting staphylococcal enterotoxins. Biological tests (feeding monkeys or injecting cats with toxic filtrates) have been replaced more and more by specific serologic procedures. The antigenicity of two of the known enterotoxins (*A* and *B*) has been demonstrated. *A* has been purified above 70% (Bergdoll, 1962), and *B* above 99% (Schantz *et al.*, 1965). Specific antisera against these two types of toxins are now available, and both toxins are demonstrable in cultures or food extracts by single and double gel diffusion methods. Quantities of 1 μg of toxin per 1 ml of culture can be detected by the slide gel diffusion test, while tube double gel diffusion can increase the sensitivity to 0.05 μg/ml (Hall *et al.*, 1963, 1965). Type *B* strains usually produce large amounts of toxin in broth cultures (over 100 μg/ml) whereas type *A* strains produce only minute amounts. Casman and Bennett (1965) reported that with 15–20 billion microorganisms per ml the production of type *A* toxin was about 2–4 μg per ml of aerated brain-heart infusion (BHI) broth. Since foods implicated in food poisoning rarely have such a great number of staphylococci, much less toxin would be expected to be present.

The demonstration of enterotoxins in foods involves two basic problems: 1) extraction; and 2) concentration to detectable quantities. Experiments have been conducted by adding known amounts of toxin in foods and then recovering them by extraction. Recoveries have been variable: Casman and Bennett (1965) reported recovering 68% of enterotoxin *A* and 48 to 72% of enterotoxin *B*; and Hall *et al.* (1965) reported recovering 48% of *A* and 75% of type *B*. Ion-exchange chromatography and gel filtration are used for selective separation of enterotoxins from broth cultures or food extracts, which are further concentrated until the concentration is high enough to be within the sensitivity limit of the gel diffusion test. At least 24 hr are necessary for the detection of toxins when their concentration is in the area of 1 μg/ml broth or food extract.

An indirect hemagglutination-inhibition procedure has been developed (Robinson and Thatcher, 1965; Brown and Brown, 1965); and a flotation system is reported which can detect enterotoxin *B* of 1 μg/ml in 2–3 hr (Hopper, 1963, 1965). Both of these methods still need more refined techniques of extraction of enterotoxins from foods.

It is obvious from the foregoing that no suitable method is available which can demonstrate enterotoxins or enterotoxic cells in foods in a short period without resorting to extractions, concentrations, and gel diffusion. Since enterotoxins react specifically with their antibodies, the fluorescent antibody technique (FAT) might be applicable for a direct detection of toxins and toxic strains in foods in a short period by the use of conjugated antienterotoxin sera. To study this possibility, experiments were first conducted with pure broth and agar cultures of toxic

and nontoxic staphylococcal strains with the intent that, if results were satisfactory, experiments would then be conducted with foods. This paper reports observations with broth and agar cultures. Very recently, Friedman and White (1965) reported the demonstration of cell-associated staphylococcal enterotoxin *B* by immunofluorescence. That report appeared in the form of a note, providing few details.

MATERIALS AND METHODS

Staphylococcal strains. Used throughout were the following staphylococcal strains, falling into five groups:

Toxigenic strains
- Type *A*: 196-E, 238, 239, 246-3A, 265
- Type *B*: 243
- Type *A* and *B*: S-6

Nontoxigenic strains
- Coagulase-positive: No. 1, Wood 46, 242, 305
- Coagulase-negative: 14, 17, 28

All of these strains were tested for enterotoxicity in an earlier study (Genigeorgis, 1963), and the results agreed with their previous history.

Type *B* antienterotoxin serum and purified enterotoxin *B* were obtained through the courtesy of Dr. M. S. Bergdoll (University of Chicago). Purified enterotoxin *B* was obtained through the courtesy of Dr. E. J. Schantz (U. S. Army Laboratory, Fort Detrick). Type *B* antienterotoxin serum was obtained through the courtesy of Dr. H. E. Hall (Robert A. Taft Sanitary Engineering Center).

Production of enterotoxin. Erlenmeyer flasks of 25-ml and 125-ml capacity, respectively containing 5 and 25 ml of brain-heart infusion (BHI) broth, pH 7.4 (Difco), were inoculated with a few drops of 18-hr trypticase soy broth cultures and incubated for different periods on a rotary shaker at 37°C. Cellophane-membrane cultures, as described by Casman and Bennett (1963), in BHI without special atmosphere were used to obtain high concentrations of toxins. A modification of this method that proved easier and less costly, was also applied and resulted in good growth and high toxin concentrations. Dialysis tubing was cut into circles of 3 cm diameter, sterilized in the autoclave, and then placed on blood agar (BA). One drop of a suspension of cells washed three times with saline was placed on each membrane, and the plates were incubated aerobically for 48–72 hr at 37°C. Four strains per plate could be tested in this way. Hallander (1965) recently reported a similar method.

Demonstration of enterotoxin by gel diffusion. For quantitative and qualitative studies of enterotoxin production, single and double gel diffusion methods were applied. Oudin's single diffusion tubes were prepared as described by Hall *et al.* (1963), using 1:40 dilutions of type *B* antiserum in 0.3% Oxoid Ionagar No. 2 (Consolidated Laboratories, Inc., Chicago Heights, Illinois) in a standard buffer (phosphate-buffered saline, pH 7.2, 0.02*M*, with 1:10,000 merthiolate). Each tube was loaded with 0.3 ml antiserum, sealed with plastelin, and kept in the refrigerator until used. To test for toxicity, 0.3 ml of culture supernatant was placed on the top of the antiserum agar column, and the tube was sealed with plastelin and then incubated for 7 days at 29–30°C. For qualitative studies, the micro-Ouchterlony test (Crowle, 1958) was also applied.

For quantitative studies, a standard enterotoxin *B* curve was constructed by adding to the above tubes 0.3 ml of a 1:1 mixture of BHI broth and standard buffer containing amounts of pure enterotoxin *B* ranging from 1 to 250 μg/ml. The tubes were sealed, incubated at 29–30°C, and checked at 24 hr and again at 7 days. The migration distance (in mm) of the toxin-antitoxin precipitin line was plotted against the log of toxin concentration (Bergdoll, 1962; Hall *et al.*, 1963). A straight-line progression was obtained for toxin concentrations ranging from 8 to 250 μg/ml at 24 hr and from 4 to 250 μg/ml at 7 days. Below these lower limits the line curved sharply downward.

Preparation of conjugated antiserum. Three different batches of specific rabbit antienterotoxin B sera obtained from the sources reported and one prepared here were conjugated and used during this study. Immune sera were conjugated with fluorescein isothiocyanate at a dye-protein ratio of 1:40, according to a method previously developed (Corstvet and Sadler, 1964). By this procedure, the dilution effect experienced during fractionation was minimum and the method was not time-consuming. Antibody activity (titration against same amount of toxin with the slide gel test) was highest in the first colored fraction which eluted from the column. This fraction was stored in the refrigerator and used for the rest of the studies. A portion of the conjugate was absorbed 3 times with cells of strain No. 1, 242, and 305, and then filtered through Sephadex G-50 (Pharcacia, Upsala) equilibrated with standard buffered saline.

Staining of fixed smears. Thin smears from BHI broth, cellophane membrane, and BA cultures were made on clean coverslips. The smears were dried for 20 min at 37°C and then fixed either by passing them twice through a low flame from a Bunsen burner or by holding them at −20°C for 1 hr in 95% ethanol or for 4 hr in acetone. After fixation, the coverslips were dried in the freezer

and kept there until staining, usually not more than 2 or 3 days. Fixed coverslips were stained with 2 drops of different dilutions of conjugate ranging from undiluted to 1:5,000. The coverslips were placed in a moist chamber for 30 min at 37°C and then washed for 10 min with standard buffered saline, air-dried, and mounted on slides with 10% buffered glycerol. The moist chamber was constructed of a large Petri dish (14-cm diameter) inside which was placed a standard Petri dish. Water was then added to a height of 3 mm. The coverslips were placed on the top of the second Petri dish, and the whole system was closed with a thick plastic sheet and sealed with paraffin.

Staining of living cells. A completely different technique was applied to test living cultures (drop technique). A drop of culture was placed on a clean coverslip and then mixed with two drops of conjugate previously adjusted to twofold dilutions ranging from 1:5 to 1:320. The three drops were mixed thoroughly, as in the agglutination test, and the coverslip was placed in the moist chamber to be incubated for 30 min at 37°C. After 30 min, the Petri dishes were removed from the incubator and placed in the refrigerator for 4 hr.

At the end of this cold incubation, each coverslip underwent the following treatment: A large Petri dish was filled with Whatman No. 1 filter paper. Millipore filter membranes of 0.22-μ pore size and 13-mm diameter were soaked with standard buffer and placed on the absorbing paper. A few more drops of buffer were then added to make sure of complete contact between the membrane and the filter paper. The fluid from each coverslip was poured onto the center of the membrane. The fluid quickly disappeared. Next, 20 drops of standard buffer were added slowly for washing purposes, and after the fluid from the last drop disappeared, the membrane was removed with forceps and an impression smear of the surface of the membrane was made on a clean slide (three such impression smears can be made per slide). After a few seconds the membrane was removed and the slides were air dried and mounted in 10% glycerol-buffer. Use of Millipore filters retained all particles greater than 0.22 μ.

All preparations were examined by Zeiss Fluorescent microscope with an Osram HBO 200 W maximal-pressure mercury-vapor arc. The UG5 filter was used as an ultraviolet excitation filter, and the combination 0/41 as a barrier filter.

RESULTS AND DISCUSSION

Preliminary experiments. Early in this work, immune conjugate diluted 1:5 with standard buffer was used to stain gentle-heat-fixed or cold-acetone-fixed smears from broth or agar cultures of the different strains. This technique did not distinguish between strains producing type B enterotoxin and strains negative for type *B*. Only coagulase-negative strains consistently stained negatively. The positive-stained cells had a smooth bright-green periphery and dark center. Culture supernatants of all strains showing classical positive FA reaction were tested for enterotoxin by the single tube gel diffusion and micro-Ouchterlony double diffusion tests without any concentration. For the respective tests the antiserum was diluted 1:40 and 1:5. All culture supernatants were found to be negative except those strains producing type *B* enterotoxin (S-6 and 243). A line of identity with pure enterotoxin *B* was obtained in the slide micro-diffusion test. When the negative supernatants were concentrated 10 times with a 30% solution of polyvinylpyrrolidone they reacted positively with type *B* antiserum, giving many precipitin lines, none of which had an identity with the line of pure enterotoxin. Normal sera from 10 chickens, 10 rabbits, and 20 monkeys were tested against the above concentrated supernatants (strains 1, S-6, 238, 243, 305) and against pure enterotoxin. All sera demonstrated the existence of a variety of antibodies against different staphylococcal antigens. None of these antibodies reacted with pure enterotoxin. Hall *et al.* (1963), reporting the existence of such antibodies, suggested that antienterotoxin serum used for gel diffusion tests be diluted at least 1:40. This treatment eliminated all nonspecific minor antibodies from their antiserum and they got only the specific line due to the enterotoxin-antienterotoxin reaction.

This method was applied to the conjugate in an effort to eliminate the cross reaction. Dilutions were made up to 1:2,000, and each higher dilution eliminated more strains as negative for green fluorescence. Strains 243 and No. 1 each stained positively with 1:2,000 dilution of conjugate. Conjugated normal rabbit serum stained at least one strain when diluted 1:500. Conjugated immune rabbit serum produced against blood proteins stained nearly all enterotoxic and nonenterotoxic strains brilliantly when diluted 1:2,000. Naturally occurring antibodies against staphylococcal antigens have been found in normal or even germ-free animals (Cohen *et al.*, 1961, 1963; Pittman and Moody, 1960), with Cohen *et al.* (1963) reporting titers of 1:1400 for conjugate from germ-free mouse sera, and 1:2100 for conventional mouse sera.

Since type *B* could not be distinguished from type *A* or nonenterotoxic strains, absorption of the conjugated antienterotoxin sera was resorted to. Conjugated serum was mixed with one-half volume of packed cells of strains No. 1, 242, and 305, incubated 1 hr at 37°C, and left in the refrigerator

overnight. This procedure was repeated three times. The cells were removed by centrifugation, and the clear supernatant was filtered through Sephadex G50 column equilibrated with standard phosphate-buffered saline. No increase of buffer molarity was necessary for elution. The clear conjugate, diluted 1:10, was used to stain smears from 12-hr BHI broth cultures of the different strains. Absorption with the nonenterotoxic No. 1 strain gave a conjugate which stained 243, stained No. 1 weakly, but did not stain S-6. Absorption with nonenterotoxic strains 242 and 305 gave a conjugate which stained nearly all strains less intensively than did unabsorbed conjugate, and there was a definite decrease in the thickness of the fluorescence of the cell walls. Absorption with lyophilized dialyzed supernatants of BHI broth cultures of strains No. 1, 242, and 305 also failed to provide the specificity desired. Because of these results, absorption of immune conjugate was not tried again.

Fixation of enterotoxin. When unabsorbed conjugate was used to stain smears from 18-hr BHI broth cultures of S-6 and 243 type *B* strains, a brighter staining was observed at the edge of smears than in the center of those fixed by heat or cold acetone. Cells appeared to have brighter and thicker walls, thick interstitial spaces, and very often abnormal surfaces with little dots on or around them. Clouds of fluorescence often covered the cells. These observations led to a consideration of the possibility that the reaction of toxin-antitoxin might produce precipitates which were not firmly associated with the cells and were being lost because of poor fixation or too much washing. If this was so, the observed fluorescence was not due to enterotoxin-antienterotoxin reaction but was actually the result of the reaction of normally present antibodies against surface staphylococcal antigens common among the different strains. Thus, these antigens resulted in an FA-positive staining.

Millipore filter membranes. These membranes were tested for their ability to catch the precipitates which might have formed and which might have eluted from the slide during washing. Results were variable, but the specific enterotoxin-antienterotoxin reaction was demonstrable for the first time. The two strains producing enterotoxin *B* showed a green halo of variable thickness, sometimes completely covering masses of cells in the form of a cloud (Fig. 1). Many times, a sky-like picture was obtained, with numerous bright precipitates in the form of stars or bridges between or around the cells. None of the type *A* and nonenterotoxic strains produced such a picture, and those cells which did stain showed only a green fluorescence with a smooth periphery and no precipitates or clouds (Fig. 4). The difference between a positive and a

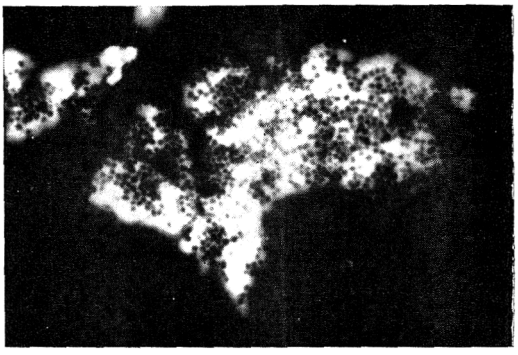

Fig. 1. Positive reaction. Living cells of *Staphylococcus aureus* strain S-6 from an 18-hr BHI broth culture (undiluted) stained with antienterotoxin *B* conjugate (diluted 1:5). All dilutions of conjugate higher than 1:5 stained only the cell walls, as the nonenterotoxic strain No. 1 in Fig. 4. Toxin content of broth 125 μg/ml. 2500×.

negative smear was obvious when 1250× magnification oil immersion was used. Negative cells were weak green, with rather thin, smooth walls. The positive showed a variety of pictures characterized by the presence of little fluorescent drops on the cells, by fluorescent precipitates in the form of halos around individual cells, by bridges joining them, or by clouds filling the interstitial spaces or covering them completely. Halos or clouds were fading in their periphery.

Lack of correlation of gel diffusion and FA. A problem encountered in demonstrating the enterotoxic cells was the lack of agreement between gel diffusion and FA tests. It was thought that the FA test could be more sensitive since it is a microscopic test, but, on the contrary, broth cultures containing over 100 μg toxin per ml often showed no precipitates around the green cells in smears fixed by heat or cold acetone. When the broth was diluted 1:5, however, precipitates could be demonstrated, indicating a positive reaction. These observations suggested that either suboptimal proportions of toxin and antitoxin or the presence of inhibitors in the broth had prevented the toxin-antitoxin reaction. Buffer was found to be a better diluent than was broth.

To test the effect of optimum proportions, dilutions from 0.02 to 250 μg/ml of pure enterotoxin were made, and one drop of each dilution was thoroughly mixed on a coverslip with two drops of dilutions of conjugate ranging from 1:5 to 1:320. The coverslips were placed in the moist chamber, incubated 30 min at 37°C, and then held 4 hr in the refrigerator. Then the content of each coverslip was poured on the Millipore filter membrane and washed slowly with 20 drops of standard buffer. Impression smears were made from the membrane on clean slides as described before. Results with

this technique were excellent. Precipitates were formed only in a rather narrow zone of antiserum dilution. High concentrations of toxin did not give precipitates when reacted with high dilutions of conjugate. When the time of cold incubation in the refrigerator was extended to 24 hr, most of the formed precipitates stuck on the coverslips and the regular 10-min washing with standard buffer did not remove them. The precipitates were more amorphous in the area of antibody and antigen excess, and they had a definite morphology in the area of optimum proportion.

After determination of optimum proportion with purified enterotoxin, the same kind of experiment was repeated, with an 18-hr BHI broth culture of strain S-6. This culture had 125 μg toxin per ml, and when one drop was mixed with 2 drops of conjugate diluted 1:5 to 1:160, it reacted positively only with the 1:5 dilution (Fig. 1). When the culture was diluted 1:5 (Fig. 2), the reaction was positive for 1:5, 1:10, 1:20, 1:40, and 1:80 dilutions of the conjugate, with the best reaction around 1:40. The cells in the region of optimum proportion were star-like, with abnormal surfaces because of the fluorescent precipitates which either covered them, occupied the interstitial spaces, or were scattered around them. Individual isolated cells frequently displayed a halo as a result of these precipitates. These fluorescent precipitates in the region of antibody or antigen excess appeared in the form of clouds that were fading or disintegrating on their periphery, suggesting that there was a loose association of toxin with the cells. This observation was confirmed when the effect of washing upon the size and brilliancy of precipitates was studied. It was found that increasing the numbers of drops of buffer used to wash the aggregates of toxin-producing cells resulted in a decrease in the size and brilliancy of precipitates around the cells, with only dull-green cells observed after excessive washing. The cells which did not produce toxin remained bright, however, and could be seen in a good impression smear. In such smears there were also dull cells with dark centers and very thin and weakly green walls. Close to these cells there were bright precipitates which obviously appeared to be casts of the cells that had separated from them. These observations suggest that the toxin is loosely associated with the cells and that when the cell does not have sufficient toxin around it, its surface antigens, being in a high concentration, react with normally existing antibodies in the conjugate and stain bright green. Additionally, when there is a high concentration of toxin around these cells, a thick precipitate is formed and the normal antibodies are prevented from reacting with surface antigens. Thus, when the precipitate is removed by excessive washing the cell appears dull, with a weakly stained wall.

Specificity of the reaction and controls. To establish the specificity of the reaction observed, the following control tests were performed. Sterile cellophane discs of 2.5 cm diameter, cut from dialysis tubing, were placed on the surface of blood agar plates. After complete contact was established by pressure, a drop of 12-hr-old BHI broth S-6 culture, previously washed 3 times with saline, was placed on top of the cellophane, and another drop was placed directly on the BA. After aerobic incubation for 48 hr, thin smears from both growths were made on coverslips, dried, fixed with heat, and then stained. The growth from the cellophane discs was strongly positive for toxin, with cells loaded with fluorescent precipitates (Fig. 3). The growth from the agar surface was negative or weakly positive, and cells were simply green, with either no precipitates or a very few small ones. Cells were harvested from the disc and from the agar and adjusted to the same optical density at 660 mμ. The cellophane disc with the growth was placed in 1 ml of standard buffered saline. Both

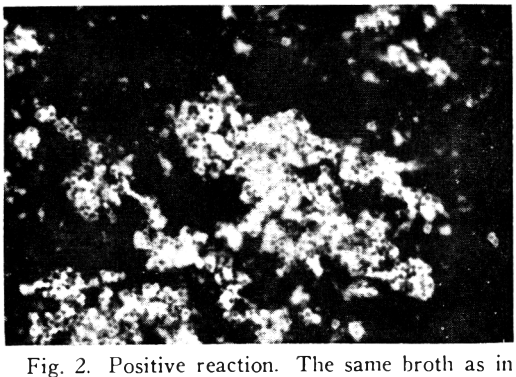

Fig. 2. Positive reaction. The same broth as in Fig. 1, but diluted 1:5 and stained with conjugate diluted 1:5 with saline. Dilutions of conjugate from 1:10 to 1:80 were also positive. 2500×.

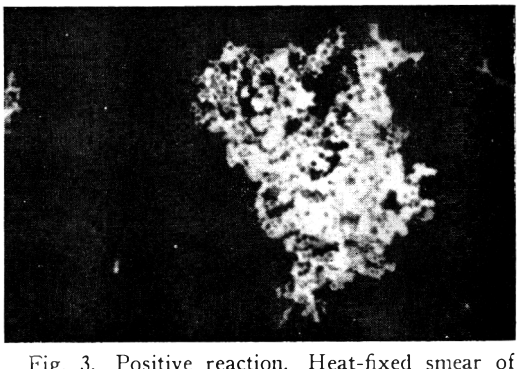

Fig. 3. Positive reaction. Heat-fixed smear of strain S-6 from cellophane membrane growth on blood agar. Conjugate diluted 1:40. 2500×.

suspensions were centrifuged, and the supernatants were checked for toxin by the single gel diffusion method. A piece of agar, 2 cm in diameter, under the area of colony growing directly on the agar was placed in a graduated centrifuge tube, and an equal volume of buffered saline was added. The mixture was homogenized, left at room temperature for 1 hr, and centrifuged, and the clear supernatant was checked for toxin by the single gel diffusion method. All three gel diffusion tubes were incubated for 7 days at 30°C. The cellophane growth and the agar extract were found to be positive, with respectively 46 and 70 μg toxin per ml. No toxin was demonstrated in the cells grown on the surface of the agar, as the FA test had also shown. A line of identity with pure enterotoxin was established when the supernatants were tested with the micro-diffusion slide method. The above test was repeated with strain 243, and the results were the same.

Strains which do not produce type *B* enterotoxin were grown in culture media under the same conditions as type *B* enterotoxin-producing strains. Smears and drops of living cells from these strains were stained and washed by the procedures previously described. None of these strains gave precipitates. The surface of the cells of many of these strains showed a 4+ fluorescence, and nothing else. The cells were round and smooth, with variations in thickness of their fluorescent walls (Fig. 4). Such cells were easily distinguished from the positive cells. Smears made from cellophane-membrane cultures of nonenterotoxic strain No. 1 and type *A* strains 196-E, 238, 239, 246-3A, 265 and stained with conjugate serially diluted from 1:5 to 1:160. Certain false-positive reactions characterized by small but typical fluorescent precipitates like those described for enterotoxin *B* producing strains were obtained with the 1:5 and 1:10 dilutions. Two out of three coagulase-negative strains gave small fluorescent precipitates with less than 1:5 diluted conjugate. All these cultures were negative for the presence of type *B* enterotoxin when tested by the micro-diffusion slide test. It was not surprising that nonspecific positive reactions were obtained with low dilutions of the conjugate, since Hall *et al.* (1963) reported similar results when they applied gel diffusion. To eliminate the nonspecific precipitin lines formed in the gel, they suggested dilution of the antiserum to 1:40 and higher. It is believed that the nonspecific reactions obtained were possibly due to staphylococcal antibodies normally existing in the serum which reacted with highly concentrated antigen.

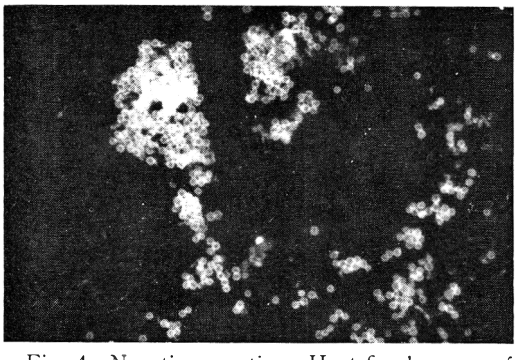

Fig. 4. Negative reaction. Heat-fixed smear of nonenterotoxic strain No. 1 from cellophane growth on blood agar. Conjugate diluted 1:40. 2500×.

Conjugated enterotoxin *B* antiserum did not react specifically with the following bacteria: *Proteus vulgaris, Aerobacter aerogenes, Escherichia coli, Pseudomonas aeroginosa, Hemophilus gallinarum, Salmonella anatum, Salmonella typhimurium, Salmonella senftenberg* (heat-labile and the heat-resistant strain 775W), *Serratia marcescens, Salmonella infantis, Pasteurella multocida.* Putrefactive anaerobe *no. 3679, Clostridium botulinum* type E, *Clostridium perfringens* type A. Certain other FA control tests suggested by Beutner (1961) were also performed. All demonstrated the specificity of the reaction observed.

Sensitivity of test. To measure the sensitivity of the test, purified enterotoxin *B* was diluted from 250 μg/ml to 0.2 μg/ml. One drop of each dilution was mixed on coverslips with two drops of conjugate doubly diluted from 1:5 to 1:320. The coverslips were placed in the sealed moist chamber, incubated 30 min at 37°C, and then kept in the refrigerator for either 4 or 24 hr. Then each coverslip was washed for 10 min in standard buffer, dried, and mounted on slides with buffered glycerol. The coverslips removed from the refrigerator at 4 hr were washed by the Millipore filter membrane procedure. This method detected toxin concentrations of over 0.8 μg/ml. There was a minimum evaporation from the periphery of the reactants, with slight nonspecific precipitation there. These precipitates interfered only with amounts of toxin below 1 μg/ml. Above this amount, specific precipitates were easily distinguished because they were numerous and of different sizes, and were scattered all over the coverslip, giving a sky-like picture. The reaction was consistently better when the coverslips remained in the refrigerator for 24 hr. In this case the precipitates were firmly adhered to the coverslip, and two washings of 5 min each with standard buffered saline did not remove them.

The following experiment was conducted to test the degree of sensitivity of the FA test for detection of enterotoxin in actual culture. Eighty ml of BHI broth were placed in an Erlenmeyer flask of 250-ml capacity. The broth was inoculated with

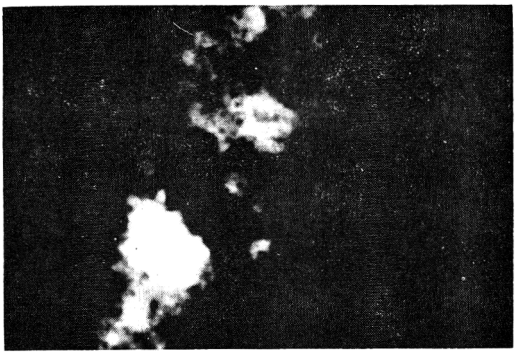

Fig. 5. Positive reaction. Enterotoxin *B* anti-enterotoxin specific precipitates from 24-hr BHI broth culture with supernatant diluted 1:3 and reacted with 1:20 diluted immune conjugate. Impression smear from Millipore filter membrane. 2500×.

cells from an overnight broth culture which had been washed three times, and the flask was placed on a shaker at 37°C. Samples of 4 ml were taken at 30-min intervals and analyzed for optical density (660 mμ), for enterotoxin content by the single gel diffusion test, and then for the presence of enterotoxin in smears by the FA test. The culture at zero time had an optical density of 0.035. After exponential growth for about 200 min, the culture entered the stationary phase, and at 210 min the growth-rate constant was zero. The first indication of toxin by the gel diffusion method was obtained at 210 min (4.5 μg toxin/ml) and then increased linearly with respect to time. The sample obtained after 6.5 hr contained 27.3 μg toxin/ml. Thin smears were made from the samples on coverslips and fixed with heat or cold alcohol. Ten slides were prepared per sample because 5 dilutions of conjugate, from 1:5 to 1:80, were used to stain. The coverslips were incubated for 30 min at 37°C and left for 4 hr in the refrigerator. They were then washed with standard buffer for 10 min, dried, and mounted on slides with buffered glycerol. All smears from the exponential time of growth were negative for precipitates or green clouds on the cells. As growth continued, there was, however, a rather gradual increase in thickness of the cell walls which gave an intensive fluorescence.

Fig. 6. Positive reaction. Enterotoxin-antienterotoxin specific precipitates from purified enterotoxin *B* (25 μg/ml) reacted with 1:20 diluted immune conjugate. Impression smear from Millipore filter membrane. 2500×.

Positive smears were obtained from samples with toxin concentrations over 15 μg/ml. When the drop technique was used to stain living cells, positive reactions were obtained with samples from the late exponential and early stationary phases having 2–4 μg toxin/ml. Smaller amounts of toxin (about 1 μg/ml) were detected in cell-free supernatants after diluting 1:2 with standard buffer. Friedman and White (1965) reported that smears from cultures of strain S-6 in the early lag phase (3 hr) contained cells with minimal green fluorescence, whereas a brilliant wide band of peripheral fluorescence was seen in unwashed cells from 8-hr cultures. We observed that, when washed three times with saline, the cells from 6.5-hr cultures gave minimum fluorescence. It was a fluorescence weaker than the fluorescence demonstrated by nonenterotoxic cells. Therefore, a slide was considered as positive only when cells were covered with fluorescent precipitates. Those showing no fluorescent precipitates were considered negative even if they had 4+ fluorescent walls.

The ease with which enterotoxin *B* and the toxic cells were demonstrated in cellophane membrane cultures (high toxin concentration around the individual cells) encouraged a study of the application of fluorescent antibody technique in detecting enterotoxin in foods. The results will be reported in a companion paper.

GENERAL DISCUSSION

Smears made from cellophane growth of strains S-6 and 243 and fixed with gentle heat reacted strongly with twofold dilutions of conjugate from 1:5 to 1:80. With this type of growth (high toxin concentration around the individual cells), the range of optimum proportion was much wider than with broth cultures under the same staining procedures. When smears from broth cultures were fixed with heat it was frequently found that only one dilution of the conjugate gave positive reaction. When drops of living cultures were used, the range of optimum proportions was intermediate. A good positive reaction apparently results from the presence of a large quantity of toxin around the individual cells, as in the cellophane culture. Good fixation, obviously, is also neces-

sary. Broth culture smears fixed by heat do not have such a high concentration of toxin around the individual cells, so the reaction is weaker and fades more, because of loss of material from the smear during washing. When one drop of living culture was mixed with two drops of conjugate dilution, the molecules of toxin were not fixed and so could form larger precipitates, resulting in intermediate fluorescence. Somehow the broth itself must also play a role in the antigen-antibody reaction, through concentration of its different compounds or pH.

Since there is a significant loss of material from the slides in washing, which is especially important for small concentrations of toxin, degree of fixation was recognized as a possible source of error. Because of this, certain fixation methods proposed for other systems were tested. Ten min of fixation of smears with 95% ethanol or with acetone at room temperature was found to affect the antigen, and the staining was dull. Cold fixation with acetone (4–5 hr at −20°C) or 95% ethanol (30–60 min at −20°C) and then drying in the freezer was found to be satisfactory. Heat fixation gave more losses of the smear during washing than did fixation with cold alcohol or acetone. It appeared that the thickness of the smear was the most important factor in getting good fixation; losses from washing were less in thin smears, so a brighter fluorescence resulted. The newly developed method of enterotoxin *B* detection by FAT is based on the demonstration of specific fluorescent precipitates around the bacterial cells and not on the presence of fluorescent cells alone. The use of different batches of antiserum and different dilutions has demonstrated that high titers are necessary to eliminate nonspecific cross reactions due to antibodies other than antienterotoxin *B*.

REFERENCES

Bergdoll, M. S. 1962. Chemistry and detection of staphylococcal enterotoxin. Proc. 14th Res. Conf., Am. Meat Inst. Foundation, Univ. of Chicago, p. 47.

Bergdoll, M. S., H. Sugiyama, and G. M. Dack. 1959. Staphylococcal enterotoxin. I. Purification. *Arch Biochem. Biophys.* **85**, 62.

Beutner, E. H. 1961. Immunofluorescent staining: The fluorescent antibody method. *Bacteriol. Rev.* **25**, 49.

Brown, G. R., and C. A. Brown. 1965. Sensitization of erythrocytes with staphylococcal enterotoxin by means of tolylene-2,4-diisocyanate for the passive hemagglutination reaction. *Bacteriol. Proc. Soc. Am. Bacteriologists* **65**, 72.

Casman, E. P. 1958. Serological studies of staphylococcal enterotoxin. *U. S. Public Health Rept.* **73**, 599.

Casman, E. P., and R. W. Bennett. 1963. Culture medium for the production of staphylococcal enterotoxin A. *J. Bacteriol.* **86**, 18.

Casman, E. P., and R. W. Bennett. 1965. Detection of staphylococcal enterotoxin in food. *Appl. Microbiol.* **13**, 181.

Cohen, J. O., G. S. Cowart, and W. B. Cherry. 1961. Antibodies against Staphylococcus aureus in nonimmunized rabbits. *J. Bacteriol.* **82**, 110.

Cohen, J. O., W. L. Newton, W. E. Cherry and E. L. Updyke. 1963. Normally occurring staphylococcal antibodies in germ-free mice. *J. Immunol.* **90**, 358.

Corstvet, R. E., and W. W. Sadler. 1964. The diagnosis of certain avian diseases with the fluorescent antibody technique. *Poultry Sci.* **43**, 1280.

Crowle, A. J. 1958. A simplified micro double-diffusion agar precipitin technique. *J. Lab. Clin. Med.* **52**, 784.

Friedman, M. E., and J. D. White. 1965. Immunofluorescent demonstration of cell-associated staphylococcal enterotoxin B. *J. Bacteriol.* **89**, 1155.

Genigeorgis, C. 1963. Comparison of staphylococci isolated from poultry meat and food poisoning outbreaks. Master's thesis. University of California, Davis.

Hall, H. E., R. Angelotti, and K. H. Lewis. 1963. Quantitative detection of staphylococcal enterotoxin B in foods by gel-diffusion methods. *U. S. Public Health Rept.* No. **78**, 1089.

Hall, H. E., R. Angelotti, and K. H. Lewis. 1965. Detection of staphylococcal enterotoxins in food. *Health Lab. Sci.* **2**, 179.

Hallander, H. O. 1965. Production of large quantities of enterotoxin B and other staphylococcal toxins on solid media. *Acta Pathol. Microbiol. Scand.* **63**, 299.

Hopper, S. H. 1963. Detection of Staphylococcus enterotoxin. I. Flotation antigen-antibody system. *J. Food Sci.* **28**, 572.

Hopper, S. H. 1965. Flotation system for detection of Staphylococcus enterotoxin in foods. *Bacteriol. Proc. Soc. Am. Bacteriologists* **65**, 72.

Pittman, B., and M. D. Moody. 1960. Staining of Staphylococcus aureus with fluorescent-labeled

globulin from non-immunized and streptococcus-immunized animals. *Bacteriol. Proc. Soc. Am. Bacteriologists* **60**, 140.

Robinson, J., and F. S. Thatcher. 1965. Determination of enterotoxin by an indirect hemagglutination inhibition procedure. *Bacteriol. Proc. Soc. Am. Bacteriologists* **65**, 72.

Schantz, E. J., W. G. Roessler, J. Wagman, L. Spero, D. A. Dunnery, and M. S. Bergdoll. 1965. Purification of staphylococcal enterotoxin B. *Biochemistry* **4**, 1011.

Ms. rec'd 12/18/65.

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Studies in Meat Tenderness. III. The Effects of Cold Shortening on Tenderness

SUMMARY

The tenderness of meat removed from the carcass in a pre-rigor condition is highly dependent on the extent of the cold shortening which occurs after excision. The relationship between shortening and tenderness is complex. A decrease of up to 20% of the initial excised length does not exert a significant effect, but toughness increases rapidly with further shortening beyond this point, reaching a peak of several times its original value (in terms of shear force required) at a shortening of about 40%. With yet further shortening, the meat becomes progressively more tender until, at about 55–60% shortening, it is cleaved about as easily as meat in which less than 20% shortening has occurred.

The presence of intact skeletal attachments does not necessarily overcome the development of shortening-induced toughness. It is shown that a pre-rigor muscle which is absolutely fixed in over-all length is still capable of appreciable shortening in one zone, with compensating lengthening elsewhere, if the application of cold is uneven along its surface.

INTRODUCTION

During the first few hours post-mortem, the components of muscle undergo an extensive series of biochemical changes culminating in rigor mortis (Bate-Smith and Bendall, 1949), and the ultimate structure of meat is determined partly by the rate and extent of events in this phase. It is rather surprising, therefore, to find a paucity of papers attempting to relate meat tenderness with conditions during rigor onset. Indeed, few reports of meat quality investigations even mention the temperature at which the experimental material was held during rigor onset, the inference being that this information is irrelevant and the rigor phase is of no importance.

There are a few indications, however, that a muscle which has been cut or excised in a pre-rigor condition may be tougher than expected following rigor onset and cooking (Lowe and Stewart, 1946; Ramsbottom and Strandine, 1949; Koonz *et al.*, 1954; Paul and Bratzler, 1955; Lowe, 1958; de Fremery and Pool, 1960). Locker (1960) suggested that the toughening might be due to a shortening of the muscle during the onset of rigor mortis, a view consistent with the turgidity observed by Lowe and Stewart (1946) when the breast muscles of roasters were cut soon after slaughter.

This rigor shortening has been studied extensively, within the temperature range 17–37°C, by Bendall (1951); and, more recently, Locker and Hagyard (1963) have examined the shortening which accompanies or precedes rigor onset at temperatures below this range. Their investigations have revealed an interesting "cold-shortening" phenomenon in which the exposure of excised fresh bovine muscles to temperatures near freezing point causes very appreciable shortening.

These two groups of observations—the first relating muscle shortening to meat toughness, the second connecting the extent of shortening to temperature—suggest that the ultimate tenderness of meat might be affected by temperature during the first few hours post-mortem, quite apart from the well-recognized temperature-dependent aging process. Locker and Hagyard's results indicate that shortening is minimal at about 15–20°C, and within limits is progressively greater the further removed is the pre-rigor holding temperature from this region in either direction. We might expect to detect toughening, therefore, in muscles which were still in a pre-rigor condition when exposed to temperatures either well above or appreciably below this intermediate range, and evidence has already been presented (Marsh, 1964) to support this view. More recently, Herring *et al.* (1965) have obtained further evidence favoring a relationship between tenderness and the shortening produced by chilling pre-rigor bovine muscles.

The effect of temperature on shortening is much greater in the lower (0–15°C) than in the upper (20–43°C) range, and in addition

the accelerated aging to be expected at higher temperatures (Wilson *et al.*, 1960) might well obscure or eliminate entirely any toughening produced during rigor onset at an elevated temperature. Furthermore, it is in the low-temperature region that practical implications might be expected, for modern refrigeration techniques have made the rapid cooling and freezing of meat economically attractive (Marsh and Thompson, 1958). For these reasons the present investigation is concerned primarily with the toughness produced by cold shortening rather than by heat shortening.

EXPERIMENTAL

Meat. Sternomandibularis muscles ("neck muscles") were obtained within 20 min of slaughter from a variety of beef carcasses. In a few experiments longissimus dorsi (LD) muscles were excised from lamb carcasses 20–30 min after death. In all cases the muscles were transported to the laboratory, trimmed, and prepared for experimental treatment within 90 min of slaughter.

General design. To eliminate inter-animal variability, all observations in any one experiment were made on the two neck muscles of only one beef carcass. Each experiment was repeated several or many times on the neck muscles of other carcasses, as will be apparent from the results, but one aim throughout this project has been to utilize to the full the quite remarkable uniformity of this muscle within any one carcass (Marsh *et al.*, 1966).

The muscles were cut into pieces of 80–200 g, the longest dimension (minimum 7 cm) being along the fiber direction. The number of samples so obtained ranged from 6 (small cow) to 45 (large bull). To permit approximate measurement of changes in length during cooling, freezing, thawing, and cooking, a small nail was inserted near each end of every sample (Locker and Hagyard, 1963).

The experimental treatments of the samples varied widely. In general the measured pieces were exposed for different periods to one or more of the following conditions: high temperature (30, 37, or 43°C), room temperature (18–26°C), 15°C, low temperature (circulating air at 2°C, still air at 5 or 10°C, or, when plastic-wrapped, water at 0, 2, or 5°C), and freezing (dry ice–alcohol at −75°C, or circulating air at −29°C). Frozen samples were stored at −14°C in plastic bags, and were thawed (usually for 16 hr in circulating air at 2°C) before being cooked. Samples which had not been frozen at any stage of their experimental treatment were held at 2°C until 48-hr post-mortem before being cooked, to ensure the completion of rigor changes.

The samples were cooked by the water-bath method described by Marsh *et al.* (1966), and their tenderness values were assessed objectively by the tenderometer of Macfarlane and Marer (1966).

In a few experiments the progress of rigor onset was followed by periodic determination of the extensibility of a vertically-mounted loaded strip of muscle (Marsh, 1954).

Estimations of pH were made by glass electrode on homogenates (Marsh and Snow, 1950) of 1–2 g muscle in 10–15 ml neutralized sodium iodoacetate solution. The "initial pH" is the pH of a sample taken 70–90 min post-mortem; the "ultimate pH" is that of the muscle after 24 hr at room temperature.

RESULTS

Cold shortening in relation to rigor onset. In a preliminary study designed to confirm and extend the work of Locker and Hagyard (1963), it was found that the extent of cold shortening is related inversely to the degree of rigor onset at the time of cold application. This is illustrated in Fig. 1, summarizing four experiments in which samples of neck muscle were held at 37°C for varying periods before transfer to a cold environment, the extensibility being determined on one strip every time a

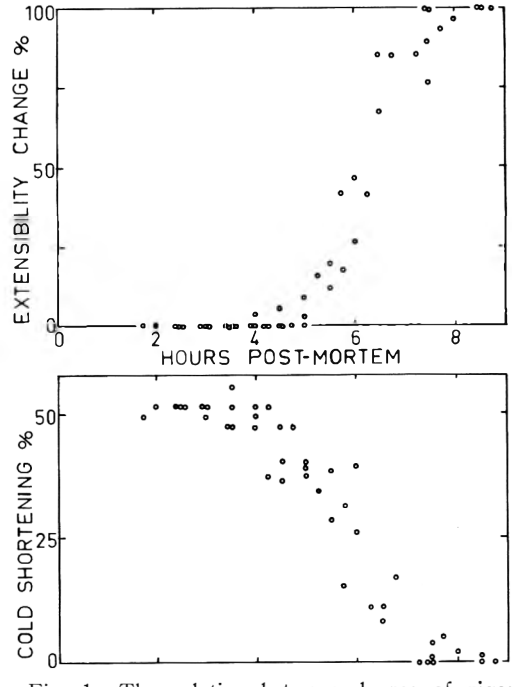

Fig. 1. The relation between degree of rigor onset at the time of cold application (upper) and extent of cold shortening produced (lower). Extensibility change as percent of total decrease in extensibility; cold shortening as percent of sample length at time of transfer from 37°C to 2°C.

sample from the same muscle was transferred to air at 2°C. The neck muscles providing these data were typical of those used throughout this project; the uniformity of these four pairs is demonstrated by their consistent extensibility behavior, cold-shortening response, relatively high initial pH values (greater than 7.1), and narrow range of ultimate pH values (5.50–5.61). There is some evidence that muscles of high ultimate pH do not follow the pattern of cold shortening and toughening displayed by more normal muscles, and for this reason results from the few atypical muscles encountered (initial pH less than 7.0, or ultimate pH greater than 6.0) are excluded from this paper.

In practice it is sometimes more convenient and instructive to relate cold shortening and allied effects to declining pH rather than to time. This procedure allows direct comparison of results when, for instance, samples from the same muscle are held at two or more temperatures before application of a cold shock. An example of this is provided in Fig. 2, illustrating an experiment performed with

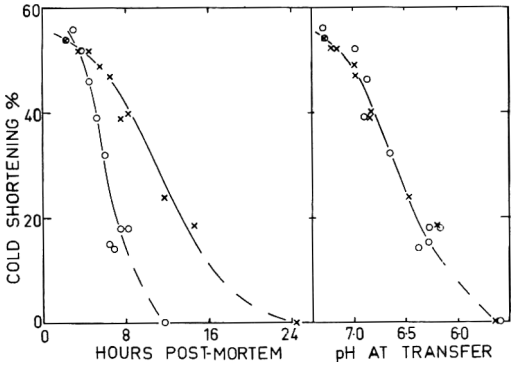

Fig. 2. The effects on extent of cold shortening of (left) temperature before cold application, and (right) pH at the time of transfer. Samples held before transfer at 18°C (×) and 37°C (○). Cold shortening as percent sample length at time of transfer to 2°C.

one pair of neck muscles. Some samples were held at 37° and others at 18°C; at intervals, samples were transferred from these holding temperatures to air at 2°C. It will be seen that a plot of cold shortening against time produces two separate curves, one for each of the holding temperatures, whereas a plot of cold shortening against the pH at the time of transfer is fitted by a single curve.

The rate, as well as the extent, of cold shortening is dependent on the degree of rigor onset at the time of cooling. This is shown in Fig. 3, one of several experiments in which samples were transferred at intervals from air at 30°C to water at 0°C, the lengths of all strips being measured every 15 or 30 min after immersion.

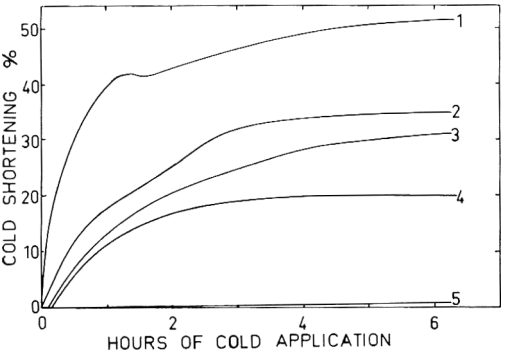

Fig. 3. The effects of time post-mortem on rate and extent of cold shortening. Times post-mortem and pH values at time of transfer from air at 30°C to water at 0°C were: 1) 2 hr, 7.20; 2) 4½ hr, 6.85; 3) 6½ hr, 6.66; 4) 8½ hr, 6.13; 5) 24 hr, 5.60. Cold shortening as percent initial excised length.

Tenderness in relation to cold shortening. In the first major series of experiments to involve tenderness determinations on cold-shortened samples, shortening values of 5 to 60% of the initial excised length were obtained by transferring samples to air at 2°C after varying periods (0–24 hr) at room temperature. Samples left at room temperature till rigor was fully, or almost fully, established before being transferred to the cold were found to be relatively tender; cold shortening had been negligible, and the shortening which accompanied rigor onset had been only slight. Samples which had been exposed to a cold environment within about 5 hr post-mortem, while still in a strictly pre-rigor condition, were also found to be quite tender despite very considerable shortening, usually exceeding 50%. Between these two situations, however, in the region where the extent of cold shortening had been restricted by partial rigor onset, a range of shortening was detected in which the resistance to shearing was higher, occasionally reaching values four times that of either the greatly shortened or the relatively unshortened samples from the same muscle. Three of the many examples of this phenomenon observed are illustrated in Fig. 4. It will be seen that in each experiment excessive toughness (upper figure) was found in those samples which had shortened by intermediate amounts (lower figure), while relatively tender meat resulted from low or very high shortening values.

This result was most unexpected, so the experiment was repeated many times with a wide variety of beef animals, including young steers, old cows, and bulls. The holding temperature before the samples were cold-shocked was also varied; in addition to the room temperature studies, others were undertaken in which samples were held at 37 and at 43°C. In all cases, regardless of age and sex of

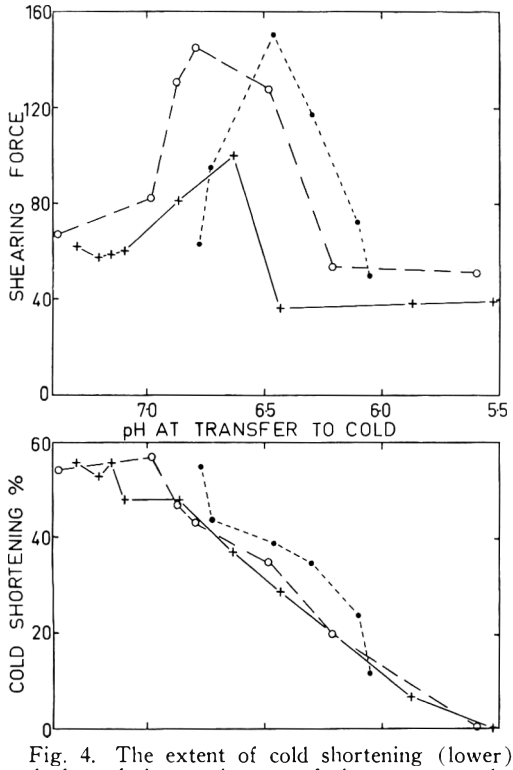

Fig. 4. The extent of cold shortening (lower) and the relative tenderness of the same samples when cooked (upper) in relation to the pH values of the raw samples when transferred to air at 2°C during rigor onset. Cold shortening as percent sample length at time of transfer.

the animal or of the temperature before cold application, only those samples shortening by intermediate amounts were excessively tough.

As will be discussed later, we believe the "background toughness" (as measured by the resistance to shearing of the unshortened muscle) to be the contribution of connective tissue to toughness, and the increase in toughness with shortening to be due to configurational changes in the contractile proteins actin and myosin. It would be expected, then, that "background toughness" would be considerably smaller in a muscle of lower connective-tissue content than in beef neck, whereas the ability to toughen with shortening would he unimpaired. This view was tested in a small series of experiments using paired lamb LD muscles excised 20–30 min after slaughter. The left muscle of each pair was frozen rapidly (dry ice-alcohol), with only momentary exposure to cold-shortening temperatures before ice crystal formation prevented shortening; the right muscle was held at 2°C for 1–2 hr before being frozen at the same fast rate. After slow thawing, the muscles were cooked and the tenderness assessed by tenderometer. The force required to shear the left muscles seldom exceeded 20 arbitrary units, whereas the right muscles occasionally required more than 100 units. In a few experiments the right muscle was six times as tough as the left, a considerably higher ratio than found in beef neck. This difference between lamb LD and beef neck appears to be due entirely to the smaller "background toughness" of the former; the magnitude of the increase in toughness with shortening is comparable in the two species.

The combined results of 13 experiments on beef neck are presented in Fig. 5. In each of them, 6–12 samples were held at room temperature before exposure to air at 2°C. Similar relationships were found for samples held for various times at 37°C (18 experiments) and 43°C (7 experiments) before cold application, but in these series scatter was rather more pronounced, an effect possibly due to accentuation of differences in aging rates at elevated temperatures. Apart from this increased scatter, we have detected no exception to the conclusion made plain in Fig. 5: beef neck muscles are invariably very tough if cold-shortened by about 40% of their initial excised length, but are very much more tender, within the shortening range 20–60%, the further the shortening is removed *in either direction* from 40%.

Before seeking an explanation for this effect, several experimental details require emphasis. In the beef neck studies described above, freezing was not introduced at any stage. All samples in any one experiment were from the same muscle, or pair

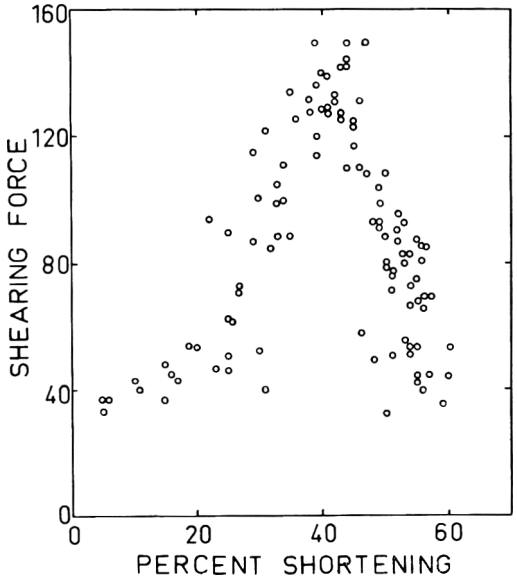

Fig. 5. Relative tenderness in relation to the shortening induced by transfer of samples from room temperature to 2°C at intervals during rigor onset. Cold shortening as percent initial excised length.

of muscles, of one carcass. The tenderness of beef neck muscle is known to be remarkably uniform if all samples from a single carcass are given the same post-mortem treatment (Marsh *et al.*, 1966), but in any case the samples were randomized to avoid any intramuscular variation. All samples were given the same cooking treatment at the same time (48 hr) post-mortem. There are only two obvious differences, then, among the samples of any one experiment: the lengths of time spent at room or elevated temperature before transfer to 2°C, and the degree of shortening produced by exposure to cold.

Attempts to explain the results by the first of these differences, invoking an aging effect, obviously fail completely, for the samples which were aged both most and least were very significantly more tender than those aged for intermediate times before transfer to the cold. This fact can be ascertained from Fig. 4, bearing in mind that the decrease in pH during active glycolysis at any one temperature is roughly proportional to time post-mortem (Marsh, 1954). It is illustrated more directly in Fig. 6 for samples of three neck muscles held at room temperature before transfer to the cold; after a relatively steady phase of up to 5 hr, toughness apparently increased very substantially with aging during the ensuing 4–8 hr.

Only the differences in shortening remain, therefore, to account for the effect, a conclusion supported by the result of the experiment illustrated in Fig. 7. Twelve samples from one muscle were held at 43°C, six being free to shorten while the remainder were clamped securely at their initial lengths. One free and one clamped sample were transferred to air at 2°C at each of six times. The clamped samples were released 48 hr post-mortem, and all 12 samples were cooked together and assessed for tenderness. It is obvious from the figure that the prevention of shortening by physical restraint has eliminated the toughening effect entirely.

The regular occurrence of peak toughness in samples which had shortened by about 40% suggested possible direct involvement of the rigor process, in addition to shortening, in toughness production. It was argued that, while shortening was clearly necessary to cause toughening, shortening *alone* could hardly be held responsible, since both minimal and maximal shortening resulted only in tender meat. On the other hand, the occurrence of cold shortening simultaneously with the formation of "rigor linkages" might be expected to result in some degree of internal strain or actual disorganization, with a consequent increased resistance to cleavage. On this reasoning, toughening would occur only when cold application coincided with the early part of the rapid phase of rigor onset; if the muscle was cooled either before the initial development of rigor linkages or when such linkage formation was nearing completion, little or no conflict between shortening and rigor would occur, and disorganization would be expected to be negligible.

Extensibility measurements appeared to lend support to this concept: in each of six experiments, the toughest sample was one of those which had

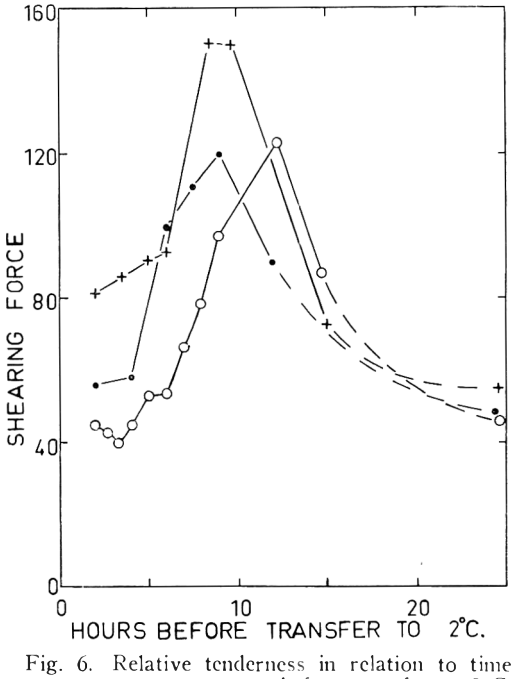

Fig. 6. Relative tenderness in relation to time spent at room temperature before transfer to 2°C. All samples cooked 48 hr post-mortem.

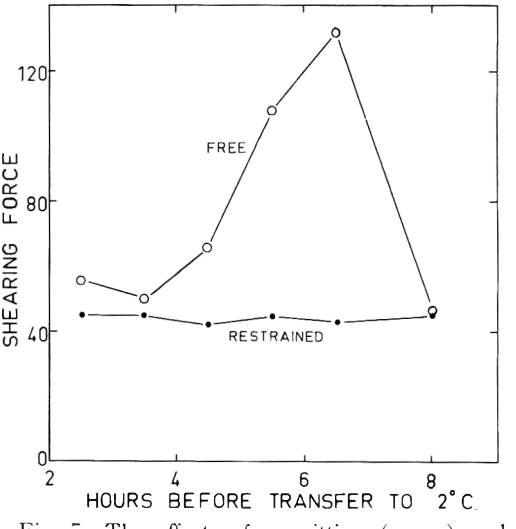

Fig. 7. The effects of permitting (upper) and preventing (lower) cold shortening on relative tenderness.

been cold-treated during the first half of the rapid phase of rigor onset. To be certain that toughening is really due to this postulated combination of cold shortening and rigor onset, however, it is necessary to establish that a peak would not appear if these two processes took place at different times. To attempt this, three series of experiments were undertaken. All failed to support the hypothesis that simultaneous cold shortening and rigor onset were necessary for toughness production. The results nevertheless provided further strong evidence for the relationship between toughness and extent of shortening.

In the first series, samples were transferred to several air temperatures between 2 and 15°C 1–2 hr post-mortem, and were left there for the entire course of both cold shortening and rigor onset. At the lower temperatures within this range, cold shortening was considerable and preceded the onset of rigor mortis, as demonstrated by Locker and Hagyard (1963); in these samples we may assume, therefore, a fairly clear separation in time between the processes of cold shortening and rigor onset. At or near 15°C, shortening accompanied rigor onset, as judged both from the time post-mortem of its occurrence and from the pH at which it took place. It may be assumed, therefore, that only rigor-associated shortening occurred in this temperature region, so in these samples too there was no possibility of simultaneous cold shortening and rigor onset. Despite this separation of the effects, however, a clearly-defined peak toughness was detected in the vicinity of 35–40% shortening, as illustrated in Fig. 8 (4 experiments).

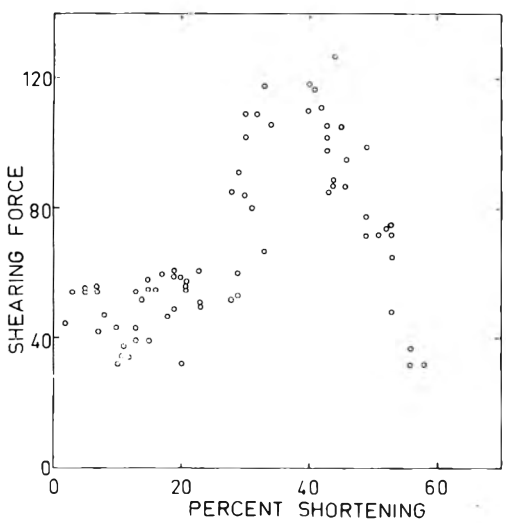

Fig. 8. Relative tenderness in relation to the shortening induced by holding samples at temperatures within the range 2–15°C throughout rigor onset. Combined cold-plus-rigor-shortening as percent initial excised length.

In the second series, cold shortening was virtually eliminated and replaced by thaw shortening. Samples were frozen at −75°C after varying periods at room temperature, and were later thawed at different rates: in air at 2 and 5°C, and in water (when plastic-wrapped) at 0, 2, and 5°C. The range of thawing temperatures was kept narrow in order to minimize any differences in aging. Once more, the dependence of tenderness on extent of shortening was demonstrated (Fig. 9); maximum toughness occurred in those samples which had shortened by about 30–35%. This value is somewhat lower than observed in the other series, and scatter is more apparent. Nevertheless it is clear that substitution of thaw shortening for cold shortening has not greatly modified the relationship between tenderness and length. Although it may be argued that thaw shortening and thaw rigor are essentially the same process, it has in fact been established (Bendall, 1960) that the shortening phase is complete before the ATP level has decayed significantly. Again in this series, therefore, a fair separation of shortening and rigor onset may be assumed, yet despite this, a significant toughness peak appears at intermediate levels of shortening.

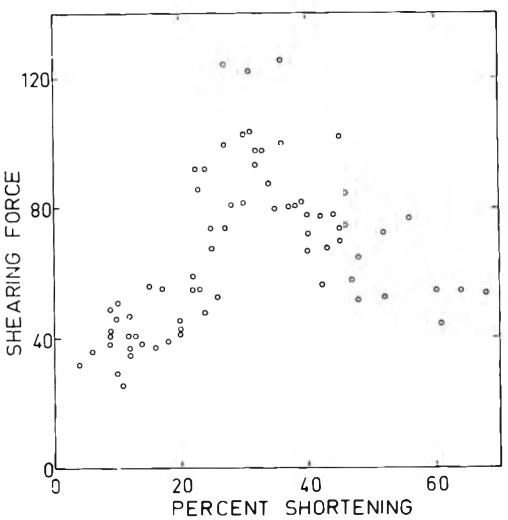

Fig. 9. Relative tenderness in relation to the shortening induced by thawing samples (previously frozen very rapidly at different stages of room-temperature rigor onset) at several rates. Thaw shortening as percent initial excised length.

In the third series, various degrees of cold shortening and thaw shortening were produced in the same samples. Muscle strips were transferred to water at 0°C for varying periods (15 min to 24 hr) and at different stages of rigor onset. Cold shortening was terminated by freezing in alcohol at −75°C, and the samples were thawed later in air

at 2°C before being cooked and assessed for tenderness in the usual way. The results obtained from seven similar experiments are illustrated in Fig. 10, and clearly show that peak toughness persists at about 40% shortening.

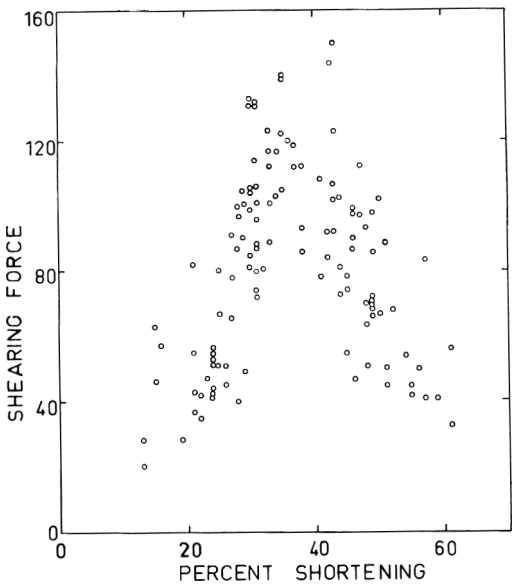

Fig. 10. Relative tenderness in relation to the total shortening induced by successive exposure to temperatures of 20, 0, —75, and 2°C. Combined rigor-, cold-, and thaw-shortening as percent initial excised length.

These three series of experiments demonstrate that a coincidence in time of onset of rigor and shortening is not an essential requirement for the development of toughness. Furthermore, they provide strong evidence, additional to that presented earlier (Fig. 5), that the extent of shortening affects tenderness very markedly, and that toughness attains a peak value at a shortening of about 40%.

This value is of special interest, for it is at about 40% shortening that stimulated fresh muscle enters the "delta state" (Ramsey and Street, 1940), a condition probably indicative of some degree of internal disorganization or disruption. It has been found (Marsh and Thompson, 1957) that strips of thaw-shortened lamb muscle enter a phase of copious fluid release at about 40–50% shortening, an observation supporting the view (Fenn, 1947) that at this shortening the elastic limit of a containing membrane is reached, further shortening being possible only after its rupture. An almost identical relationship between shortening and fluid exudation has now been found in strips of beef neck muscle air-frozen at different stages of rigor and thawed in plastic bags (to minimize evaporative losses) in air at several temperatures (2–37°C) to extend the shortening range. Although considerable variation was found in the "drip"/shortening relationship among animals, due perhaps to the wide variety of animals used, all experiments indicated that little fluid was exuded until shortening exceeded 40%. This is illustrated in Fig. 11, all the samples for which were obtained from one pair of bull neck muscles. Below about 40% shortening, "drip" exudation was low and fairly constant, but between approximately 40 and 55–60% shortening, the rate of "drip" release rose rapidly with increasing shortening. Beyond this the volume of exuded fluid was high, and increased rapidly with further shortening, but its rate of release with shortening was roughly constant. We suggest that this would be the expected effect if cell damage commenced at 40%, continued with further shortening, and was complete at 55–60%. Further shortening would then cause the release of fluid by continued compression of an already maximally-damaged structure.

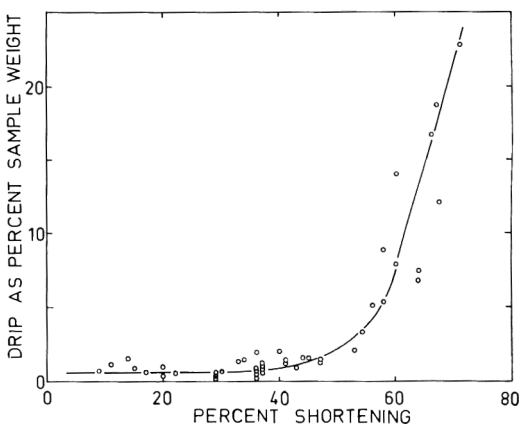

Fig. 11. The relation between "drip" exudation (as percent frozen sample weight) and extent of thaw shortening (as percent initial excised length).

It was shown earlier (Fig. 7) that strips of muscle, clamped to prevent shortening, showed no toughening trend as a result of transfer to a cold environment, regardless of the stage of rigor onset at which this transfer took place. It might be assumed from this result that, in muscles firmly attached to the skeleton, toughening of the cold-shortening type could not develop because of the supposed impossibility of muscle shortening. The treatment received by the clamped strips, however, differed appreciably from that undergone by intact muscles on a carcass during chilling. In particular the strips were uniform in cross-section, they were free of superficial fat (variations in the thickness of which could have delayed cooling in some parts of the strip), and they were cooled uniformly by directing air at 2°C over their entire surfaces,

except for the small areas covered by the restraining clamps. In view of these appreciable differences between clamped muscle strips and intact skeletally-attached muscles, a study was undertaken to assess the possible development of zones of shortening within a muscle of fixed length.

In each experiment, a beef muscle was clamped vertically in a metal frame at its initial excised length, and lines were drawn on it at 1-cm intervals across the fiber direction. The upper and lower thirds of the muscle were then wrapped lightly with several thicknesses of paper, and the clamped, partly-insulated muscle was transferred to moving air at 2°C about 6 hr post-mortem. The expected cold shortening occurred only in the exposed section, the lightly-insulated zones lengthening by a corresponding amount. The muscle was removed from the restraining frame 48 hr post-mortem, cooked by the water-bath method, and assessed by tenderometer. In all cases the zone exposed to the cold air was appreciably tougher than the insulated sections, in one experiment by a factor of 3.5. An example is illustrated in Fig. 12; in this case the central exposed zone shortened by 26% on average, but small areas within it attained shortening values of up to 47%. The bulk of the compensating length change occurred in the lower of the two insulated sections. The *over-all* length change of the muscle was zero, yet the midsection was three times as tough as either end.

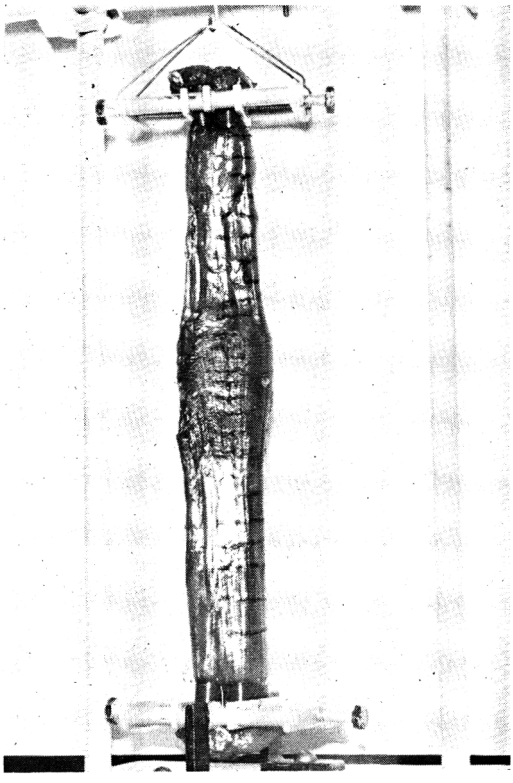

Fig. 12. Photograph of a beef neck muscle in which cold shortening was confined to the central zone by light insulation of the end thirds. Lines originally 1 cm apart. Muscle restrained at its initial excised length.

DISCUSSION

The results confirm a preliminary report from this laboratory (Marsh, 1964) that the extent of shortening during the early post-mortem period affects meat tenderness to a remarkable degree. In addition the study has shown the relationship between tenderness and shortening to be a most interesting and unexpected one. A decrease in length of up to about 20% causes little or no toughening, but from 20 to roughly 40% shortening the toughness increases severalfold. Beyond 40% the meat becomes rapidly more tender, and at 60% shortening it is cleaved about as easily as meat in which almost no shortening had occurred. Thus there appear to be three critical shortening values—approximately 20, 40, and 60%—which divide the tenderness/shortening plot into reasonably symmetrical zones.

We are unable to relate the initial excised length, on which all our shortening values have been based, to the true resting length of the muscle in the animal; indeed, for the sternomandibularis muscle, which changes its length considerably with every head movement, the term "resting length" is perhaps meaningless. Nevertheless there is a strong possibility that excision and trimming of the muscle strips caused some degree of shortening before measurement of the initial length (Locker and Hagyard, 1963). Herring *et al.* (1965) reported shortenings of 20–25% for bovine semitendinosus and of 10–20% for psoas major muscles upon excision shortly after death. Our critical shortening values may therefore be too low. If true resting length was 20% greater than our measured initial excised length, then the supposed critical values of 20, 40, and 60% shortening would in fact correspond to shortenings of ⅓, ½, and ⅔ of the true resting length—an interesting possibility, even though unsupported at this stage by direct measurement.

It seems clear that the relationship between tenderness and shortening is not related to connective tissue, for any shortening-induced thickening or passive folding in this component would be expected to result in a toughening which would increase regularly with shortening, a pattern quite unlike that detected here. It appears, then, that we are concerned with a consequence of actin-myosin shortening, and that it is the contractile material itself which is responsible for the observed tender-tough-tender sequence. However, any attempt to explain the mechanism of the effect in molecular terms would be premature at this stage.

A possible lead to further investigation is provided by the relationship (Fig. 11) between fluid exudation and extent of shortening during thaw rigor. If actual cell damage is slight at 40% shortening but maximal at 60%, as the results suggests, then the range of 40-60% shortening could be considered a zone of "progressive rupturing." It is not difficult to visualize a rapid decrease in internal strain in this phase, with consequent realignment of previously-distorted "cleavage planes."

Finally, we must consider the relevance of these results to present and future commercial practice. The very appreciable toughening reported here is dependent on shortening, and it may well be asked to what extent such shortening can occur when most skeletal attachments are still intact at the time of chilling. A few relatively unimportant muscles, like the sternomandibularis used in this study, are severed during dressing, and could certainly shorten appreciably; this may be partly responsible for the general belief that the neck muscle is excessively tough. Other muscles, notably the important LD, are able to shorten because of their method of attachment, the fibers being firmly anchored to the skeleton at one end only. Even muscles which are fixed absolutely in length by firm attachment to the skeletal framework would be capable, as demonstrated above (Fig. 12), of shortening in part of their length, provided the cold application does not reach the entire span of the muscle simultaneously. In practice it would require only a slightly greater thickness of fat, the proximity of a bone, or a protective shielding of part of a muscle from cold moving air by overlying tissue, to activate this mechanism. Such an activation may well prove to be the rule rather than the exception in carcasses exposed to a cold environment before the onset of rigor mortis.

REFERENCES

Bate-Smith, E. C., and J. R. Bendall. 1949. Factors determining the time course of rigor mortis. *J. Physiol.* **110**, 47.

Bendall, J. R. 1951. The shortening of rabbit muscle during rigor mortis: relation to the breakdown of adenosine triphosphate and creatine phosphate and to muscular contraction. *J. Physiol.* **114**, 71.

Bendall, J. R. 1960. Post-mortem changes in muscle. *In:* Structure and function of muscle. Academic Press, New York and London, **3**, 227.

de Fremery, D., and M. F. Pool. 1960. Biochemistry of chicken muscle as related to rigor mortis and tenderization. *Food Research* **25**, 73.

Fenn, W. O. 1947. Contractility. *In:* Physical chemistry of cells and tissues. Churchill, London, p. 453.

Herring, H. K., R. G. Cassens, and E. J. Briskey. 1965. Sarcomere length of free and restrained bovine muscles at low temperature as related to tenderness. *J. Sci. Food Agr.* **16**, 379.

Koonz, C. H., M. I. Darrow, and E. O. Essary. 1954. Factors influencing tenderness of principal muscles composing the poultry carcass. *Food Technol.* **8**, 97.

Locker, R. H. 1960. Degree of muscular contraction as a factor in tenderness of beef. *Food Research* **25**, 304.

Locker, R. H., and C. J. Hagyard. 1963. A cold shortening effect in beef muscles. *J. Sci. Food Agr.* **14**, 787.

Lowe, B. 1958. Experimental Cookery. Wiley, New York, p. 226 (4th ed).

Lowe, B., and G. F. Stewart. 1946. The cutting of the breast muscles of poultry soon after killing and its effect on tenderness after subsequent storage and cooking. Unpublished data, Iowa State College. Cited by Lowe, B., in *Advances in Food Research* **1**, 232 (1948).

Macfarlane, P. G., and J. M. Marer. 1966. An apparatus for determining the tenderness of meat. *Food Technol.* In press.

Marsh, B. B. 1954. Rigor mortis in beef. *J. Sci. Food Agr.* **5**, 70.

Marsh, B. B. 1964. Meat quality and rigor mortis. *In:* Carcass composition and appraisal of meat animals. C.S.I.R.O.., Melbourne, Paper No. 12.

Marsh, B. B., and A. Snow. 1950. A simple tissue homogenizer. *J. Sci. Food Agr.* **1**, 190.

Marsh, B. B., and J. F. Thompson. 1957. Thaw rigor and the delta state of muscle. *Biochim. et Biophys. Acta* **24**, 427.

Marsh, B. B., and J. F. Thompson. 1958. Rigor mortis and thaw rigor in lamb. *J. Sci. Food Agr.* **9**, 417.

Marsh, B. B., P. R. Woodhams, and N. G. Leet. 1966. Studies in meat tenderness. I. Sensory and objective assessments of tenderness. *J. Food Sci.* **31**, 262.

Paul, P. C., and L. J. Bratzler. 1955. Studies on tenderness of beef. II. Varying storage times and conditions. *Food Research* **20**, 626.

Ramsbottom, J. M., and E. J. Strandine. 1949. Initial physical and chemical changes in beef as related to tenderness. *J. Animal Sci.* **8**, 398.

Ramsey, R. W., and S. F. Street. 1940. The isometric length-tension diagram of isolated skeletal muscle fibers of the frog. *J. Cell. and Comp. Physiol.* **15**, 11.

Wilson, G. D., P. D. Brown, C. Pohl, C. E. Weir, and W. R. Chesbro. 1960. A method for the rapid tenderization of beef carcasses. *Food Technol.* **14**, 186.

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