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# Journal of FOOD SCIENCE

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# Biochemical Changes in Fish Muscle During Rigor Mortis

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(Manuscript received April 16, 1962)

**SUMMARY**

**Mullets *(Mugil dussumieri)*, Bombay ducks *(Harpodon nehereus)*, and groupers *(Epinephalus malabaricus)* were stored in crushed ice at about 2°C, and changes in pH, glycogen, lactic acid, inorganic phosphorus, creatine phosphate, ATP, acid-soluble and Ba-acetate non-precipitable ribose, and TMA were studied. Though glycogen decreased and lactic acid increased throughout, no direct relation was observed, and measurable quantities of glycogen were present at the end of the experiment. Inorganic phosphorus increased slowly, and creatine phosphate and ATP decreased, the former at a more rapid rate. The Ba-acetate non-precipitable ribose and the ratio of non-precipitable ribose to acid-soluble ribose increased continuously. TMA values rose slowly, but were quite low even at the end of the experiment.**

## INTRODUCTION

Biochemical changes in fish muscle shortly after death have been described by numerous investigators, but comparatively few attempts have been made to correlate these changes with the course of rigor mortis. Reay and Shewan (1949) noted that the ultimate pH depended on the amount of lactic acid produced, and Reay *et al.* (1943) concluded that the ultimate pH for fish was definitely higher than that reported for mammals. Macleod and Simpson (1927), Macpherson (1932), Sharp (1934), Amano *et al.* (1953), Noguchi and Yamamoto (1955), and Frazer *et al.* (1961) studied changes in glycogen and lactic acid content in fish muscle post-mortem. Tomlinson *et al.* (1961) examined the changes in lactic acid content of fish muscle in relation to rigor mortis.

The organic phosphates are rapidly broken down after death, resulting in an increase in inorganic phosphorus (Cutting, 1953). Golovkin and Pershina (1957) reported that in fish muscle stored between 0 and 6°C, the acid-labile phosphorus decreased for 2–3 days post-mortem, then increased slightly for a day or so, and then again decreased. Other workers (Reay and Shewan, 1949; Noguchi and Yamamoto, 1955; Jones and Murray, 1961; Nakano, 1961a) have shown a continuous decrease in ATP content after death. Tomlinson *et al.* (1961) found that the acid-labile phosphorus content in unexercised trout and flounders was maintained for a few hours post-mortem, but if these fishes were exercised or exhausted before death (and also in sockeye salmon caught under commercial fishing conditions) the acid-labile phosphorus content decreased from time of death. Results similar to those of Tomlinson *et al.* (1961) were obtained by Nakano (1961b,c) and Partmann (1961), respectively working with unexercised gibels and with trout and carp.

Another change that takes place in fish after death is an increase in Ba-acetate non-precipitable ribose from 0 to 80% in 6 days (Shewan and Jones, 1957). Tarr (1954b), working with lingcod and rock cod, showed that the muscles of these fishes contained enzyme systems capable of degrading nucleic acids and certain nucleosides, nucleotides, and ribose-5-phosphate to free ribose. Tomlinson and Creelman (1960), working with lingcod, came to the conclusion that ribomononucleotides and not RNA were the main precursors of free ribose.

In the present work we investigated some of the biochemical changes that take place during the first few hours after death in 3 varieties of Indian fish. No data on these

lines are available for Indian fish. The fish were examined at regular intervals to obtain some idea of the manner in which the above-mentioned constituents changed from death until a few hours after the resolution of rigor.

## MATERIALS AND METHODS

Mullets (*Mugil dussumieri*, average weight 100 g), and Bombay ducks (*Harpodon nehereus*, average weight 50 g), caught close to the shore, were brought alive to the laboratory within 15 minutes of being caught, and killed by a blow on the head. Groupers (*Epinephalus malabaricus*, average weight 500 g) were held alive at the Taraporewala Aquarium for a few days until a sufficient number were collected, and then brought to the laboratory and killed as above. Four fish were taken for immediate analysis, and the rest were placed in crushed ice at an ambient temperature of about 2°C, as suggested by Jones and Murray (1957).

**Observation of rigor mortis.** The onset and duration of rigor mortis were observed by feeling the firmness and elasticity of the flesh as well as by holding the fish by the head and noting the rigidity of the body as described by Cutting (1939).

**Chemical methods of estimation.** At regular intervals of storage (1 hr for Bombay ducks, and 2 hr for mullets and groupers), three or four fishes were taken out of the ice; the muscle samples of all the fishes were pooled together, and half was extracted with 30% KOH (for glycogen) and the rest in ice-cold 5% TCA (for lactic acid, ATP, creatine phosphate, inorganic phosphorus, acid-soluble and Ba-acetate non-precipitable ribose and trimethylamine).

*Glycogen.* Glycogen was extracted with 30% KOH as described by Good *et al.* (1933). The extracted glycogen was estimated by Montgomery's method (1957) with phenol and sulfuric acid.

*Lactic acid.* The extract for color development was prepared as suggested by Van Slyke (1917), and the lactic acid was estimated by Barker and Summerson's method (1941).

*Inorganic phosphorus, creatine phosphate (CP), and ATP.* The procedure described by Griswold *et al.* (1951) was followed for extraction and determination of these substances. In this method CP was estimated as molybdate-acid-labile phosphorus and ATP as readily acid-hydrolyzable phosphorus.

*Trimethylamine (TMA).* TMA was estimated by Dyer's picrate method (1945), the yellow color produced on the addition of picric acid being estimated at 420 mμ.

*Acid-soluble ribose and Ba-acetate non-precipitable ribose.* Acid-soluble ribose was estimated from the TCA extract by Mejbaum's method (1939) with orcinol and ferric chloride. The Ba-acetate non-precipitable ribose was determined by treating the TCA extract with Ba-acetate in 80% ethanol at pH 8.2, the non-precipitable ribose being estimated in the filtrate.

## RESULTS AND DISCUSSION

Both mullets and groupers were fairly stiff one hour after death and passed into full rigor in about two hours. Bombay ducks, on the other hand, started stiffening within a few minutes of death, reaching full rigor within an hour. The mullets, Bombay ducks, and groupers respectively remained in full rigor for 9, 4, and 11 hours, and the resolution of rigor took 6, 5, and 6 hours more. The time between hauling and the end of rigor was 16, 10 and 18 hours, respectively, less than the times reported for other fish by Cutting (1939) and Tomlinson *et al.* (1961). Amlacher (1961) recently reviewed rigor mortis data for selected fishes. These fishes were asphyxiated, whereas in our experiments they were killed by stunning on the head. The duration of rigor mortis reported for fish stored at about 2°C was usually higher than the values found in our investigation. This may be due to the different families and habitat from which the fishes used in our experiments were selected.

The results are presented in Table 1. The pH of mullets, Bombay ducks, and groupers immediately after death was respectively 6.8, 6.7, and 6.9. The pH decreased slowly to about 6.4–6.5 when the muscle had passed into full rigor, and later, during its resolution, after which the pH rose slightly, to about 6.5–6.6. Reay *et al.* (1943) stated that the pH of freshly killed fish dropped from 7.0–7.3 to 6.2–6.6 at the onset of rigor mortis. Similar results were obtained by Cutting (1953): from about 7.0 to between 6.1 and 6.9 when rigor mortis had set in. Noguchi and Yamamoto (1955) stated that the pH of *Scomber japonicus* and *Pagrosomus major* respectively decreased in 9 hours from 6.6 and 6.3 immediately after death to 5.5 and 5.7.

Freshly killed mullets, Bombay ducks, and groupers respectively contained 219, 20,

Table 1. Post-mortem changes[a] in three fish (mg per 100 g of fresh muscle).

| Time post-mortem (hr) | State[b] | pH | Glyc | LA | ASR | Ba ANPR | *R* | IP | PCP | P of ATP | TMA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Mullet* | | | | | | | | | | | |
| 0 | | 6.8 | 219 | 273 | 127 | 2.3 | 1.82 | 106 | 15.8 | 30.1 | 0.03 |
| 1 | 2 | 6.7 | 167 | 296 | 127 | 2.4 | 1.91 | 110 | 11.4 | 25.2 | 0.04 |
| 2 | 1 | 6.7 | 130 | 319 | 127 | 2.6 | 2.04 | 110 | 9.0 | 20.6 | 0.04 |
| 4 | 1 | 6.7 | 108 | 357 | 126 | 3.4 | 2.68 | 115 | 7.3 | 15.7 | 0.04 |
| 6 | 1 | 6.6 | 97 | 387 | 125 | 4.2 | 3.37 | 108 | 5.8 | 13.9 | 0.05 |
| 8 | 1 | 6.6 | 88 | 386 | 118 | 5.0 | 4.19 | 112 | 4.9 | 12.1 | 0.07 |
| 10 | 1 | 6.5 | 74 | 414 | 116 | 6.3 | 5.46 | 114 | 3.7 | 10.2 | 0.08 |
| 12 | 3 | 6.5 | 63 | 425 | 115 | 7.7 | 6.68 | 116 | 2.4 | 8.2 | 0.09 |
| 14 | 3 | 6.5 | 50 | 435 | 114 | 9.1 | 7.94 | 119 | 1.2 | 9.0 | 0.11 |
| 16 | 3 | 6.6 | 39 | 436 | 113 | 10.8 | 9.56 | 118 | 0.5 | 7.9 | 0.15 |
| 18 | 4 | 6.6 | 30 | 442 | 112 | 12.4 | 11.09 | 120 | … | 7.6 | 0.17 |
| 20 | 4 | 6.6 | 26 | 448 | 111 | 14.4 | 12.88 | 123 | … | 7.0 | 0.20 |
| *Bombay ducks* | | | | | | | | | | | |
| 0 | | 6.7 | 20 | 20 | 99 | 1.9 | 1.97 | 60 | 6.7 | 38.2 | 0.14 |
| 1 | 1 | 6.7 | 13 | 34 | 96 | 2.1 | 2.18 | 60 | 4.8 | 35.6 | 0.15 |
| 2 | 1 | 6.7 | 7 | 41 | 96 | 2.0 | 2.09 | 60 | 3.1 | 33.4 | 0.19 |
| 3 | 1 | 6.7 | 7 | 46 | 95 | 2.3 | 2.42 | 61 | 1.2 | 30.1 | 0.19 |
| 4 | 1 | 6.6 | 6 | 51 | 93 | 2.6 | 2.83 | 61 | 0.3 | 28.2 | 0.19 |
| 5 | 1 | 6.6 | 6 | 53 | 93 | 2.9 | 3.12 | 61 | 0.3 | 27.2 | 0.19 |
| 6 | 3 | 6.5 | 5 | 61 | 91 | 3.3 | 3.57 | 61 | 0.1 | 24.8 | 0.19 |
| 7 | 3 | 6.5 | 5 | 61 | 90 | 3.6 | 4.06 | 62 | … | 20.8 | 0.22 |
| 8 | 3 | 6.5 | 4 | 61 | 90 | 4.3 | 4.82 | 63 | … | 17.2 | 0.24 |
| 9 | 3 | 6.5 | 4 | 61 | 89 | 5.3 | 5.90 | 64 | … | 14.0 | 0.24 |
| 10 | 3 | 6.5 | 4 | 62 | 89 | 6.3 | 7.11 | 65 | … | 11.2 | 0.27 |
| 11 | 4 | 6.5 | 3 | 61 | 88 | 7.3 | 8.32 | 67 | … | 7.7 | 0.32 |
| 12 | 4 | 6.6 | 3 | 58 | 86 | 8.2 | 9.54 | 68 | … | 4.9 | 0.34 |
| *Groupers* | | | | | | | | | | | |
| 0 | | 6.9 | 393 | 168 | 223 | 3.6 | 1.63 | 125 | 4.1 | 17.9 | 0.04 |
| 1 | 2 | 6.9 | 308 | 204 | 224 | 4.2 | 1.89 | 128 | 3.3 | 15.5 | 0.05 |
| 2 | 1 | 6.8 | 261 | 253 | 221 | 4.7 | 2.11 | 130 | 2.1 | 13.2 | 0.05 |
| 4 | 1 | 6.7 | 234 | 304 | 218 | 5.5 | 2.54 | 132 | 1.3 | 11.9 | 0.05 |
| 6 | 1 | 6.7 | 202 | 315 | 216 | 8.4 | 3.89 | 134 | 0.6 | 9.6 | 0.06 |
| 8 | 1 | 6.6 | 181 | 367 | 212 | 10.9 | 5.13 | 135 | 0.3 | 7.8 | 0.07 |
| 10 | 1 | 6.5 | 165 | 408 | 208 | 14.0 | 6.74 | 137 | 0.1 | 5.4 | 0.08 |
| 12 | 1 | 6.5 | 149 | 438 | 204 | 18.1 | 8.89 | 137 | 0.1 | 4.9 | 0.10 |
| 14 | 3 | 6.4 | 134 | 466 | 200 | 22.8 | 11.42 | 137 | | 3.7 | 0.11 |
| 16 | 3 | 6.4 | 119 | 489 | 196 | 27.8 | 14.16 | 140 | | 3.0 | 0.13 |
| 18 | 3 | 6.4 | 105 | 504 | 192 | 32.4 | 16.85 | 141 | … | 3.1 | 0.14 |
| 20 | 4 | 6.5 | 95 | 510 | 194 | 37.6 | 19.42 | 143 | … | 3.1 | 0.16 |

[a] Glyc, glycogen; LA, lactic acid; ASR, acid-soluble ribose; BaANPR, barium-acetate non-precipitable ribose; R, $\frac{\text{non-precipitable ribose}}{\text{acid-soluble ribose}} \times 100$; IP, inorganic phosphorus; PCP, phosphorus of creatine phosphate.

[b] 1, full rigor; 2, almost full rigor; 3, softening; 4, soft.

and 393 mg of glycogen per 100 g of fish muscle. Glycogen content decreased rapidly during the first 2 hr after death, by which time full rigor had set in, and more gradually later on. Measurable quantities of glycogen were present even after rigor had been completely resolved. Glycogen content was lower in Bombay ducks than in mullets and groupers. This was probably not the result of violent struggling or exhaustion, since the lactic acid content of Bombay ducks was also low (20 mg/100 g of muscle immediately after death). Mullets and groupers, on the other hand, contained appreciable amounts of lactic acid: 273 and 168 mg/100 g. The lactic acid content of all the three species increased throughout the period when the fish was in full rigor and during its resolution. By the time rigor was fully resolved, the lactic acid content had risen almost to its maximum, and further increase was very slow. In Bombay ducks, the lactic acid content decreased slightly after rigor had been resolved.

Bate-Smith and Bendall (1949), working on rabbits, rats, and bullocks, and Benson (1928), working on fish, reported that glycogen and lactic acid contents determined the time of onset and resolution of rigor mortis. Bate-Smith and Bendall (1949) have stated that the glycogen reserve cannot delay rigor mortis beyond definite limits. As pH decreases (through the formation of lactic acid), enzymes of the glycolytic system become progressively inactive. Sharp (1934) reported that glycogenolysis, which sets in rapidly after death, was arrested in fish that had a high initial glycogen content (0.6–0.85%). In fish with a high glycogen content at time of death, the equilibrium values for lactic acid were 0.4–0.45%. In our experiments it was noticed that in mullets and groupers, the total decrease in glycogen, of 193 and 298 mg, respectively, was roughly balanced by an increase in lactic acid of 175 and 342 mg, respectively. In Bombay ducks, however, the lactic acid increase (about 40 mg) was much greater than the total glycogen decrease (17 mg). Tarr (1954a) stated that, frequently, no relation between glycogen and lactic acid was observed, and that it was possible that the latter arose through the degradation of some other carbohydrate. Frazer *et al.* (1961) also found higher values for lactic acid than glycogen in cod muscle stored at both 9 and 25°C. However, at 0°C the decrease in glycogen and increase in lactic acid were roughly of the same order.

Freshly killed mullets, Bombay ducks, and groupers contained 127, 99, and 168 mg of acid-soluble ribose per 100 g of muscle. These values decreased gradually on storage in ice, to about 86–88% of their original value (Table 1). The Ba-acetate non-precipitable ribose was present in rather small amounts in freshly killed fish, and this value increased throughout storage, as did the ratio of non-precipitable ribose to acid-soluble ribose. The $R$ value (non-precipitable ribose/acid-soluble ribose) × 100, was respectively 1.82, 1.97, and 1.63 for freshly killed mullets, Bombay ducks, and groupers, and these rose gradually to 9.56, 7.11, and 16.85 by the time rigor had been resolved.

Jones and Murray (1957) reported that the total ribose content of codling decreased on storage. Tarr and Bissett (1954) reported that sea fish usually contained 0.1–0.3% of total ribose, probably combined in the form of nucleic acids in living fish. On storage at 0°C, free ribose developed in concentrations varying from 0.02 to 0.08% in some varieties, whereas in others (e.g., salmon and halibut), little or no free ribose was formed on storage. Tarr (1954a) reported the presence of free D-ribose in quantities exceeding 800 µg/g in the muscles of some fish post-mortem. Shewan and Jones (1957) observed that the ratio of Ba-acetate non-precipitable ribose to total ribose rose steadily in freshly killed cod in a straight line from 0 to 80% in 6 days. As can be seen in Table 1, increase in the ratio $R$ and non-precipitable ribose throughout the storage period indicated a decrease in phosphorylated compounds that could be precipitated by barium acetate (in 80% ethanol at pH 8.2).

In all three fish (Table 1) the inorganic phosphorus content gradually rose during storage. The phosphorus of creatine phosphate (CP-P) decreased. This compound was present in much greater amounts in mullets than in Bombay ducks and groupers.

Another difference observed was that while the creatine phosphate (CP) had been broken down completely in Bombay ducks and groupers while these fishes were still in full rigor, it persisted in mullets until rigor was completely resolved. The creatine phosphate was broken down at a faster rate in the initial stages, and more gradually later on. Groupers contained the least amount of ATP. In Bombay ducks the ATP decreased at an almost regular rate throughout, but in mullets and groupers it decreased more rapidly in the beginning and at a somewhat slower rate during the resolution of rigor. In all the three species, however, a small amount of ATP was present till the end of the experiment.

Tomlinson *et al.* (1961) found that rigor in unexercised trout became established over a decreasing range of concentration of acid-labile phosphorus, similar to the observations of Bate-Smith and Bendall (1947) and Bendall (1951) in rabbit psoas muscle, whereas in exhausted trout and other species rigor was established over a somewhat lower range. Full rigor was established only after the acid-labile phosphorus had decreased to a very low value. Jones and Murray (1961) stated that exhausted fish contained only 0.27 μmole/g, but even these fish did not go into rigor for some time. They further reported that codling, whether rested or exhausted, passed into rigor at an ATP level of about 0.20–0.23 μmole/g, and full rigor was established at a value of about 0.18 μmole/g, which represented only 5% of the level in the rested living animal. In our experiments, groupers passed into full rigor when the acid-labile phosphorus content was about 13 mg/100 g of fish muscle, only slightly higher than the values reported by Tomlinson *et al.* (1961) and Jones and Murray (1961). In mullets and Bombay ducks however, full rigor became established while much higher concentrations of acid-labile phosphorus were present in the muscle.

Like glycogen and lactic acid, ATP and creatine phosphate also play important roles in determining the onset and duration of rigor mortis. Bate-Smith and Bendall (1947), working with rabbits, found that the disappearance of ATP was directly related to the stiffening observed in rigor mortis, and Bendall (1951) and Lawrie (1953), respectively working with rabbits and horses, reported that the quantity of creatine phosphate and glycogen at death determined the interval between death and the onset of rigor mortis. Reay and Shewan (1949) stated that the organic phosphates are rapidly broken down after death, the total inorganic phosphorus content reaching values ranging from 0.10–0.15%. According to Bate-Smith (private communication to Reay *et al.*, 1943), phosphocreatine, hexose monophosphate, and ATP were broken down within a few hours of death, to inorganic orthophosphate in amounts estimated to be 0.16% for haddock and 0.11% for skate; but in more recent experiments, Jones and Murray (1957, 1961) and Tarr and Leroux (1962) demonstrated the presence of ADP, AMP, and IMP in fish muscle post-mortem. The latter authors have also shown the presence of hexose phosphates and free glucose in fish muscle. Fujimaki and Kojo (1953) stated that true inorganic phosphorus increased slightly in fish killed instantaneously, but in fish killed after struggling, this value increased to about twice the original within 4 hr of death. ATP disappeared within the same period. Noguchi and Yamamoto (1955) stated that the ATP content of two fish analyzed by them decreased from 41.7 and 41.3 mg/100 g at death to 0.0 mg within 9 hr. Jones and Murray (1961) showed that there was no maintenance of a constant ATP level in codling for several hours post-mortem at 0°C, while Tomlinson *et al.* (1961) reported maintenance for a few hours in rested trout and flounders. However, maintenance was not observed either in these fishes if exercised or exhausted before killing or in sockeye salmon caught under commercial fishing conditions. Nakano (1961b,c) and Partmann (1961), respectively working on unexercised gibels and on trout and carp, independently reported similar results.

The respective TMA contents immediately after death were 0.03, 0.14, and 0.04 mg/100 g for mullets, Bombay ducks, and groupers. These values rose slowly during storage, and by the time rigor had been resolved had reached 0.15, 0.27, and 0.14

mg/100 g. It was noted that the TMA content began to increase very shortly after death, and rose gradually throughout the storage period, though even at the end of the experiment the values were quite low.

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# Polarographic Studies on Storage of Meats. XXII. Influence of Proteolytic Enzymes on the Polarographic Wave of Beef Protein Solutions

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**SUMMARY**

**Investigation was made of the influence of pepsin, irradiated pepsin, trypsin, and papain on the polarographic wave of native and denatured beef protein solutions. Beef protein solutions incubated with pepsin or irradiated pepsin show a characteristic change in the second waves of their polarograms. This change is quite similar to that observed with a water extract from stored irradiated beef. Pepsin subjected to low-level irradiation causes a more pronounced polarographic change in beef protein solutions than does unirradiated pepsin, and has a greater proteolytic action. Beef protein solutions incubated with trypsin or papain show less significant polarographic changes than those incubated with pepsin.**

## INTRODUCTION

Relations between polarographic waves of protein solutions and proteolytic enzymes have been reported (Brdicka, 1937; Carrathers, 1947; Hamamoto, 1942; Jühling *et al.,* 1939). Hata and Matsushita (1952) reported a polarographic study of the hydrolytic process of digestion of crystalline ovalbumin by purified pepsin and trypsin, and suggested that the difference between changes in polarographic waves caused by papain and trypsin might be due to the difference between the proteolytic processes of the enzymes.

The shape of the polarographic wave of meat extracts varies considerably with the kind of meat and method of extraction (Hata, 1951; Ito, 1959; Kikuchi *et al.*, 1959; Tachi, 1954; Tropp *et al.*, 1939). Factors influencing the polarographic waves of extracts of unirradiated meats during storage (Obara and Ogasawara, 1959a,b, 1960a,b,c), and effects of $\gamma$-ray irradiation on beef (Obara and Ogasawara, 1960d,e,f,g) have been reported from this laboratory. When meats irradiated with $\gamma$-rays are stored, there is a remarkable change in the shape of the polarographic wave of their extracts. For example, a water extract from beef irradiated at about $200 \times 10^4$ r had a pronounced variation in the shape of its polarogram, and the maximum of the second wave was much higher than that of the first wave (Obara and Ogasawara, 1960h). The causes of these observed changes are not known.

The present paper reports the influence of pepsin, irradiated pepsin, trypsin, and papain on the polarographic wave of native and denatured beef protein solutions.

## EXPERIMENTAL METHODS

**Preparation of samples.** *Enzymes.* A commercial crystalline pepsin (Merck) was dissolved in 20% (v/v) ethanol and adjusted to pH 4.0. The solution was then filtered. The filtrate was brought to pH 1.8, and was inoculated with crystals of pepsin. After standing at 20°C for three days, recrystallized pepsin was obtained (Northrop, 1946). The pepsin thus obtained was used.

Crystalline trypsin or papain (Merck) was dissolved in water. The solution was then filtered. The filtrate thus obtained was used.

*Water extract from beef.* To 1 g of the beef was added 50 ml of water, and the mixture was ground. After 30 min at 0°C, it was then centrifuged 10 min at 6000 rpm. The supernatant (360 $\mu g$ protein per ml) was used as a native water

extract from the beef (Obara and Ogasawara, 1960d,h).

Ten ml of 1*N* NaOH solution was added to 40 ml of the native water extract, and the mixture was kept for 10 min at 30°C. After adjusting to the proper pH (pH 2.0 for pepsin hydrolysis, pH 8.0 for that of trypsin, and pH 7.2 for that of papain), each solution was made to 80 ml with a buffer of pH 2.0, 8.0, or 7.2. These solutions were used as denatured water extracts of beef.

*Casein solution.* Using commercial casein (Merck), 0.6% casein solutions, adjusted to pH 2.0, 8.0, or 7.2 were prepared with each buffer.

**Assay of enzyme activity.** After 3 min at 30°C, 5 ml of a substrate solution, e.g., a water extract from the beef or the 0.6% casein solution, was mixed with 1 ml of the enzyme solution, and the mixture was kept for 0–180 min at 30°C. The final mixture contained 0.5% casein or 300 $\mu g$ of beef protein per ml. The optimum pH value of the reacting mixture was 2.0 for pepsin, 8.0 for trypsin, and 7.2 for papain hydrolysis. In the case of the estimation of the papain activity, $4.66 \times 10^{-3} M$ of KCN, used as an activator, was contained in the reacting solution. The reactions were stopped by adding 5 ml of 0.44*M* trichloroacetic acid, and the mixture was filtered. The amount of tyrosine liberated in the filtrate was determined spectrophotometrically with Folin reagents (Akabori, 1956). From comparisons to standard tyrosin solutions, each enzyme activity was calculated.

**$Co^{60}$ gamma-ray irradiation.** One percent pepsin solution was sealed tightly in ampoules. It was exposed to gamma-ray of $Co^{60}$ for 20 hr at 12°C at various distances from the source. A 1000-curie $Co^{60}$ source was used. Each sample was irradiated with the following total doses: 0, $84 \times 10^4$, $240 \times 10^4$, and $800 \times 10^4$ r. Immediately after irradiation, each assay was carried out.

**Estimation of polarographic wave of proteins.** A definite volume of each protein solution was placed in a buffer with the following composition: $10^{-3}M$ $CoCl_2$, 0.1*M* $NH_4Cl$, and 0.2*M* $NH_4OH$. The protein wave of each sample was estimated under the conditions of 25°C; sensitivity = 0.08 $\mu A/mm$; $m = 1.107$ mg/sec; and $t = 4.2$ sec/drop. The final protein concentration in the solution used for estimation of the protein wave of the water extract from beef was 56 $\mu g$ per ml of the buffer solution. The wave height was estimated by a method of Hata (1951) and Tachi (1954).

**Assay of protein content.** The protein content of each sample was determined by measuring the absorption at 750 m$\mu$ after reaction with Cu-Folin reagent (Lowry *et al.*, 1951).

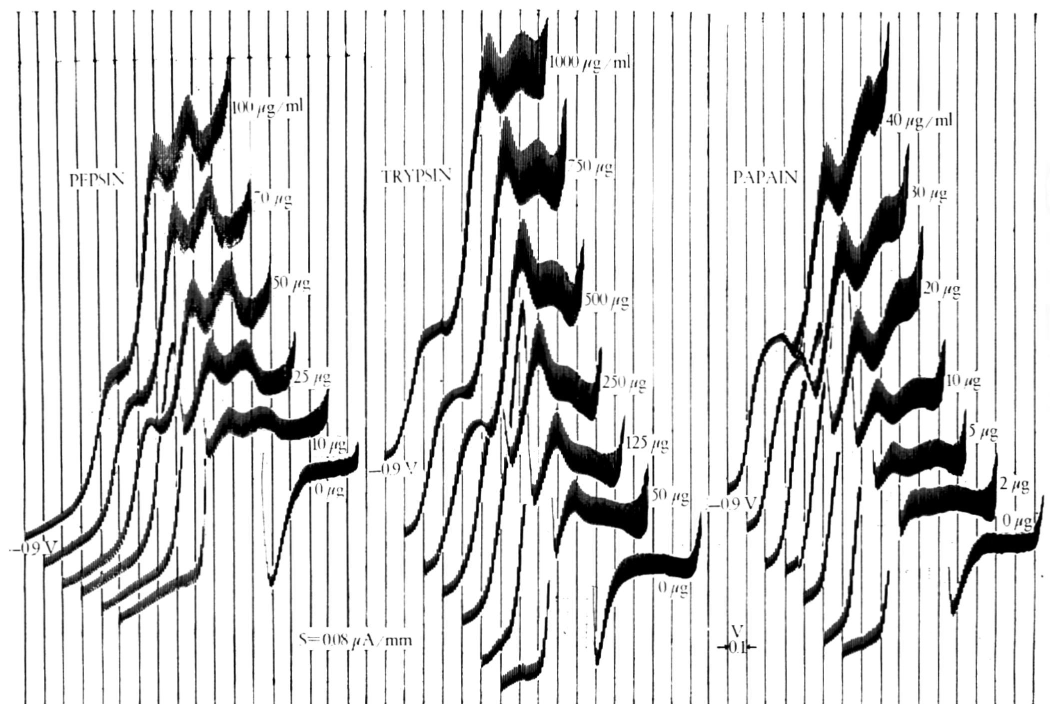


Fig. 1. Protein waves of pepsin, trypsin, and papain in various concentrations of protein expressed with $\mu g$ per ml of the buffer solution.

## RESULTS

**Polarographic wave of proteolytic enzymes.** Fig. 1 shows polarographic waves of pepsin, trypsin, and papain, measured in buffers of the same composition but containing the enzymes at various concentrations. Polarographic waves of pepsin, trypsin, and papain differed greatly from each other, and changed with concentrations. The crossing points of these protein waves, expressed as protein per ml of the buffer solution, were located at about 25 $\mu$g for pepsin, about 1000 $\mu$g for trypsin, and about 5 $\mu$g for papain. At higher protein concentrations the heights of the second waves were greater than those of the first waves. However, the amounts of enzymes added to beef solutions in the subsequent study were very minute, so that any effect of the polarographic waves of these enzymes themselves on the waves of beef solutions are considered negligible.

**Ultraviolet absorption spectra and proteolytic activity of irradiated pepsin.** The ultraviolet absorption spectra of the pepsin proteins irradiated with doses of 0, 84 $\times$ 10⁴, 240 $\times$ 10⁴, or 800 $\times$ 10⁴ r are shown in Fig. 8. The maximum was located at about 270 m$\mu$, and there was an increase in the height of the maximum with increasing doses of $\gamma$-ray.

Irradiated pepsin was added to a casein solution or the water extract of the beef. The mixture was kept at pH 2.0 for 0–180 min at 30°C, and

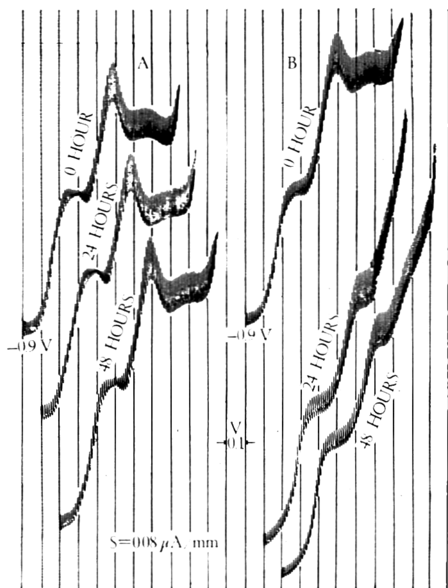


Fig. 2. Changes in protein waves of the water extracts of beef kept at pH 2.0 for 0–48 hours at 30°C without pepsin (A) or with pepsin (B) of 1.8 $\mu$g per ml of the reacting mixture.

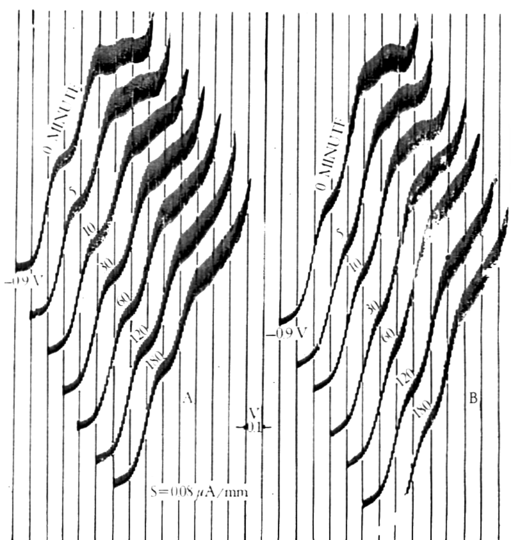


Fig. 3. Changes in protein waves of native (A) and denatured (B) water extracts of beef kept at pH 2.0 for 0–180 minutes at 30°C with pepsin of 5 $\mu$g per ml of the reacting mixture.

the activity of the pepsin was estimated. The results are shown in Fig. 9. Pepsin irradiated at 84 $\times$ 10⁴ r had a greater activity than unirradiated pepsin, whereas pepsin irradiated at 240 $\times$ 10⁴ r had a lower activity than unirradiated pepsin. In the case of the pepsin irradiated at 800 $\times$ 10⁴ r there was a remarkable decrease in activity. Thus the activity of pepsin was increased by $\gamma$-ray irradiation at a suitable dose level, but decreased at higher levels.

**Polarographic waves of beef solution incubated with pepsin or irradiated pepsin.** *Polarographic waves of beef solution.* The polarographic wave of the water extract adjusted to pH 2.0 was measured after 0, 24, and 48 hr at 30°C. The results are shown in Fig. 2-A. There was no remarkable variation in the shapes of the waves, and this confirmed our previous results (Ohara and Ogasawara, 1959b).

*Effect of unirradiated pepsin.* When a minute amount of pepsin was added to a water extract, kept under conditions identical to those just above (Tachi, 1954), there was a pronounced increase in the height of the second wave compared to that of the first wave (Fig. 2-B). Moreover, it was very difficult to distinguish the location of the maximum of the second wave. It is interesting to note that similar changes were observed with the irradiated beef solution (Obara and Ogasawara, 1960b).

Fig. 3-A shows the changes with time of the polarographic waves of beef solution incubated

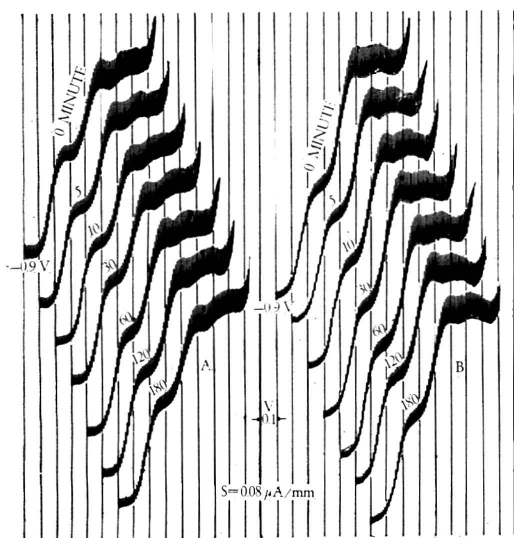


Fig. 4. Changes in protein waves of native (A) and denatured (B) water extracts of beef kept at pH 8.0 for 0–180 minutes at 30°C with trypsin of 5 μg per ml of the reacting mixture.

with pepsin. After 30 min at 30°C, the maximum of the second wave was difficult to locate and there was a remarkable change in its shape.

The same experiment was carried out with denatured beef solution (Fig. 3-B). The change in the polarogram of denatured beef solution was more prominent than that of undenatured beef solution.

The amounts of proteins in the native or denatured water extracts hydrolyzed by pepsin under identical condition (as in Fig. 3) were measured (Fig. 7). There was a remarkable increase in the rate of hydrolysis in the denatured water extract compared to that in the native one.

*Effect of irradiated pepsin.* Fig. 10 shows changes in polarographic waves of water extracts of beef treated with irradiated pepsin. Pepsin irradiated with doses of less than $240 \times 10^4$ r showed a change in the shapes of the polarographic waves of the water extracts with increasing periods of incubation with enzyme, and the height of the second wave was above that of the first wave. But there were no pronounced differences in the shape of each protein wave at a given period of incubation due to changes in γ-ray dosage of the pepsin. In the case of pepsin irradiated with high-level doses like $800 \times 10^4$ r, there was no particular change in the shape of the protein wave of the water extract from the beef incubated with enzyme up to 180 min at 30°C.

**Polarographic waves of beef solution incubated with trypsin or papain.** Under conditions identical with the experiments with pepsin mentioned above, the effects of trypsin and papain were studied. Very small amounts of trypsin or papain were added to the native or denatured water extract from the beef. The enzyme reactions were carried out for 0–180 min at 30°C in mixtures adjusted to pH 8.0 for trypsin or to pH 7.2 for papain. In the papain reaction, KCN solution was added as an activator. The results are shown in Figs. 4 and 5.

When trypsin was added to native or denatured water extract from the beef, there was no remarkable change in shape of the protein wave of the water extract up to 180 min at 30°C (Fig. 4). This point was greatly different from that in the case of pepsin.

When papain was added to native or denatured water extract, there was a prominent variation in the shape of the protein wave after 10 min at 30°C, and the height of the second wave was considerably greater than that of the first wave. The second wave in this case gave very indistinct maxima (Fig. 5).

We examined the influence of various KCN concentrations on the protein wave from the water extract without papain, and the effect of time of reaction on the protein wave from the water extract with KCN of a constant concentration but without papain. These results are shown in Fig. 6. From these it was clear that the shape of the protein wave of the water extract was greatly influenced by KCN. In the case of the addition of KCN of constant concentration, the shape of

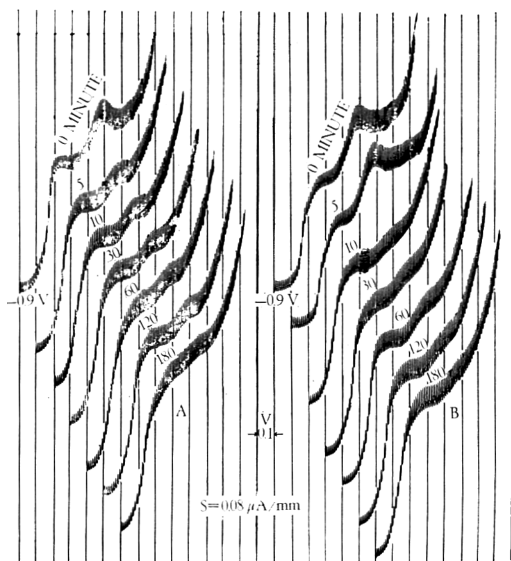


Fig. 5. Changes in protein waves of native (A) and denatured (B) water extracts of beef kept at pH 7.2 for 0–180 minutes at 30°C with papain of 5 μg per ml and KCN of $4.66 \times 10^{-3}$M in the reacting mixture.

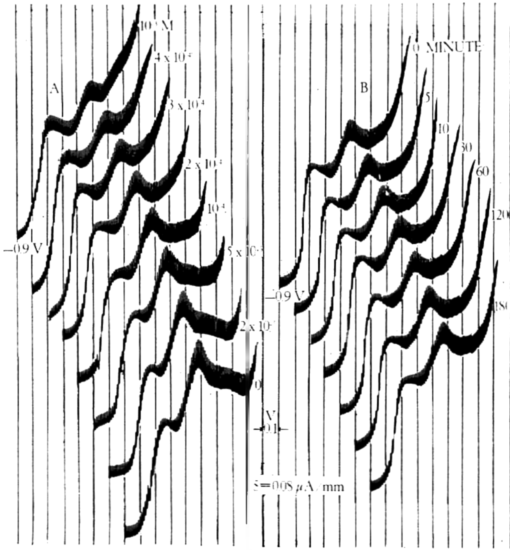


Fig. 6. A: Change in the protein wave of the native water extract of beef kept at pH 7.2 for 5 minutes at 30°C with KCN of $0-10^{-3}$M but without papain. B: Change in the protein wave of the native water extract of beef kept at pH 7.2 for 0–180 minutes at 30°C with KCN of $10^{-4}$M but without papain.

the protein wave was little affected by varying times from 10 to 180 min. Consequently, it seemed that the greater part of the change in the shape of the protein wave in Fig. 5 occurred from the effect of KCN itself. But comparing both protein waves after 0 min reaction and 5 or 10 min reaction at 30°C, their shapes were obviously different. Therefore, a portion of the change in the shape of the protein wave was brought by the papain reaction.

As shown in Fig. 7, the amounts of hydrolyzed proteins were greater in denatured water extracts than in the native ones.

## DISCUSSION

Proteins denatured by heat are more susceptible to proteolysis than native proteins

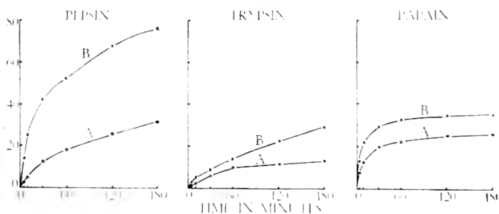

Fig. 7. Amount of tyrosine liberated from proteins in native (A) and denatured (B) water extracts of beef kept at pH 2.0, pH 8.0, or pH 7.2 for 0–180 minutes at 30°C with pepsin, trypsin, or papain of 5 $\mu$g per ml of the reacting mixture.

(Haurowitz *et al.*, 1945; Lineweaver and Hoover, 1940). Hata and Matsushita (1952) observed that ovalbumin in the native state was hardly hydrolyzed by either papain or trypsin, and the polarographic waves were hardly changed. In the present study, changes in the polarographic waves of denatured beef solution incubated with pepsin were more pronounced than those of native beef solution incubated with pepsin; this can be attributed to the higher degree of hydrolysis in the denatured one.

Enzymes in cells or cell particulates may in some instances show an increase in activity following irradiation. This phenomenon is believed to be due to a liberation of the enzyme from inactive complexes within the cell. The phenomenon has been observed following X-irradiation of invertase and phosphoglucomutase in chloroplasts (Sissakian, 1955) and alkaline phosphatase in yeast cells (Meissel, 1955). This can also be attributed to changes from inactive forms of enzymes to active states.

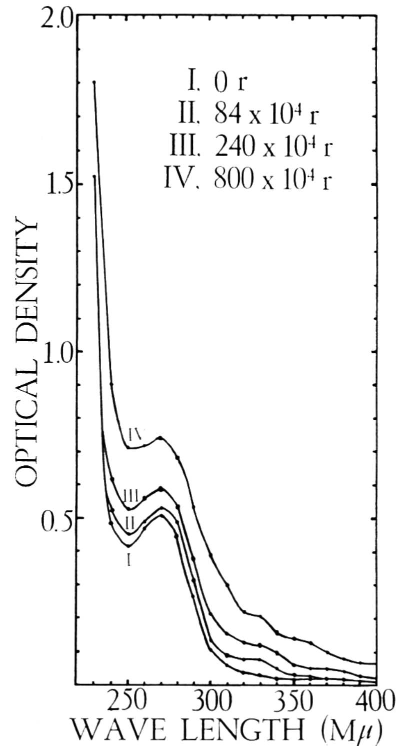


Fig. 8. Ultraviolet absorption spectra of irradiated pepsin of 500 $\mu$g per ml.

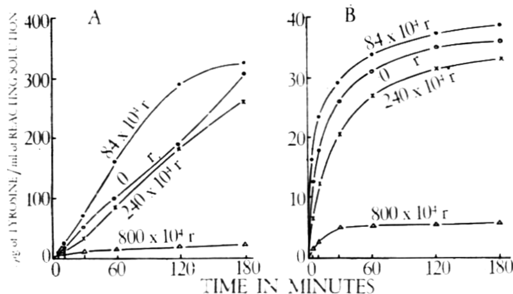


Fig. 9. Amount of tyrosine liberated from 0.5% casein solution (A) or from proteins in the native water extract of beef (B) kept at pH 2.0 for 0–180 minutes at 30°C with irradiated pepsin of 5 $\mu g$ per ml of the reacting mixture.

When beef samples irradiated with $148 \times 10^4$ to $200 \times 10^4$ r were stored at 20°C, there was a pronounced and characteristic change in the polarographic wave of their water extract (Tachi, 1954). This change is quite similar to that observed with beef protein solutions incubated with pepsin, particularly irradiated pepsin. Therefore, the polarographic change with the irradiated stored beef (Obara and Ogasawara, 1960b) may be partly explained by the increased activity of proteolytic enzymes in beef that are accelerated by the $\gamma$-ray irradiation of about $148 \times 10^4$ to $200 \times 10^4$ r. This suggests that the irradiation activates the natural proteolytic enzymes in beef, resulting in more rapid proteolysis. However, this hypothesis is not supported by the finding that beef protein solutions incubated with papain, an enzyme similar to cathepsins, which are the natural proteolytic enzymes in beef, showed less significant polarographic changes than similar solutions incubated with pepsin.

The identification of protein components in beef solution responsible for the characteristic polarographic changes is now under investigation.

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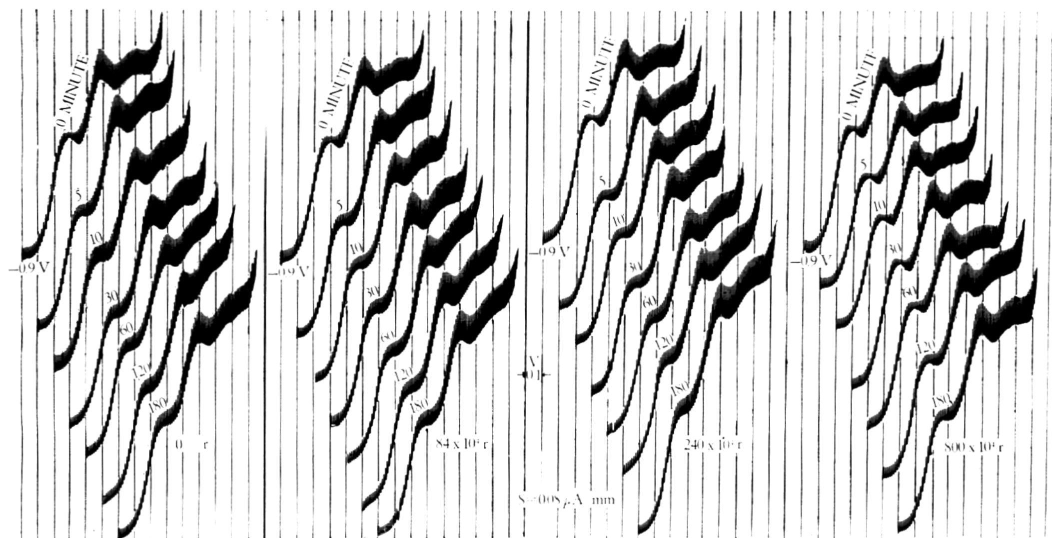


Fig. 10. Change in the protein wave of the native water extract of beef kept at pH 2.0 for 0–180 minutes at 30°C with irradiated pepsin of 5 $\mu g$ per ml of the reacting mixture.

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# Post-Mortem Changes in Chilled and Frozen Muscle

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**SUMMARY**

**The conception of Huxley concerning the structural basis of muscular contraction is universally accepted. It shows a close correlation between ATP and the fibrillar proteins, also existing during the first phase of the post-mortem changes. Rigor development has been followed in whole fish and isolated beef muscles by measuring the torsion elasticity. Often there are great individual deviations in rigor development within a single species. Generally, at corresponding temperatures, rigor development lasts longer in mirror carp than in the gastrocnemius of beef; rosefish needs a longer time to reach maximum rigor than cod. Evidently, the rapid phase of ATP breakdown and increasing rigidity of muscles is initiated by inactivation of the Marsh-Bendall factor in the post-mortem period. Normally, contraction occurs when ATP is added to fiber fragments of aged meat. This implies that the actomyosin complex formed during rigor development becomes dissociated, or at least may become dissociated easily, in aged meat, and that tenderness changes in the aging period are correlated to this process. ATP breakdown in fish muscle is highly activated by freezing and thawing ("biochemischer Verletzungseffekt") and seems to be caused by inactivation of the relaxing factor. Fish (whole fish or fillets) frozen under normal commercial conditions immediately after death show an insignificant degree of thaw contracture. In cod and rosefish no significant difference has been found in the extractability of the actomyosin fraction, if the time passing between death and freezing was considered. In frozen muscle tissue stored below −18°C, ATPase activity and contractability decrease very slowly. This shows that the actin and myosin filaments are not subject to great structural changes by freezing and thawing. In the freezer-burn area of muscle tissue the structure proteins lose the ability to contract on ATP addition more and more with increasing storage time; finally, even the plasticizing effect of ATP on the fibrillar proteins disappears, the fibrils scarcely change their original orientation.**

In dealing with meat we normally think of the skeletal muscles of higher vertebrates like warm-blooded animals and fish. The typical skeletal muscle is enclosed in a sheath called the perimysium and permeated by the connective tissue, often containing fat deposits. The main portion of the muscles consists of fibers with a diameter of 10 to 100 microns. Observations in the light microscope show that the muscle fibers are cross-striated. Electron micrographs reveal that the cross-striations are associated with the subunits of the fibers, the myofibrils (Fig. 1). The sarcoplasm between the myofibrils contains a highly organized tubular or vesicular system: the sarcoplasmic reticulum and, in close connection to it, the mitochondria and microsomes, which apparently produce and release the relaxing factor (Marsh-Bendall factor) (Nagai *et al.*, 1960).

The pattern of the cross-striations is repeated regularly every few microns. The unit is called a sarcomere. The boundaries of the sarcomere are the two narrow Z-lines. In the middle is a dense anisotropic band, the Q-band. Here is localized myosin, one of the two main structural proteins of muscle fibers. In the middle of the Q-band we often find a less dense zone, called the H-zone. Alternating along the fibril with the Q-bands are the isotropic I-bands. During



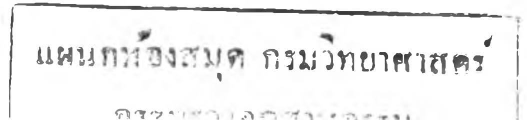

Fig. 1. Muscle fiber of the diaphragma of a calf aged 1 day at +4°C, fixed for 1 hr in buffered 2% $OsO_4$. Embedding: 90% butyl methacrylate and 10% methyl methacrylate, polymerization at 60°C. Magnification × 10,000.

stretch and contraction of the fibrils the Q-bands remain at approximately constant length whereas the I-bands change in length. Of special importance is the fact that the H-zones are longer in stretched muscle than in contracted muscle. From histological and biochemical evidence, Huxley developed his "sliding filament model" of striated muscle with two sets of protein-filaments (Huxley, 1956) (Fig. 2).

The thicker myosin filaments of the Q-band do not change their length during contraction and extension. In the resting state the thin actin filaments, starting at the Z-line, terminate before they reach the center of the Q-band. The presence of the active relaxing factor besides ATP inhibits the contraction and is the reason that actin and myosin remain dissociated. Excitation causes depolarization of the membrane, and probably the release of $Ca^{++}$ and in consequence, inactivation of the relaxing factor (Brecht and Pauschinger, 1961). Thereupon, the interaction of ATP, myosin, and actin will occur. It leads to the formation of the highly active actomyosin-ATPase and to contraction, which means—according to our model—that the two sets of filaments slide past each other. With this association of actin and myosin a decrease in the length of the I- and H-zones is effected (Huxley and Hanson, 1954; Huxley, 1956).

The existence of two overlapping sets of filaments in muscle, which at first was doubted by other workers, was later demonstrated very clearly in excellent electron micrographs by Huxley (1957) and recently by Stenger and Spiro (1961).

Hence, the sliding model-system, which has been confirmed by biochemical measurements, explains the changes in the visible band-pattern of muscle and its changes in length during contraction and stretch. Certainly, this model will be completed and improved in the near future to fulfill the conditions required by some new findings of Carlsen *et al.* (1961).

The following will try to show that some of the important post-mortem muscle changes show a close correlation to the interactions between ATP, actin, and myosin that take place in living muscle during contraction and extension.

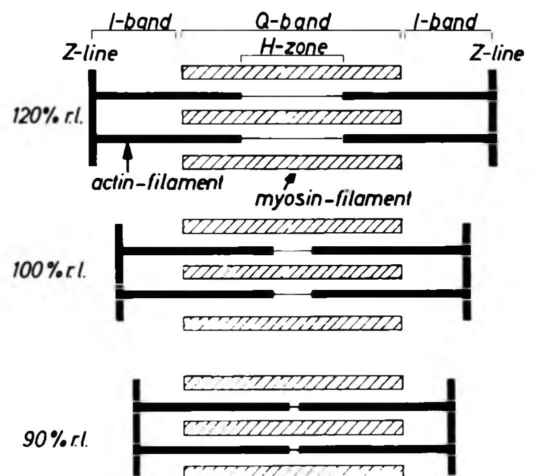


Fig. 2. Sliding filament model of Huxley (1956); localization of actin- and myosin-filaments at varying muscle length. r.l. = rest length.

Before this problem is discussed it may be useful to complete the outlined conception of Huxley by some statements from recent research work: In the presence of ATP the active relaxing factor in resting muscle fibers inhibits the enzymatic and structural interaction of actin and myosin. Association of actin and myosin, leading to contraction, will occur when the relaxing factor is inactivated. By this association the scarcely active L-myosin-ATPase is converted in the highly active actomyosin-ATPase. Contraction in muscle fibers and model systems will be produced only if the mechanism of ATP-splitting is based on the interaction between actin and L-myosin (Bárány and Jaisle, 1960).

With the association of actin and myosin during contraction, the resistance to stretching increases (Bárány and Jaisle, 1960). This means that the modulus of elasticity changes. The increase in cohesive forces between actin and myosin filaments may be caused by the attachment of bridges from the myosin filament to the actin filament (Huxley, 1957) during contraction.

**RIGOR-MORTIS**

After the slaughter of warm-blooded animals and fishes the muscles become contracted and rigid. The extent of contraction depends on temperature (Bendall, 1951; Marsh, 1953). Erdös (1943) and, independently, Bate-Smith (1948) and Bendall (1951) showed that the disappearance of ATP is the reason for rigor-mortis development. The newer results concerning rigor development and the biochemical processes involved have been reviewed by, among others, Bate-Smith (1948), Partmann (1954a), and, recently, Whitaker (1959). Therefore, I will largely restrict this report to my own recent results.

There is no doubt that the course of the main biochemical processes leading to rigor-mortis is the same in muscles of a great variety of species of fish and warm-blooded animals. Differences were found for the ultimate pH values reached during rigor development and the glycogen residues in full rigor. It is surprising that, in horse muscles, a considerable amount of residual glycogen (e.g., 1715±142 mg/100 g diaphragma muscle) has been found at high ultimate pH value (e.g., 5.91±0.04 for diaphragma muscle) (Lawrie, 1955). General experience is that pH 5.3 is the limiting value, beyond which glycolysis is completely inhibited even though glycogen may still be present.

In warm-blooded animals it is known that, during rigor-mortis development, the changes in creatine phosphate, nucleotides, pH, and extensibility may vary considerably in different muscles. We found this is valid for fish, too. In cod (*Gadus morrhua* L.) and rosefish (*Sebastes marinus* L.) rigor development had normally already begun in the dorso lateral body muscles, whereas the muscles moving the radii branchiostegi in living animals were still excitable by electric current. In 2 of 40 rosefish this muscle could be stimulated to movement at 90 V even after 6 days of ice storage. Furthermore, Lawrie (1960),

Fig. 3. Original laboratory tool to measure the changes of the torsion elasticity of muscle.

studying the post-mortem behavior of the longissimus dorsi muscle of pigs, found consistent differences in ultimate pH and composition at different locations. These results show that it may be very difficult to estimate the exact time of the onset of rigor-mortis and of full rigor in an animal after death.

Resistance to stretching could be measured to follow the course of rigor development in muscle strips. This method is unsuitable for following rigor changes in a whole fish. For this reason we developed a new method and an instrument that allowed us to estimate the torsion elasticity in isolated muscles and whole fish. In the first laboratory model of the apparatus (Fig. 3) a constant force to induce a torsion-moment on the fish or the isolated muscle was produced by putting weights on a scale of a balance moving a small cord drum. The cord drum worked precisely under normal laboratory conditions, but was not suitable for measurements under rough conditions at sea. Therefore, in a new model (Fig. 4) a spring was used to produce the torsion moment. This instrument worked very satisfactorily in the laboratory and on board our research vessel, "Anton Dohrn," in the Icelandic Sea in February 1961. This device is described by Nemitz (in press).

The torsion angle $\Psi_0$ measured immediately after death divided by the torsion angle $\Psi_z$ after the time $z$ tells us how many times greater the torsion modulus has become at time $z$ in comparison to the value at time zero. This relation $S_z$ was used as a measure of the relative rigidity change reached at time $z$.

A curve for the isolated musculus gastrocnemius may serve as an example for the rigidity changes in beef muscle (Fig. 5). The curves differed somewhat for fishes of different species (Fig. 6). As Fig. 5 and 6 show, the beef and fish meats are not characterized by a systematic difference in the time course of rigidity changes after death: Under corresponding temperatures—somewhat unexpectedly—rigor development lasts even longer in mirror-carp (*Cyprinus carpius* L.) than in the gastrocnemius of beef. Often there are great individual deviations in rigor development within a single species, depending on the physiological state of the single animal. For instance, in rosefish (*Sebastes marinus* L.) and cod (*Gadus morrhua* L.) caught and handled carefully and iced immediately after the fillet was taken from one side, the time at which excitation by electric current ceased showed considerable deviations (Fig. 7). But in testing 50 fishes or more of the same species it became evident that, on the whole, the muscle tissue of cod "dies" earlier than the muscle tissue of rosefish.

Results were similar when rigidity changes in the same iced fishes were determined with a simple penetrometer described by Messtorff (1954). Used as a measure of rigidity of the surface layers of the fish was the depth of penetration of a punch when a constant spring force was applied (Fig. 8). However, since measurements show high average deviations, an increase

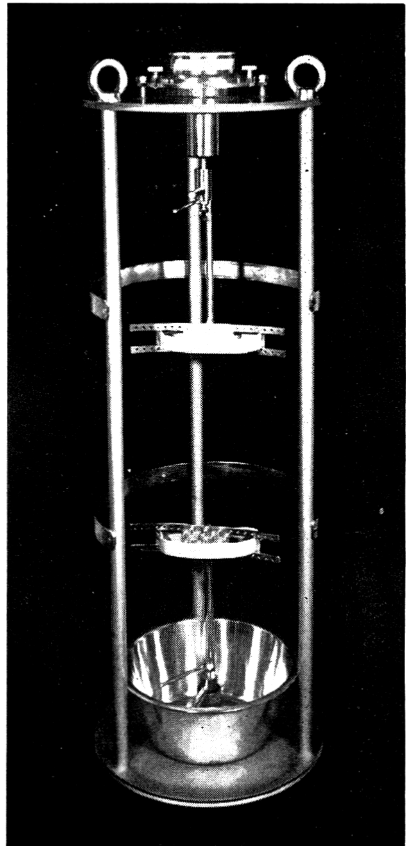

Fig. 4. Instrument for measurement of the elasticity changes of muscle using a spring to induce the torsion moment.

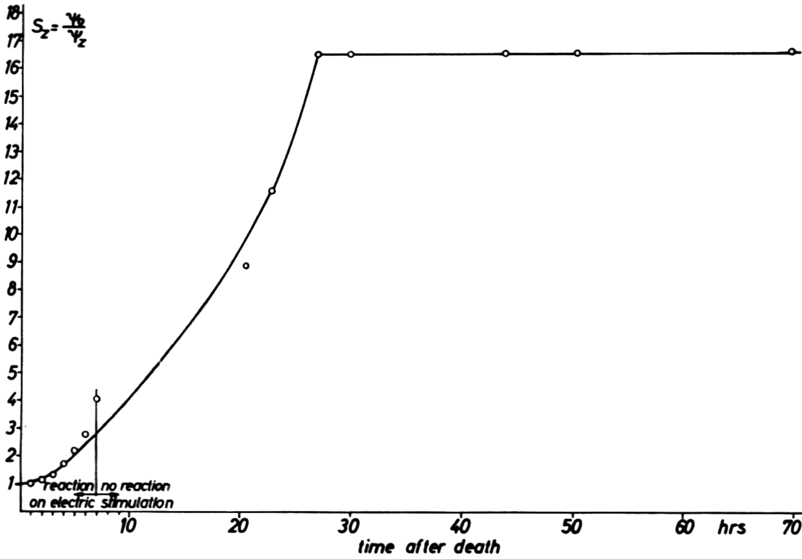


Fig. 5. Changes in rigidity of the gastrocnemius muscle of beef at +5°C in dependence of the time after slaughter.

in rigidity in each fish during rigor development is not clear enough in this figure. Most of the cod reached maximum rigor on the second or third day after death, and the majority of rosefish on the fourth or fifth day.

In well-fed and rested animals the length of the delay before rigor begins is, in our opinion, determined in great part by the time that elapses until the Marsh-Bendall factor becomes more or less inactivated, and hence the rapid phase of ATP breakdown begins. This inactivation could be due to

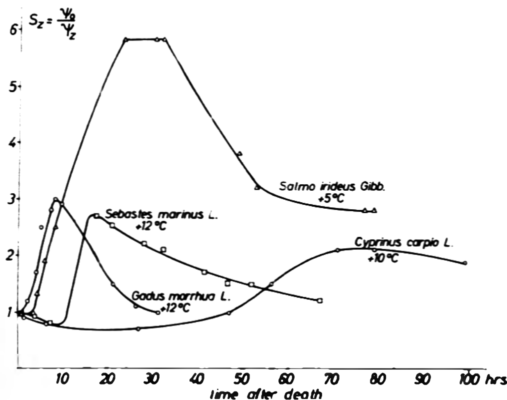


Fig. 6. Changes in rigidity of rainbow trout (*Salmo irideus* Gibb.), carp (*Cyprinus carpio* L.), rosefish (*Sebastes marinus* L.), and cod (*Gadus morrhua* L.) with time after slaughter.

the release of sufficient $Ca^{++}$ by the fibrillar muscle proteins. Hasselbach (1957) observed that the calcium content of the structure proteins from muscles in rigor-mortis (5 $\mu M$/g protein) amounts to only half the calcium content of the structure proteins from fresh muscle. According to Howard and Lawrie (1956), preslaughter injections of beef with $Ca^{++}$ hastened the onset of rigor-mortis considerably, and removal of the $Ca^{++}$ by injection of EDTA delayed the onset of rigor-mortis. This release of $Ca^{++}$ followed by inactivation of the relaxing factor seems to be correlated with membrane changes of the fiber, since the final signs of excitability by electric current disappeared at the beginning of the rapid phase of increasing rigidity, acidification, and ATP breakdown. However, in these investigations with fish, the muscles of the head and tail region were not stimulated, because of the stretch of the animals in our measuring device.

Interpretation of the course of rigor development is still not satisfactory, however. We have to consider that until depletion of the glycogen reserve or a pH value of 5.4 in the muscle tissue is reached, both breakdown of ATP and its resynthesis by the glycolytic cycle will take place. These counteracting processes run to the end of rigor development.

Thus the striking differences among animal species in the course of rigor development might be caused by: a) variations in membrane resistance against autolytic processes or increasing acidification, b) deviations in post-mortem release of $Ca^{++}$ and other ions by muscle proteins, and c) differences in the relation between the velocities of glycolytic ATP resynthesis and its breakdown. The initial glycogen and energy-rich nucleotide content, and the initial pH value do not explain why, e.g., a mirror-carp normally needs considerably more time to reach maximum rigor than does beef muscle or the pectoralis major muscle of the chicken (Partmann, 1961).

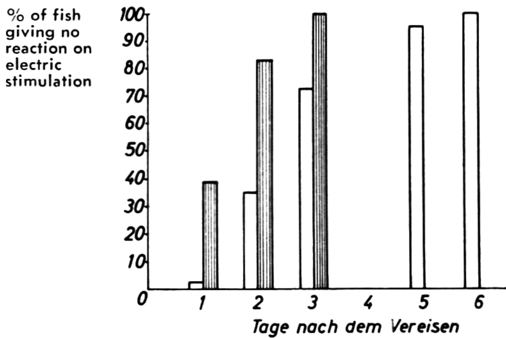


Fig. 7. End of reaction on electric stimulation in the muscles of the radii branchiostegi of cod and rosefish □ during ice-storage.

Obviously, some work still has to be done before the influence on rigor development of the single factors mentioned can be assessed. But it seems to be well established that rigor mortis and muscular contraction have the same mechanism. However, both kinds of muscle action differ considerably in the processes leading to their initiation and the amount of work produced in their course. It is clear that many biochemical changes and even a part of the changes in elasticity or hydration properties observed in rigor muscle cannot be said to be caused by "muscular contraction." To be considered are, among others, the pH decrease caused by glycolysis and the change in ion binding to the muscle proteins and the changes in phosphate-containing compounds (Hamm, 1960).

**RESOLUTION OF RIGOR-MORTIS**

After rigor reaches maximum, the elasticity values stay generally constant for some time. Important tenderization will occur in isolated beef muscle, but we observed only a small decrease in "rigidity" values during the normal aging period. Similar results were reported by Bate-Smith and Bendall (1956) for rabbit and beef muscle, and by Fremery and Pool (1960) for chicken muscle (pectoralis major).

Researchers have often attempted to demonstrate that the reason for the increase in tenderness is proteolysis by the enzymes of the tissue itself. In *long-term* storage of irradiated sterile meat and fish at high temperature, the tissue may actually soften as a result of autolysis. But, under the aging conditions usually applied (low temperature, limited time, good hygiene) the proteolytic changes are extremely small (Wierbicki *et al.*, 1954; Locker, 1960; Dvorak, 1960). If proteolysis plays an important part in normal post-mortem aging of beef, at least the number of end groups of the structural proteins should increase considerably during storage. But, as Locker (1960a) found, storage appears to cause no significant increase in both myosin and actin endgroups.

Sometimes it has been assumed that the changes in tenderness brought about by aging might be due to conversions of the connective tissue. Steiner (1939) and later

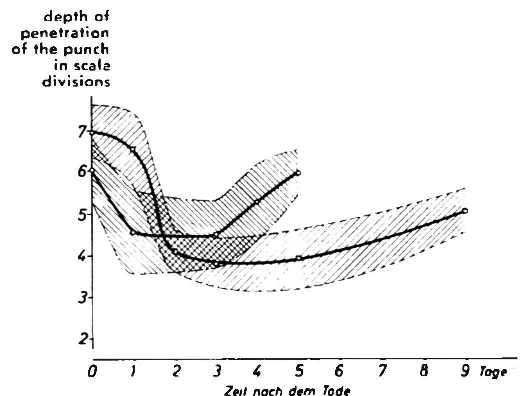


Fig. 8. Changes in muscle hardness of rosefish (40 animals) □, and cod (47 animals) ○ during ice-storage in dependence of the time post-mortem.

Table 1. Comparison of tenderness of diaphragma strips of beef (breadth ~6 cm) aged in loaded or unloaded state at +6°C (scale for panel-scores from 1 to 10, tenderness increasing with increasing notes).

| Sample | Aging time (days) | Load (kg) | Average values of tenderness: Squeezing force (kg) Loaded | Squeezing force (kg) Unloaded | Panel scores on consistency Loaded | Panel scores on consistency Unloaded |
|---|---|---|---|---|---|---|
| 1 | 2 | 2 | ............ | ............ | 5.7±1.00 | 4.3±1.34 |
| 2 | 4 | 1 | 7.2±1.33 | 15.9±1.87** | ............ | ............ |
| 3 | 4 | 1 | ............ | ............ | 7.0±0.71 | 5.4±1.32 |
| 4 | 4 | 1 | 5.8±0.77 | 10.9±1.49** | ............ | ............ |
| 5 | 7 | 2 | 5.1±0.58 | 12.9±2.34** | 8.3±0.58 | 5.5±0.58** |
| 6 | 3 | 2 | 9.3±0.75 | 23.5±2.15** | 7.2±0.87 | 5.2±0.87* |

* Difference significant ($t_{0.05}$).
** Difference highly significant ($t_{0.01}$).

Whitaker (1959) came to the conclusion that changes in connective tissue do not appear to be closely associated with aging. Probably the increased hydration of proteins (caused by, among others, the increase in pH in the course of autolysis), movement of cations, and breakdown of nucleotides influence the ripening of meat.

But, coming back to our sliding-filament model, another possibility might explain tenderness changes at least in part. It is known that relaxed muscles are more tender than partly contracted muscles (Locker, 1960). Hence, it might be possible to get a tender meat from dissociation of the actomyosin complex or if the association of actin and myosin during rigor development is impeded or partly so. To prove this, immediately after death the diaphragma muscle of beef was dissected. Strips about 6 cm wide of one half of the diaphragma were allowed to age as controls, and corresponding muscle strips were loaded with weights and forced to age in the stretched state under the same conditions. Organoleptic testing and measurements with the tenderometer developed by Wolodkewitsch (Grünewald, 1957) showed that the unloaded muscle strips were significantly tougher than the stretched strips after the same time (Table 1).

Of course, the resolution of rigor mortis is not strictly a reversal of the events that produce rigor mortis. Too many changes have occurred or are still taking place in the dead tissue, changing the whole reaction system. Furthermore, the already mentioned small decrease in the modulus of elasticity in beef muscle during resolution of rigor has been taken as an argument against the idea of actomyosin dissociation as a basic process leading to tenderization (Marsh, 1954; Bate-Smith and Bendall, 1956).

Nevertheless, there is one important fact in favor of the theory that the actomyosin complex formed during rigor development becomes dissociated normally or at least may become dissociated easily, in aged meat, as already proposed by other workers (Sokolow, 1951; Wierbicki and Kunkle, 1954; Weinberg and Rose, 1960): If completely aged muscle tissue of beef or fish is carefully homogenized in $0.16M$ KCl solution, a suspension of fiber fragments is obtained; when ATP not exceeding $10^{-2}M$ is added to the reaction mixture, all the fiber fragments contract (unpublished results).

Our observations were recently confirmed by Hamm (1962), who also found that the aged beef muscle tissue of certain animals shows no contraction. The reason for this unusual behavior is still unknown.

According to most experimental results the assumption seems allowable that the structural proteins have not changed very much during the whole post-mortem aging period; otherwise the fiber fragments would not contract. This view is supported by the findings of only small proteolytic and microscopic changes in aged muscle. To explain the insignificant changes in elasticity during the aging period of muscles from warm-blooded animals, other biochemical changes influencing tenderness increase have to be considered. Surely, much work is still nec-

essary before the whole problem of meat ripening will be elucidated.

### THAW RIGOR

After discussion of some of the problems connected with glycolysis of meat at temperatures above 0°C, the question arises as to how subzero temperatures may affect the processes that lead to ATP breakdown. If muscle tissue is frozen immediately after the death of a warm-blooded animal, the glycolytic processes slow down and become practically completely inhibited at a temperature low enough (e.g., below −30°C). The ATP still present at that moment will persist. When the temperature increases sufficiently during thawing, the ATP will be split quickly under contraction of the tissue, producing the thaw rigor, followed by a high drip loss. In muscle blocks of whales, the drip, containing considerable portions of proteins and salts, amounts to about 30–40% of tissue weight (Bendall and Marsh, 1951; Sharp and Marsh, 1953; Tanaka and Tanaka, 1955). The question of thaw rigor is being intensively investigated since the freezing of fish at sea has become of increasing importance in recent years.

Our own investigations revealed some interesting results, which may be mentioned briefly: when strips of muscle tissue of warm-blooded animals or fish were frozen in liquid air no losses of labile energy-rich phosphate were observed; on the other hand, in fish muscles the energy-rich nucleotides not bound to structure proteins were completely split after being thawed in air at +20°C. In the same time (½ hr after death) the ATP content had decreased only slightly in unfrozen muscle strips of comparable size kept at +10°C. In beef muscle strips quick-frozen in liquid air, ATP breakdown during thawing was slower than in fish muscle: the acceleration of ATP breakdown in the psoas muscle of beef by freezing and thawing was about 5-fold, whereas in the lateral body muscles of fish it was about 50- to 200-fold.

The ATP of the white muscle tissue of carp and rainbow trout was split at temperatures near 0°C, e.g., at −2°C, more quickly than in unfrozen muscle at +10°C. This activation of the enzymatic ATP breakdown at the beginning of freezing was not found in the muscle tissue of beef and chicken. From the temperature dependence of the rate of reaction—measured in the range from +20° to −8°C, starting at the higher temperature—it is evident that in all muscles the mechanism of the ATP splitting changes at the moment when freezing of the tissue begins (Figs. 9, 10).

In the freezing range the reaction velocity of the ATP breakdown starts to fall

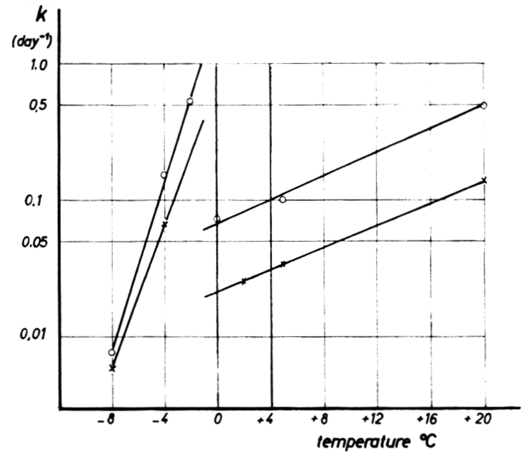


Fig. 9. Rate-constant (k) for the ATP breakdown (taking the course of a first-order reaction) in the white muscles of mirror carp (x) and rainbow trout (o) in dependence of temperature.

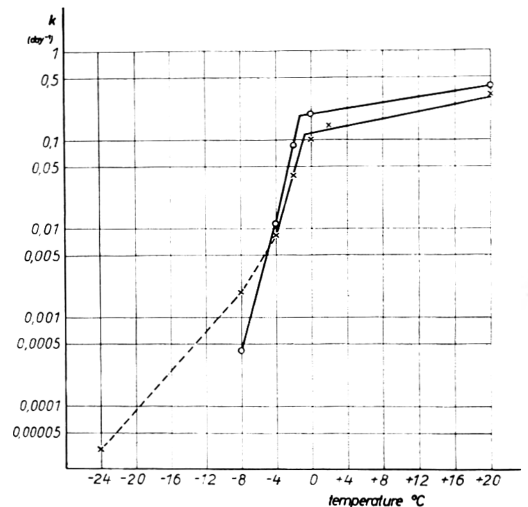


Fig. 10. Rate-constant (k) for the ATP breakdown (taking the course of a first-order reaction) in the musculus extensor digitalis communis of beef (x) and in the musculus pectoralis major of chickens (o) in dependence of temperature.

quickly with decreasing temperature. As an example it may be mentioned that the energy-rich nucleotides in beef, chicken, rainbow trout and carp muscle were split in 10–25 days at −8°C, whereas at −24°C, 70–100% of the initial ATP content is normally still present after 6 months (Partmann, 1961).

An activation of ATP splitting similar to that with freezing and thawing of fish muscle and with thawing of frozen beef and chicken muscle is obtained when dissected muscle tissue is ground immediately after slaughter and stored at temperatures higher than 0°C; it is also well known that rough mechanical handling of chicken before development of rigor-mortis accelerates the breakdown of energy-rich nucleotides. We have interpreted this acceleration of the ATP splitting—whether caused by mechanical means, by freezing, or by irradiation—as the result of a "biochemical injury effect" on the tissue, or, in German: "biochemischer Verletzungseffekt" (Partmann, 1961). The reason for its occurrence seems to be a very quick destruction or inactivation of the relaxing factor. Here again, the actin and myosin filaments associate, forming the highly active actomyosin-ATPase. Apparently the processes leading to inactivation of the relaxing factor by injury proceed more quickly and more completely in fish muscle than in the muscles of warm-blooded animals.

This is supported by the observations that it is more difficult to extract pure myosin from fish muscle than from the muscles of warm-blooded animals (Hamoir, 1955) (and under certain conditions even impossible). Connell (1958), on the other hand, succeeded in extracting myosin from prerigor cod muscle (I = 0.3; pH = 6.5 and 7.5). Though he found no myosin in extracts of post-rigor muscle or in extracts of cod muscle frozen rapidly prerigor and then extracted during thawing. The actomyosin fraction in the pectoralis major of young chicken increased in the first hours after death, but free myosin was still present in the post-rigor extracts (Weinberg and Rose, 1960). In my opinion, these results confirm the conception given above. By rigor development and by freezing "in the prerigor state" in fish muscle a rather complete formation of the actomyosin complex has taken place under simultaneous splitting of the ATP. In "rapidly" frozen cod muscle, inactivation of the relaxing factor is obviously relatively quick and complete compared with that in the muscles of warm-blooded animals.

Inactivation of the relaxing factor by freezing of the tissue is probably not caused by destruction of the relaxing factor itself, for the microsomes seem to be resistant against repeated freezing and thawing (Gergely *et al.*, 1959). Apparently, the inhibition of the relaxing factor is induced by the release of $Ca^{++}$ (Hamm, 1958) during freezing and thawing.

Because of the extremely quick breakdown of ATP under commercial freezing conditions—even if carp and rainbow trout are handled carefully and frozen immediately after death as single fish—only about half of the initial ATP content is present after freezing (Partmann, 1961). In rosefish and cod fillets carefully handled, dissected, and frozen within two hours of death, only about 12% of the fish contained 50% or more of the initial ATP content after normal blast-freezing.

On the average, fillets of longer and heavier frozen cod had higher contents of energy-rich P than smaller cod. Thus, it seems that the injury effect of freezing that leads to inhibition of the relaxing factor is less severe in cod that are 1 m long or more than in cod of small and medium size.

The expectation was confirmed by volume and drip measurements that fish frozen under normal commercial conditions immediately after death would show an insignificant degree of thaw contracture, because of the low ATP content at the time of thawing. Contrary to this, the drip loss caused by freezer storage increased significantly with storage time or elevated storage temperature (Partmann and Gutschmidt, in press). But these changes are not based on biochemical processes; they are caused by increasing denaturation of the fibrillar muscle proteins.

### DENATURATION OF STRUCTURAL MUSCLE PROTEINS

Denaturation of protein was measured by the extractability of the actomyosin fraction (extraction agent was a solution containing 0.8$M$ KCl and 0.03$M$ $NaHCO_3$) after the albumin fraction has been removed from the tissue. In cod and rosefish no significant difference has been observed in the extractability of the actomyosin fraction if the time between death and freezing was taken into consideration; the fishes were frozen either within 2 hr of death or after having reached rigor maximum or after the resolution or rigor-mortis during ice storage. The results were the same with both fish fillets and whole fish.

As expected the extractability of the structure proteins of rosefish and cod decreased at higher storage temperatures and with advancing storage time during freezer storage. Quantitatively, the differences between the two fish species have been considerable. Especially, at temperatures below −20°C the decrease in extractable structure proteins was considerably smaller in rosefish than in cod, although at these low temperatures the changes in cod muscle were not as large as one might expect from evaluation of the consistency by our taste panel. These results (Partmann and Gutschmidt, in press) are in agreement with those reported by Dyer and co-workers (1956), and Luijpen (1957).

In recent years it has become more and more evident that the insolubility of the structural fish muscle proteins does not always show a close correlation with the toughness of frozen fish as evaluated by organoleptic tests. We hoped to provide a measure of denaturation by estimating the loss in ATPase activity during freezer storage. But in tench (*Tinca vulgaris* Cuv.) we found no loss of activity during 3 months at −18°C. Furthermore, no loss in contractability after addition of ATP to muscle fiber suspensions had occurred in fresh-water fish and rosefish during this time (Partmann, 1954b; 1955a). On the other hand, at −3.5°C loss of ATPase activity and contractability was considerable in 3 months (Partmann, 1957). These results on ATPase activity were recently confirmed by Connell (1960) on cod; it may be concluded from his work that, at the point where the protein becomes almost completely insoluble, about half of the original ATPase activity is still present. The contradictory results mentioned, and the unexpected finding that there is no large change in either "easily-reactable" or total sulfhydryl groups during prolonged freezer storage of cod (Connell, 1960), reveal that considerable work has to be done before the mechanism and significance of protein denaturation during freezer storage of fish will become elucidated.

Evidently, maintenance of the ability to contract after ATP addition implies that the actin and myosin filaments may associate and hence are not subject to great structural changes by the freezing and thawing procedure. From these facts and microscopic observations we feel that the conceptions dealing with gross structural changes in normally aged and in frozen and thawed muscle tissue sometimes found in the literature will be corrected in the future.

### FREEZER BURN

The above does not mean that no histological changes can be observed in the regions of muscle tissue where freezer burn occurred. In such damaged surface areas of rosefish and chicken, structural differences have been great, obviously largely

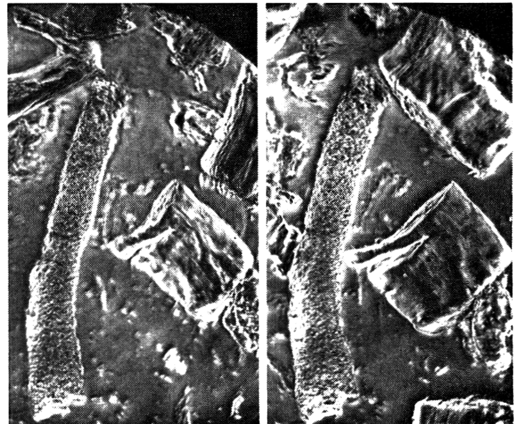

Fig. 11. Fiber fragments from the freezer burn area of rosefish before and after the addition of ATP.

Fig. 12. Freezer burn area of the muscle tissue of a hare after thawing.

depending on the process of dehydration. Microscopic examinations revealed that pores have been formed by sublimated ice. They may be seen as channels between the fibers, penetrating the fibers, or causing a lateral excavation or shrinkage of the fibers. Apparently, the higher the degree of ice sublimation, the higher is the shrinkage of the fiber membranes (Partmann, 1959).

The best-preserved fiber fragments from this region showed, on the addition of ATP, still slight contraction and a large increase in fiber diameter. Fibers from surfaces with the mentioned great structural changes reacted neither with shortening nor with swelling on addition of ATP (Fig. 11) (Partmann, 1955b; 1959). The swelling of the less damaged fibers on addition of the nucleotide is apparently caused by a loss of ATPase activity and a deficiency in association of the actin and myosin filaments; since the ATP present will be split very slowly, it acts as a plasticizer (Engelhardt and Ljubimova, 1939), causing an increase in hydration (Hamm, 1956). In the heavily damaged fibers the structure proteins are obviously altered in such a way that the added ATP has neither its contracting nor its plasticizing effect on the fibrillar proteins.

Nevertheless, electron micrographs of fiber sections from the freezer burn area of the muscle tissue of hares (Fig. 12) showed that the fibrils have scarcely changed their original orientation. Even the pattern of cross-striation, although a bit disintegrated, is not lost. Changes have taken place in the microstructure of the endoplasmatic reticulum, the sarcosomes, and the filaments of the fibrillar proteins (Fig. 13) (unpublished results).

It is well known that both freezer burn and freeze-drying are caused by ice sub-

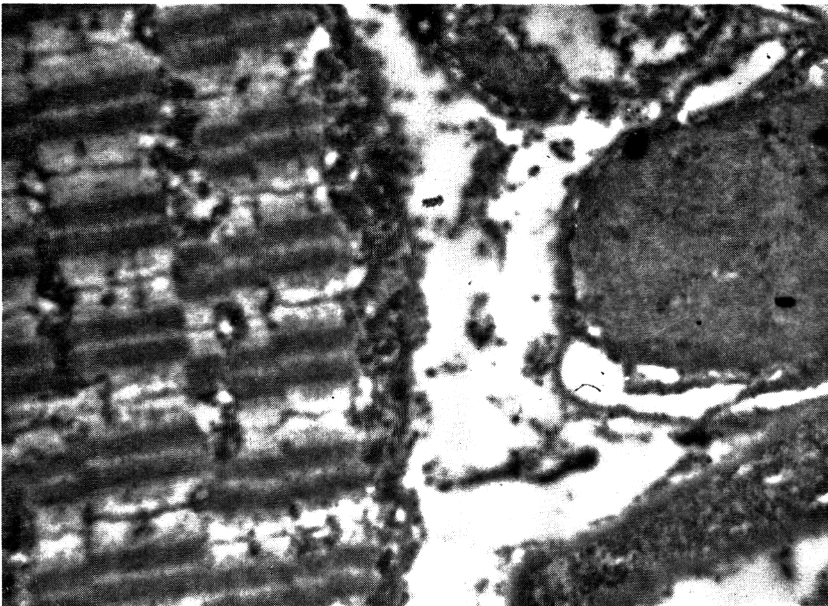

Fig. 13. Boundary of a muscle fiber from the freezer burn area of a hare. Preparation and magnification as in Fig. 1.

limation. It is not easy to understand why freezer burn produces a high degree of irreversibility whereas freeze-drying normally yields a product with highly reversible characteristics. We believe that the highly differing periods during which a critical water content is passed in both processes is responsible for the quality changes taking place in the product. In carp muscle such a broad minimum of stability seems to be in the range of a water content of 20% (Partmann and Nemitz. 1959). Although this water content is passed relatively quickly during freeze-drying at low temperatures, it may remain for a very long time during freezer burn development. But this problem also needs still more experimental work.

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# Nucleotide Degradation in the Muscle of Iced Haddock *(Gadus aeglefinus)*, Lemon Sole *(Pleuronectes microcephalus)*, and Plaice *(Pleuronectes platessa)*

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**SUMMARY**

**The muscle of trawl-caught haddock, lemon sole, and plaice contained little adenosine 5′-triphosphate (ATP) and much inosine 5′-monophosphate (IMP) at death. ATP, adenosine 5′-diphosphate (ADP), and adenosine 5′-monophosphate (AMP) changed rapidly after the fish died. IMP was lost from the muscle more slowly, with liberation of inosine, which was, in turn, degraded to hypoxanthine. A little adenine was formed by an alternative pathway of ATP degradation in lemon sole. A relatively high initial level of guanine was found in plaice muscle. Traces of xanthine were detectable in spoiling muscle from the three species. Implications of the findings are discussed in relation to quality testing and flavor changes in iced fish.**

Adenosine 5′-triphosphate (ATP) concentrations of flesh foods have been of interest for many years in relation to the rigor phenomenon, with its attendant difficulties in handling and processing. The disappearance of ATP from fish muscle has usually been followed by the estimation of acid-labile phosphate or that of phosphate after the fractionation of extracts with barium (e.g., Tarr, 1950; Fujimaki and Kojō, 1953; Noguchi and Yamamoto, 1955; Partmann, 1960; Tomlinson *et al.*, 1961; Fraser *et al.*, 1961; Nakano, 1961). Such procedures to follow nucleotide degradation have been extended to use beyond the immediate post-rigor period. Thus Golovkin and Pershina (1957) estimated acid-labile phosphorus in fish muscle stored up to 10 days, and Shewan and Jones (1957) obtained a measure of total nucleotide dephosphorylation in chill-stored cod muscle by the barium fractionation of extracts and ribose estimation. Sawant and Magar (1961) recently reported analogous changes in frozen fish.

It has become increasingly apparent that the products of ATP degradation also merit the attention of the food scientist. Solov'eva (1952) suggested that breakdown products of nucleotides contributed to the taste and aroma of ripened meat. The preparation and use of inosine 5′-monophosphate (IMP) salt as a condiment has been the subject of a Japanese patent (Yoshida and Kageyama, 1956). Oishi *et al.* (1959) related the quality of "katsuwo-bushi" (dried bonito) to the concentration of IMP. Saito (1960) and Jones (1960a) reviewed nucleotide degradation in relation to fish flavor and technology generally. Wood (1961) commented on the "meaty" flavor of IMP in relation to a study on ox-muscle extracts. Doty *et al.* (1961) implicated IMP and its immediate degradation products in the flavor-precursing complex of beef. Kazeniac (1961) indicated that IMP "makes a major contribution to mouth satisfaction, intensifying the flavor effects of other compounds" in relation to chicken flavor, and that hypoxanthine and inosine are bitter. Jones (1961) showed that much of the loss of sweet, meaty flavor during the early chill storage of cod results from the disappearance of glucose, the hexose phosphates, and IMP from the muscle:

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a progressive increase in bitterness is atributable to hypoxanthine liberated from IMP.

Recent chromatographic and electrophoretic studies have accumulated a few data on the course of mononucleotide degradation in fish species (Jones and Murray, 1957, 1960, 1961a, b, 1962; Saito and Arai, 1957, 1958a, b; Murray and Jones, 1958; Saito *et al.* 1959b; Tomlinson and Creelman, 1960; Creelman and Tomlinson, 1960). Together with analytical investigations on changes in ribose concentration (e.g., Tarr, 1954; Jones, 1958; Tarr and Leroux, 1962a, b) and in that of ribose phosphates post-mortem (Burt and Jones, 1961; Burt, 1961; Tarr and Leroux, 1962a, b), they have clarified the origins of the important pentose participants in discoloration reactions producing off-flavor in processed fish and shellfish muscle (e.g., Tarr, 1953; Jones, 1959; Nagasawa, 1960; Nagayama, 1960).

The estimation of nucleotide catabolites has provided the basis of new indices of fish quality. Shewan and Jones (1957) estimated a combined inosine + ribose fraction as a measure of early autolytic change, whereas Saito *et al.* (1959a) estimated an analogous dephosphorylation of IMP from the combined inosine + hypoxanthine concentration. It has been pointed out, however, that experience in this laboratory has indicated that the estimation of hypoxanthine alone may provide a better index (Reay, 1960; Jones, 1960b). Earlier, Solov'eva (1952) studied several chemical changes in meat and found a relation, maintained over a wide range of storage temperatures, between ripening, as judged by tasting tests, and the formation of hypoxanthine from nucleotides. More recently, Howard *et al.* (1960) supported Solov'eva's claim of correlation between the tenderness of meat and its hypoxanthine concentration.

This paper presents data on nucleotide degradation in a further three species. It discusses the implications of differing courses of catabolism.

## MATERIALS AND METHODS

Haddock (14–18 in.), lemon soles (13–16 in.), and plaice (12–14 in.) were caught by trawl by the Station's research vessel. They were eviscerated, stored in ice in boxes in the fish room, and landed within 24 hr. The boxes were transferred ashore to a refrigerated room simulating conditions in an average trawler's fish room (2.5°C) and re-iced periodically. Control lemon sole were taken from the "cod-end" of the trawl and immersed immediately in liquid nitrogen. They were transferred ashore in solid carbon dioxide and extracted immediately for analysis. Owing to adverse weather conditions, controls for the other species were frozen directly in solid carbon dioxide.

**Reference nucleotides, inosine, purines, and pyrimidines.** These were purchased (Sigma Chemical Co., St. Louis, Mo.; L. Light and Co. Ltd., Colnbrook, Bucks.).

**Preparation of muscle extracts.** Iced fish were filleted immediately on withdrawal from storage. Muscle from the anterio-dorsal portion of the fillet was extracted with 0.6*N* perchloric acid as described previously (Jones and Murray, 1961a). Control samples were not allowed to thaw before extraction. Aliquots of neutralized extracts from individual fish of batches were combined to obtain mean analyses.

**Nucleotide separation.** Two procedures were used. a) Extract (pH 6.5, equivalent to 10 g muscle) was passed through a column (18.2 × 1.3 cm) of Dowex 1 × 8 (formate) resin (200–400-mesh) refrigerated to 3±1°C. The column was washed with water until $E^{1 cm}_{260 m\mu}$ <0.01. Effluent and washings were retained (Fraction A) for the analysis of bases and nucleoside. Nucleotides retained by the column were eluted with formate according to the program illustrated in Fig. 1. This three-stage, stepwise, non-linear gradient separation is a simplification of that developed by Hurlbert *et al.* (1954). Elution of nucleotides was monitored automatically by passage through a Uvicord ultraviolet absorptiometer (L.K.B. Produkter, Stockholm) synchronized to a fraction collector. Separated peaks were identified as reported previously (Jones and Murray, 1960).

b) Extract (pH 6.5, equivalent to 10 g muscle) was passed through a column (40 × 1.6 cm) of Dowex 1 × 8 (chloride) resin (200–400-mesh) refrigerated to 3±1°C, and the column was washed as above. Nucleotides were eluted by a single non-linear gradient of 0.34*M* potassium chloride, containing 0.068*N* hydrochloric acid, into a constant 1000-ml volume of, initially, water. Nicotinamide adenine dinucleotide (NAD) and adenosine 5′-monophosphate (AMP) were eluted together under these conditions. Adenosine 5′-diphosphate (ADP) and IMP were eluted in reverse order as compared with the formate separation. ATP was eluted last.

**Estimation of AMP, ADP, ATP, and IMP.** Fractions of individual peaks were combined and evaluated spectrophotometrically (Jones and Murray, 1960).

**Estimation of NAD.** Combined fractions of the peak were evaluated spectrophotometrically in the presence of 1*N* cyanide (Ciotti and Kaplan, 1957).

**Separation and estimation of free bases and nucleoside.** A procedure developed for cod muscle (Jones, 1960b) was followed except that no initial nucleotide removing stage was required for effluents retained under procedure (*a*) above. A typical separation of a Fraction A is illustrated in Fig. 2.

**Confirmation of the presence of xanthine and xanthosine.** Fractions adjacent to inosine in the base separation (above), suspected of containing traces of these compounds, were subjected to adsorption onto charcoal, eluted with ammoniacal aqueous ethanol, and partitioned with chloroform (Jones and Murray, 1960). The resulting aqueous extract was lyophilized, taken up in 500 $\mu$l water, and chromatographed on acid-washed Whatman No. 1 paper in the solvents recommended by Wyatt (1955) for purines. After drying, papers that had not been in contact with a solvent containing the acid were sprayed lightly with 1*N* HCl and dried carefully. They were irradiated under a Chromatolite lamp (Hanovia, Slough, Bucks.) for 30 min, by which time intensely ultraviolet-absorbing brown spots appeared in the presence of xanthine

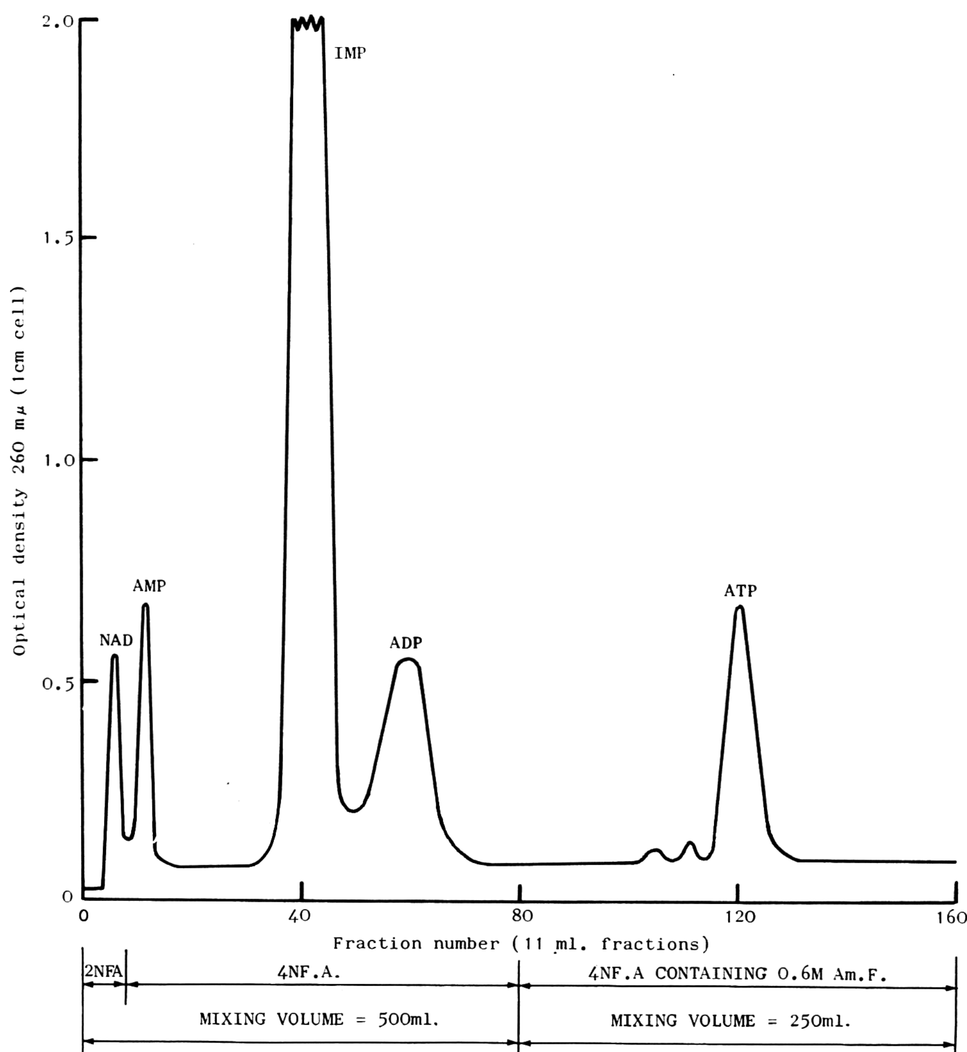


Fig. 1. Separation of nucleotides from fresh plaice muscle on Dowex 1 × 8 (formate). (Formic acid, F.A.; ammonium formate, Am.F.)

or xanthosine. This procedure is considerably more sensitive for detecting xanthine-containing compounds than the conventional visual examination of papers in ultraviolet light.

## RESULTS

Figs. 3, 4, and 5 illustrate the patterns of nucleotide degradation in the haddock, lemon sole, and plaice. The muscles of all three species had low initial ATP concentrations and high IMP. IMP disappeared from the muscle of plaice within 11 days, and more slowly in lemon sole. A transient increase in concentration preceded loss of IMP from haddock muscle. After rapid initial changes in adenine nucleotide concentration, low, relatively stable values were observed. Resolution between ADP and IMP was poor in Dowex 1 × 8 (chloride) separations of muscle extracts from haddock stored in ice for 3, 7, and 20 days. These extracts were separated for analysis on Dowex 1 × 8 (formate).

The course of IMP catabolism varied considerably with species. In haddock, the concentration of inosine increased for about 10 days and then fell. That of hypoxanthine increased from death, but more rapidly after 10 days, to a peak at 17 days. Traces of guanine were detectable throughout chill storage. Xanthosine and xanthine were also detectable after 14 days.

Inosine was present only in traces throughout 23 days' chill storage of lemon sole. Hypoxanthine increased to peak concentration at 12–14 days. Traces of guanine were detectable throughout chill storage, and of adenine up to 2 days. Xanthine was detectable at 12 and 23 days.

In plaice, maximum concentrations of inosine were measured at 7 days, and of hypoxanthine at 14 days. Guanine was measured in quantity during early chill storage. Xanthine and xanthosine were detected in traces ($< 0.1$ $\mu$mole/g) after 14 days.

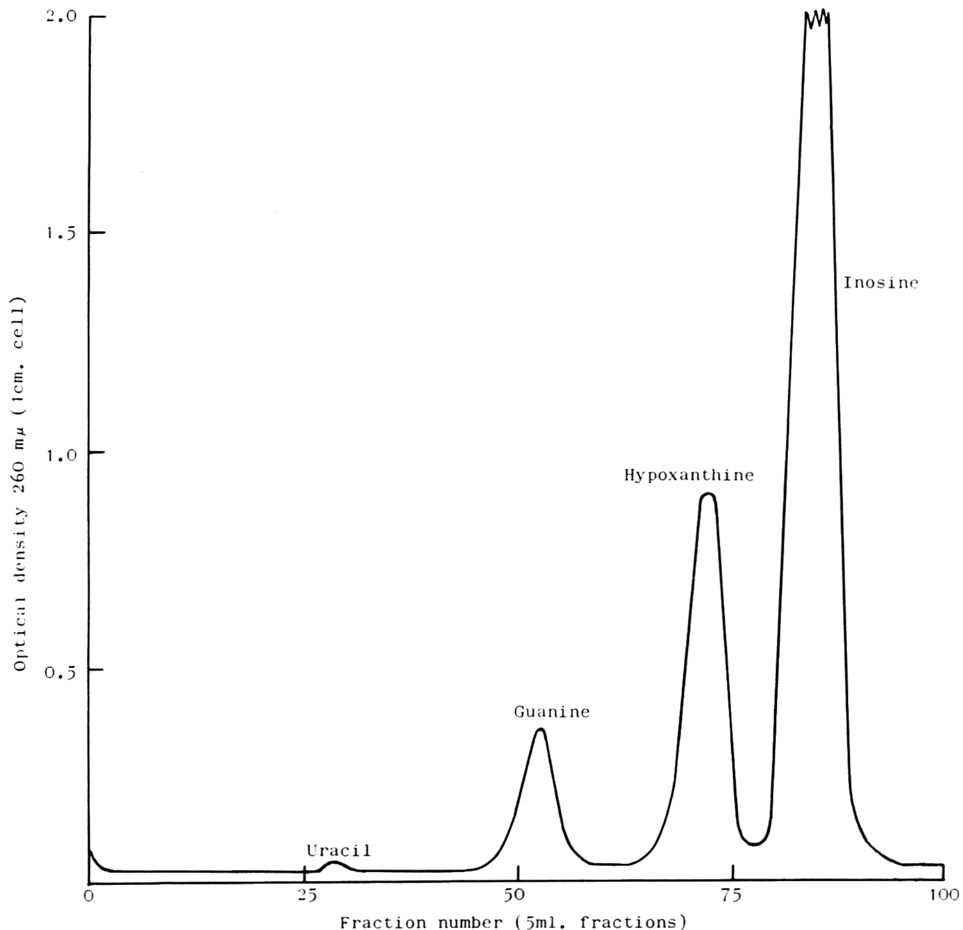


Fig. 2. Separation of bases and nucleoside from plaice muscle (5 days iced) on Dowex 1 × 8 (chloride).

## DISCUSSION

Figure 6 illustrates a schematic course of the major pathways of ATP degradation in fish muscle (see below).

The low ATP values measured in the control fish of the three species are of the same order as those observed in trawl-caught cod (Jones and Murray, 1957, 1961a) and they supplement data on acid-labile phosphorus contents, etc., of exhausted fish as determined by workers to whom we refer in the introductory section. The high IMP concentrations at death resulted from the dephosphorylation and deamination of ATP during the struggle.

Loss of IMP from the muscle of trawled plaice during chill storage proceeded at a rate similar to that observed in iced cod (Murray and Jones, 1958; Jones and Murray, 1961a, 1962) but more rapidly than those in lemon sole and haddock. It was considerably slower than that from iced trawl-caught spring salmon (Creelman and Tomlinson, 1960) and from the red lateral muscle of rainbow trout stored at 0° (Saito *et al.*, 1959b). In haddock, the small initial transient increase in IMP concentration corresponded to the much larger initial increases recorded for rested cod (Jones and Murray, 1961a) and the dorsal muscle of rainbow trout (Saito *et al.*, 1959b). Analogous small changes, corresponding to initial conversions of adenine nucleotide, may have occurred between our first and second measurements on trawled cod, plaice, and lemon sole.

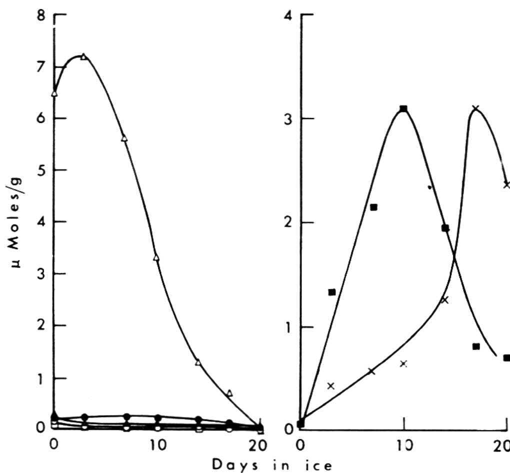


Fig. 3. Nucleotide degradation in iced haddock muscle. ATP, ▲; ADP, ●; AMP, ○; NAD, □; IMP, △; inosine, ■; hypoxanthine, ×. Points are mean values for 6 fish.

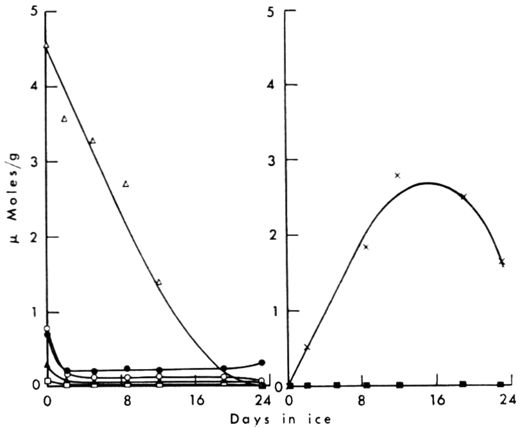


Fig. 4. Nucleotide degradation in iced lemon sole muscle. ATP, ▲; ADP, ●; AMP, ○; NAD, □; IMP, △; inosine, ■; hypoxanthine, ×. Points are mean values for 6 fish except at 23 days (4 fish).

After taking into account "back reaction," which is possible where low ribosidase activities allow a build-up in inosine concentration, it is apparent that 5′-nucleotidase activity varies considerably with species. Even greater variations occur in the rates of cleavage of inosine by muscle riboside hydrolases and phosphorylases (Tarr, 1955, 1958a). Creelman and Tomlinson (1960) have demonstrated the very small losses of inosine during the early chill storage of spring salmon. Our present investigation indicates an extremely rapid turnover of the nucleoside in lemon sole: hypoxanthine increased in concentration rapidly whereas only traces of inosine were detectable throughout the experiment. Intermediate activities have been reported of other species examined to date (Saito *et al.*, 1959b; Creelman and Tomlinson, 1960; Jones and Murray, 1962).

Eventually, the hypoxanthine produced by the phosphorolysis or hydrolysis of inosine is also lost from the muscle. In cod (Jones and Murray, 1962) and haddock, net loss was observed after the muscle had become inedible (~15–16 days) but concentrations of the purine fell a little before this point in

lemon sole and plaice. Our detection of traces of xanthine during advanced spoilage indicates that hypoxanthine is lost by bacterial oxidation. Experiments on relative rates of disappearance of the purine from cod muscle suspensions incubated in the presence and absence of known species of spoilage microorganisms (Avery *et al.*, 1962) substantiate this view. Leaching also results in some loss of constituents of low molecular weight from iced fish muscle.

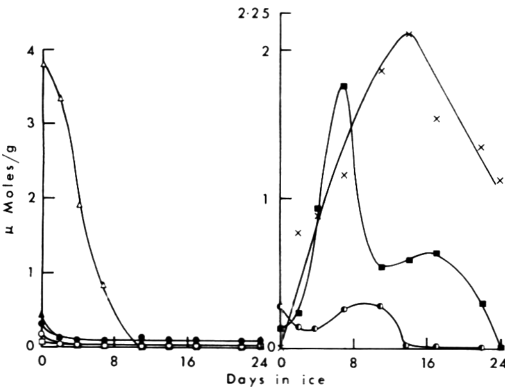


Fig. 5. Nucleotide degradation in iced plaice muscle. ATP, ▲; ADP, ●; AMP, ○; NAD, □; IMP, △; inosine, ■; hypoxanthine, ×; guanine, ◐. Points are mean values for 3 fish.

The rate of hypoxanthine liberation in gadoids (which have a low ribosidase activity in the fresh muscle relative to that of *Pleuronectes* species, as indicated above by the analyses) increased considerably during the period of the proliferation of the spoilage microflora (e.g., Reay and Shewan, 1949). In this relation it seems significant that data on *Oncorhynchus tschawytscha* (Creelman and Tomlinson, 1960), which lacks initial ribosidase activity, indicate an increase in concentration between the 6th and 10th days of chill storage. The appearance of significant quantities of guanine in plaice muscle, but not in that of other species stored at 0°, is of interest. This purine appears in larger quantities (e.g., 0.88 μmole/g after 62 weeks at −14°) in the muscle of frozen cod (Jones and Murray, 1961b) but has been detected only occasionally in iced cod in high concentration (Jones, 1960b; Jones and Murray, 1962). The origin of this compound is uncertain. Equivalent concentrations of guanine nucleotide or nucleoside are not found in the fresh material, and the presence of free pyrimidines or pyrimidine nucleotides in quantities indicative of considerable nucleic acid degradation in the stored material (*vide* Tarr, 1958b), has been observed in no species examined in this laboratory to date. It seems reasonable to suppose from analogous mammalian systems that the guanine derives from hypoxanthine by oxidation and amination possibly involving a rapid turnover of guanosine 5′-triphosphate. We have indications from our recent work on cod that there are seasonal variations in the incidence of the enzymic mechanism of guanine biosynthesis in muscle.

The presence of adenine in fresh lemon sole muscle was unusual also. Although the purine was measured in trace concentrations only, it indicates that over-all deamination occurs at a slower rate in this species than in the others examined to date.

Analytical studies by different groups of workers (Jones and Murray, 1957, 1960, 1961a,b, 1962; Saito and Arai, 1957, 1958a,b; Saito *et al.*, 1959b; Murray and Jones, 1958; Tomlinson and Creelman, 1960; Creelman and Tomlinson, 1960; Sawant and Magar, 1961, present paper) on nucleotides and bases, similar studies on the liberation of ribose and ribose phosphates (Tarr, 1954; Jones, 1958; Burt and Jones, 1961; Burt, 1961), and a number of enzymic investigations (Tarr, 1954, 1955, 1958a, b; Jones and Murray, 1961a; Martin and Tarr, 1961; Jones, 1962a; Tarr and Leroux, 1962a) support the pathways of nucleotide degradation postulated in Fig. 6. This represents a curtailment of the scheme proposed by Shewan and Jones (1957) in that it is now apparent that a suggested additional pathway to IMP via inosine 5′-triphosphate, such as occurs in mammalian tissue (Bendall and Davey, 1957), is of little importance. Current evidence enables additions to the original scheme beyond hypoxanthine and ribose 1-phosphate. It differs from the recent partial scheme of Sawant and Magar (1961) [which is essentially similar to that proposed by Saito and Arai (1957)] in that it indicates the importance of myokinase action on ADP rather than direct dephos-

phorylation to AMP. It also indicates a small contribution to the IMP pool from nicotinamide adenine dinucleotide via adenosine 5′-diphosphate ribose (Jones, 1962b).

It is now clear that the free ribose of fish muscle derives predominantly from ATP (*vide* Shewan and Jones, 1957) rather than ribonucleic acid (Tarr, 1958b). The recent reports of Tarr and Leroux (1962a, b) confirmed the findings of a number of groups in this respect, but questioned the identification of ribose 1-phosphate in chill-stored cod (Burt and Jones, 1961; Burt, 1961). The procedure of ion-exchange separation on Dowex 1 columns followed by Tarr and Leroux was essentially a modification of that employed in this laboratory, and it seems unlikely that discrepancies derive from this source. Rather, some difference in technique during the preparation and neutralization of extracts may be responsible. Dr. Burt recovered some 90% of ribose 1-phosphate added to muscle before extraction: this compares favorably with 0–22% (Tarr and Leroux, 1962a) or 0% (Tarr and Leroux, 1962b).

Tarr and Leroux (1962a) suggested further that the presence of ribose 1-phosphate in quantity was unlikely since the equilibrium of phosphoribomutase action favors ribose 5-phosphate. Such a situation would be expected to apply only where the effective concentration and activity of enzyme was such as to convert immediately any 1-phosphate formed (from inosine) in excess of the equilibrium level. Otherwise, both phosphates would increase in concentration, with the 1-phosphate predominating if its production were rapid enough. On the failure of the supply of the precursor, inosine, the relative proportion of 5-phosphate would progressively increase and it would ultimately exceed that of the 1-phosphate. This is the situation demonstrated in chill-stored cod by Burt and Jones (1961). Similar situations with respect to other catabolites are reported in the present paper. Tarr and Leroux (1962a, b) did not extend their studies on intact chill-stored fish beyond 2 lingcod respectively held for 3 and 6 days at 0°.

The effects of the multiplicity of factors affecting the over-all course of nucleotide degradation in different species are reflected both in the rates of change of catabolite concentrations and in the peak concentrations. This may be of some significance to species variations in flavor. We pointed out in the introductory section that IMP and its catabolites have characteristic flavorous attributes, the nucleotide itself having the additional property of enhancing the flavor of other compounds. A loss of this compound (some-

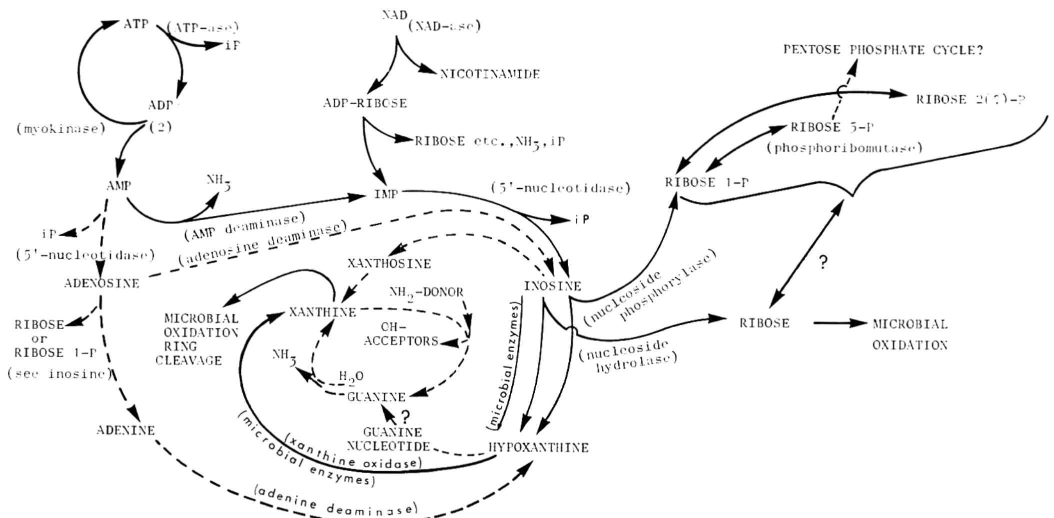


Fig. 6. Pathways of degradation of ATP in fish muscle. (Broken lines indicate routes of minor importance. i P = inorganic phosphate. Named enzymes have been studied directly. ? indicates speculative pathway.)

times preceded by a transient rise consequent on adenine nucleotide degradation) has been found consistently during early chill storage, with specific variations in rate. This is in accord with a general thesis that loss of flavor during the early chill storage of fish is attributable in part to loss of IMP (Jones, 1961). Recent organoleptic assessment of sterile cod muscle stored at 0° in this Station has also correlated well with the course of autolytic nucleotide degradation (Cann *et al.*, 1962) in both the disappearance of initial flavor and the appearance of bitterness deriving from hypoxanthine liberation. Thus we have a new experimental basis for early postulates (e.g., Fieger and Friloux, 1954) that tissue autolysis is implicated in the early loss of flavor from sea foods. From the variations in the rates of IMP degradation it may also be postulated that off-flavor, resulting from ribose-amino reactions during the canning, dehydration and subsequent storage of reasonably fresh fish, may also be expected to vary considerably from species to species.

Consideration of the earlier paragraphs of this discussion allows a reappraisal of quality tests based on nucleotide breakdown in fish (Shewan and Jones, 1957; Saito *et al.*, 1959a). Both types of test reported upon to date measure essentially the initial dephosphorylation of IMP. Insofar as they determine directly the loss of an important flavorous constituent from the fresh flesh, and give a measure of storage time also, they are doubly useful. They have the advantage over currently available tests based solely on bacterial activity in that they are maximally sensitive at a period when procedures such as the estimation of trimethylamine give only the information that active microbial spoilage is not in train. Reasonably precise information of the storage history within this period would be a useful adjunct to the preparation of frozen fish of good quality ashore (*cf.* Reay *et al.*, 1950). However, for general use, they must be considered as complementary to tests based on microbial activity in that dephosphorylation is often complete well within the period of edibility. Obviously there is a requirement for a test measuring both loss of flavor during early chill storage and the appearance of an off-flavor deriving partly, at least, from bacterial action later. It was indicated earlier from this laboratory (Jones, 1960a, b; Reay, 1960) that the determination of hypoxanthine, deriving from IMP, meets these requirements for a number of species. Rapid procedures have been devised for the measurement of the purine in fish muscle, and their evaluation is the subject of a report in preparation.

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# Gelation of Egg Yolk

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**SUMMARY**

**A study of the effects of freezing and thawing on egg yolk showed that there was a rapid increase in viscosity (25°C) during the first few hours of storage at −10° and −14°C. The effectiveness of sucrose and of sodium chloride as inhibitors was determined, and the influence of urea on the viscosity of native and of frozen and thawed yolk was investigated. Paper electrophoresis indicated that freezing and thawing caused electrophoretic changes of the yolk lipoproteins.**

During the freezing and thawing of egg yolk, an irreversible alteration in fluidity takes place (Romanoff and Romanoff, 1949). This phenomenon is termed gelation. Moran (1925) indicated that yolk temperature must be below −6°C before gelation occurs. This critical temperature is below the freezing point, −0.65°C for the yolk. However, regardless of temperature, ice crystal formation, in contrast to supercooling, has been reported to be a requisite for gelation (Moran, 1924).

Storage time and rate of thawing influence the degree of yolk gelation. Moran (1924) stated that the pastiness of thawed yolk in whole eggs frozen at −11°C increased in proportion to time of storage up to about 24 hr. Pearce and Lavers (1949) observed that the viscosity of frozen (−18°C) and thawed yolk increased up to the eighth month of storage under certain conditions. Yolk frozen in liquid air becomes pasty upon thawing at room temperature, but if thawed in mercury at 30°C reverts completely to its normal fluidity (Moran, 1924). Pearce and Lavers (1949) showed that an increase in thawing time between 0.03 and 24 hr increases the viscosity of yolk frozen at −12°C.

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Investigations have indicated that yolk gelation can be inhibited by mechanical and chemical treatment of yolk before freezing. Pearce and Lavers (1949) and Lopez *et al.* (1954) found that homogenization and colloid milling of native yolk minimized gelation to some extent. The first reported chemical inhibitor of gelation was sucrose (Moran, 1925); in the presence of 10% sucrose, yolk, upon freezing and thawing, showed no appreciable change in fluidity. Other sugars, as well as sodium chloride and glycerol, have been reported to inhibit gelation (Thomas and Bailey, 1933; Lesser, 1948; Lopez *et al.*, 1954). In 1932, Tressler (1932) was granted a patent on a method of inhibiting gelation by treating native egg yolk with some types of proteolytic enzymes. An extensive study of the ability of various types of enzymes to inhibit gelation was reported by Lopez *et al.* (1955). Papain was found to be the most satisfactory enzyme from the standpoint of both inhibition of gelation and lack of off-flavor development.

Very few investigations have been directed toward study of the gelation mechanism. Moran (1925) suggested that salts of yolk become concentrated at temperatures below −6°C, whereupon the irreversible precipitation of lecitho-vitellin takes place. The presence of lecithin in a water extract of gelled yolk was the basis for the hypothesis of Urbain and Miller (1930) that the coagulation of lecithin was responsible for

gelation. Evidence accrued by Fevold and Lausten (1946) indicated that lipovitellenin, a highly soluble lipoprotein fraction of yolk, was involved in the gel formation of yolk. Enzymic studies have strengthened the thesis that lipoproteins are implicated in the gelation of egg yolk. Feeney *et al.* (1954), from studies with crotoxin-treated yolk, concluded that gelation is related to changes involving lipoproteins. Those investigators observed that lecithinase added to yolk before freezing prevented gelation. Studies on the inhibition of gelation by some proteolytic enzymes (Tressler, 1932; Lopez *et al.*, 1955) are consistent with the lipoprotein-gelation relationship.

The broad purpose of this study was to obtain more basic knowledge on yolk gelation and its inhibition. All published research on the increase in gelation during freezer storage has been conducted with large quantities of yolk and no consideration of uniform heat removal. Moreover, the rate of viscosity change during the early stages of gelation has not been reported. Consequently, experimentation was undertaken to obtain quantitative data on the rate of apparent viscosity increase of yolk frozen for short periods at various temperatures. In all cases, the heat energy was removed uniformly from yolk samples. Furthermore, since the mechanism of lipoprotein alteration during the freezing-thawing process has not been examined extensively, preliminary experiments were conducted to obtain some information that would serve as a basis for more detailed investigations on the lipoprotein-gelation relationship.

## EXPERIMENTAL METHODS

**Materials.** Hen eggs, infertile and not more than 24 hr old, were used either immediately or stored at about 5°C for no longer than 12 hr.

Liquid yolk was prepared according to the following procedure. Each yolk was removed from the albumen, carefully washed in cold water to remove the traces of albumen, and dried by rolling on absorbent paper towels. After the chalazae were removed, the vitelline membrane of each yolk was broken and the liquid squeezed out. The yolk was mixed slowly and thoroughly to provide a homogeneous mass without excessive incorporation of air bubbles.

**Freezing of yolk.** A method was devised to freeze yolk samples uniformly and rapidly. Rectangular tin-coated metal pans $8\frac{1}{2} \times 5\frac{1}{2}$ in. and $\frac{3}{4}$ in. deep were used as containers. A copper-constantan thermocouple was located about 1/16 in. from the bottom at the center of each pan. After 90 g of yolk were spread evenly on the bottom of each pan to form a thin layer, polyethylene film was stretched across the top to prevent moisture loss. Pans, with egg yolk, were placed on metal shelves in a temperature-controlled air-blast freezer, and the temperature of the yolk in each pan was recorded by a Brown electronic potentiometer. After the desired holding temperature was reached, temperature fluctuations of the yolk samples were less than 0.5°C. Each yolk sample was thawed rapidly by placing the pan in a water bath at 30°C. Apparent viscosity readings of two yolk samples were averaged for each treatment.

Since yolk commences to gel at or below −6°C, the amount of time between −6°C and the storage temperature must not be ignored. Zero storage time was defined arbitrarily as the time at which the temperature of the yolk reached −6°C. The come-down time was regarded as the time required for lowering the temperature of yolk from −6°C to the storage temperature.

**Addition of urea.** Since the temperature of yolk decreased during the incorporation of urea, a standard procedure was adopted for rapidly increasing the temperature of each urea-yolk mixture to 25°C. Powdered urea was added to a small glass jar with 80 g of liquid yolk at about 25°C. After the jar was placed in a water bath at 38°C, the yolk mass was stirred immediately by a stirrer rotating at 30 rpm. When the urea-treated yolk approached 25°C, the jar was transferred to a water bath at 25°C. Ten minutes after the addition of urea, was chosen as zero time. At this time the temperature of each urea-yolk mixture was 25°C. Apparent-viscosity readings for two yolk samples were averaged for each treatment.

**Crude lipoprotein fractions.** A crude lipovitellin fraction was prepared by centrifuging yolk, diluted with 2 volumes of distilled water, in a Sharples centrifuge at about 40,000 rpm (Alderton and Fevold, 1945). The sedimented material was dispersed in distilled water (1 to 3 by wt.) and the slurry was recentrifuged.

The supernatant, obtained after high-speed centrifugation of diluted yolk to remove the crude lipovitellin, was regarded as a crude lipovitellenin fraction. Further fractionation of the proteins by ether treatment (Fevold and Lausten, 1946) was not conducted, since Evans and Bandemer (1946) found that the paper electrophoretic mobility of lipovitellenin was altered by ether extraction.

**Analytical methods.** To determine the viscosity changes of treated yolk quantitatively, the Brook-

field Synchro-lectric viscometer, model RVF, was used. The viscosity of 80-g yolk samples was determined at 25°C with spindle No. 6 or No. 7, and with a constant spindle speed of 20 rpm. Three readings, taken within the first 20 sec of spindle rotation in yolk, were averaged for each sample. The apparent viscosity of the yolk samples is expressed in poises.

Paper electrophoresis was used to separate the proteins in frozen and unfrozen yolk. Frozen samples were prepared by freezing 50-g portions of yolk for 24 hr at about −23°C in 50-ml beakers with vapor-proof aluminum caps. Before application of the yolk samples on the paper strips, a 10-g portion of each yolk sample was diluted with 10 ml of 10% NaCl solution. To ensure homogeneity, the diluted samples were mixed frequently during several hours at room temperature. The Spinco Model R paper electrophoresis system was used. To each paper strip (S and S No. 2043A), a 0.01-ml portion of diluted yolk sample was applied by an applicator. A phosphate buffer of pH 6.5 and 0.1 ionic strength was used exclusively. Each electrophoretic run was carried out for 24 hr at about 25°C with a constant voltage of 120 volts. The strips were dried 30 min at about 120°C in a forced-air oven. The proteins were stained by immersing the strips for 30 min in an alcoholic bromophenol blue solution (1 g of dye in 1 L of methanol). The excess dye was removed by rinsing the strips three times in 5% acetic acid solution. The strips were dried for 15 min at about 120°C. The lipid was located by placing strips in a 60% ethanol solution saturated with Oil Red O for about 16 hr (Durrum *et al.*, 1952). The strips were rinsed with distilled water.

The major bromophenol-blue-stained lipoprotein bands were cut from some electrophoretograms for a dye-elution study. Dye from each paper section was eluted in 20 ml of 0.5% $Na_2CO_3$ solution, and the optical density of each dye solution was measured at 590 mμ in a Bausch and Lomb Spectronic 20 colorimeter. Migration distance refers to the distance in mm between the origin and the front of the specific band. Migration distance and optical-density values reported in this paper are averages for two electrophoretograms of the same electrophoretic run.

## RESULTS

**Experiments with yolk.** *Effect of storage temperature and time.* Quantitative determination of the apparent viscosity of yolk previously subjected to temperatures below −6°C for short periods and thawed rapidly under constant condition was expected to give insight into the gelation mechanism. Thermocouple records indicated that the come-down time from −6°C to all storage temperatures of yolk was less than 30 min. Fig. 1 shows that the apparent viscosity of frozen-thawed yolk is dependent on the freezing temperature to which the yolk was lowered and the storage time. The viscosity of thawed yolk frozen at −7°C, did not increase appreciably with storage periods up to 750 minutes. On the other hand, the alteration of yolk viscosity by freezing at either −10 or −14°C was marked initially. A semilogarithmic plot of the apparent viscosity of thawed yolk with storage time (after a 30-min come-down time) gave a straight line in the initial storage period for temperatures of −10 and −14°C.

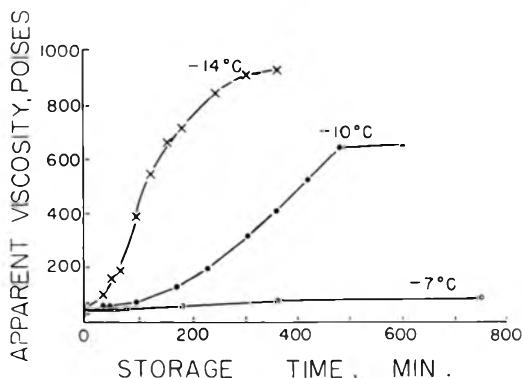


Fig. 1. Effect of storage temperature on the viscosity of yolk.

Fluffy white masses were noted immediately after the ice crystals in the yolk melted. The size of these masses appeared to be related to temperature and time of storage. The masses disappeared after a few minutes in the thawed state.

*Inhibition of viscosity changes by sugar and sodium chloride.* The alteration of yolk viscosity due to freezing and thawing was reduced by adding sucrose, a polar compound, to the native yolk. Table 1 indicates that the degree of viscosity change was decreased as the sucrose concentration was increased from 0.001 to 0.014 moles per 100 g of yolk. Even with a concentration as high as 0.014 moles/100 g, however, the viscosity change was not completely inhibited. A semilogarithmic plot of the viscosity ratio, $B/A$, and sucrose concentration between 0.001 and 0.014 moles per 100 g resulted in a straight line with a slope $[(d \log B/A)/dc]$ of about $10.3 \times 10^{2}$.

Table 2 shows that the viscosity of native egg yolk increased markedly as the concentration of NaCl was increased. The findings are in accord with data of Jordan and Whitlock (1955). The degree of gelation inhibition by NaCl is expressed by the value of the viscosity ratio in Table 2.

Table 1. Inhibitory effect of sucrose on the viscosity change of yolk[a] during freezing and thawing.

| Sucrose conc. (moles/100 g yolk) | Apparent viscosity (poises at 25°C) | | | Viscosity ratio | |
|---|---|---|---|---|---|
| | Not frozen | Frozen at −14°C | | | |
| | | 90 min | 120 min | | |
| | *A* | *B* | *C* | *B/A* | *C/A* |
| 0 | 20 | 275 | 510 | 13.7 | 25.5 |
| 0.001 | 18 | 240 | 408 | 13.2 | 22.7 |
| 0.003 | 18 | 130 | 293 | 7.2 | 16.6 |
| 0.006 | 15 | 82 | 86 | 5.4 | 5.7 |
| 0.008 | 14 | 50 | 61 | 3.6 | 4.3 |
| 0.011 | 14 | 31 | 33 | 2.2 | 2.4 |
| 0.014 | 14 | 18 | 22 | 1.3 | 1.6 |

[a] Final pH of all samples was about 6.2 at 25°C.

Under the conditions of the experiment, the viscosity change of yolk upon freezing and thawing was apparently retarded almost completely by the inclusion of 0.1 moles of NaCl per 100 g of yolk. On a molar basis, the ionic NaCl is not as effective an inhibitor as the polar sucrose.

*Influence of cysteine.* Since yolk proteins possess significant amounts of sulfur-containing amino acids, it was speculated that intramolecular S—S linkages in proteins of yolk could be ruptured in the presence of cysteine, with the ultimate result of inhibiting gelation. For preparation of a cysteine-treated yolk, 5 ml of a cysteine solution, adjusted to 6.1 with NaOH solution, was added to 80 g of yolk with a pH of 6.1. The concentration of cysteine in the treated-yolk mixture was 0.0055 moles per 100 g, and the final pH was 6.1. Sodium chloride was added to the yolk control to provide the same NaCl concentration as that in treated

Table 2. Inhibitory effect of sodium chloride on the viscosity change of yolk[a] during freezing and thawing.

| NaCl conc. (moles/100 g yolk) | Apparent viscosity (poises at 25°C) | | Viscosity ratio *B/A* |
|---|---|---|---|
| | Not frozen | Frozen at −14°C for 150 min | |
| | *A* | *B* | |
| 0 | 24 | 628 | 26.2 |
| 0.01 | 56 | 320 | 5.7 |
| 0.02 | 88 | 155 | 1.8 |
| 0.03 | 103 | 165 | 1.6 |
| 0.05 | 143 | 227 | 1.6 |
| 0.07 | 205 | 235 | 1.2 |
| 0.10 | 280 | 285 | 1.0 |
| 0.14 | 410 | 411 | 1.0 |

[a] Final pH of all samples was about 6.2 at 25°C.

yolk. As shown in Table 3, the apparent viscosity of the cysteine-treated yolk, frozen at −14°C for 165 min, was lower than that of frozen-thawed yolk control under the same conditions.

*Effect of urea.* Since some investigators (Dyer, *et al.*, 1951; Husaini and Alm, 1955; Sawant and Magar, 1961) have suggested that certain proteins in aqueous suspension or solution are denatured during freezing and thawing, a study was initiated on the viscosity changes of yolk containing a denaturing agent (urea). Above a certain concentration, urea was capable of increasing the viscosity of the native unfrozen yolk. Fig. 2 shows the changes in apparent viscosity of urea-treated yolk with time and concentration of urea. With a urea concentration of 0.166 moles per 100 g of yolk, no appreciable change in viscosity was obtained within 355 min; with higher concentrations of urea, however, the viscosity of yolk progressively increased with storage time. The apparent viscosity curves of yolk treated with urea, particularly 0.333 moles per 100 g, were somewhat similar to those of yolk frozen at −10 and −14°C. At the urea level of 0.416 moles per 100 g, yolk was converted into a stiff gel in 85 min. The stiff gels were opaque whereas the viscous yolk-urea mixtures were semitransparent. When the stiff gels were heated to 60 and 100°C, they did not lose their gel structure; the softer gels, however, were converted to a fluid state at 38°C.

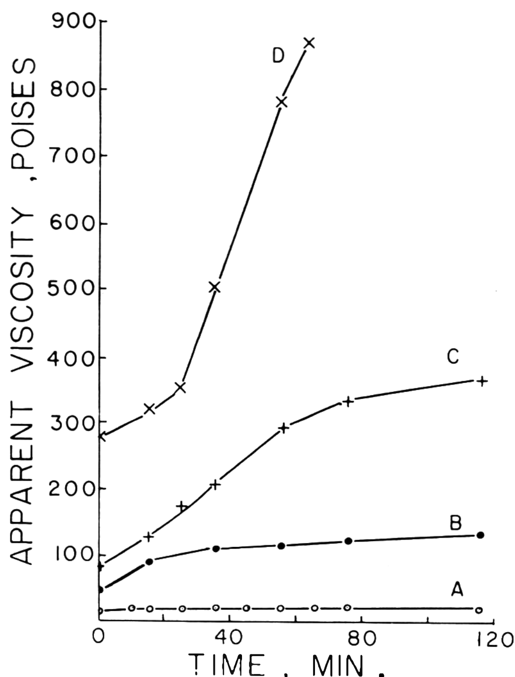


Fig. 2. Effect of urea on the viscosity of yolk. Urea concentration in moles per 100 g: A, 0.166; B, 0.250; C, 0.333; D, 0.416.

Table 3. Influence of cysteine on yolk gelation.

| Yolk sample | Apparent viscosity (poises at 25°C) Not frozen | Frozen 165 min at −14°C |
|---|---|---|
| Cysteine-treated [a] | 13 | 115 |
| Untreated control [b] | 6 | 285 |

[a] 0.0055 moles of cysteine per 100 g of yolk. Final pH was about 6.1 at 25°C.
[b] Same pH, moisture content, and NaCl concentration as in cysteine-treated yolk mixture.

Although 0.166 moles of urea per 100 g of yolk did not increase the viscosity of native yolk appreciably, a viscosity change of frozen-thawed yolk containing such a urea concentration was considered possible since the protein molecules may be reoriented and more susceptible to urea denaturation. Table 4 shows that urea, when added to thawed yolk frozen at −4.5°C for either 40 min or 20 hr, produced no increase in viscosity. In contrast, the apparent viscosity of yolk stored 40 min at −14°C increased after the addition of 0.166 moles of urea per 100 g of yolk, from 80 poises to 200 poises in 63 min. With a longer freezer storage period of 70 min at −14°C, the thawed yolk was transformed from a fluid mass to a stiff gel after 63 min with the same urea treatment.

*Effect of sucrose in the presence of urea.* The inhibitory effect of sucrose on the viscosity increments of yolk due to the presence of urea has been demonstrated (Table 5). However, even with 0.014 moles of sucrose per 100 g of yolk, inhibition of viscosity change was not complete. A plot of the logarithm of the viscosity ratio $B/A$ against sucrose concentration yielded a straight line with a slope $[(d \log B/A)/dc]$ of about $10.1 \times 10^2$.

Table 4. Effect of urea on the viscosity of frozen-thawed yolk.

| Storage condition | Apparent viscosity (poises at 25°C) No urea | Urea treatment[a] 3 min | 33 min | 63 min |
|---|---|---|---|---|
| Not frozen | 29 | 22 | 22 | 22 |
| Frozen at: | | | | |
| −4.5°C | | | | |
| 40 min | 30 | 23 | 23 | 23 |
| 20 hr | 30 | 23 | 23 | 23 |
| −7°C | | | | |
| 40 min | 30 | 24 | 24 | 24 |
| 180 min | 41 | 25 | 25 | 25 |
| −14°C | | | | |
| 40 min | 80 | 160 | 190 | 200 |
| 70 min | 207 | 375 | 425 | stiff gel |

[a] Urea concentration was 0.166 moles per 100 g of yolk. Temperature of urea-yolk mixture was brought up to 25°C in a 38°C water bath with constant mechanical stirring (30 rpm). Urea reaction time was measured after the addition of urea.

*Electrophoretic studies on lipoproteins.* Paper electrophoresis was employed to detect differences in the electrophoretic mobilities of lipoproteins in native and frozen-thawed yolk. Although veronal buffer, pH 8.6, has been reported to be satisfactory for the separation of yolk proteins by paper electrophoresis (Evans and Bandemer, 1957), a buffer with a pH value close to that of native yolk was desired. With phosphate buffer (pH 6.5), two major lipoprotein complexes, $L_1$ and $L_2$, for native yolk were separated by paper electrophoresis (Fig. 3). The lipoprotein $L_1$ was non-mobile and was found to be the major lipoprotein complex in a crude lipovitellin fraction. The major lipoprotein complex in a crude lipovitellenin fraction corresponded in mobility to the lipoprotein $L_2$, which migrated toward the cathode.

Table 6 shows the migration distances of the two major lipoprotein complexes in yolk samples and the optical densities of solutions with dye, eluted from paper sections. The electrophoretograms for frozen-thawed yolk (Fig. 3) possessed a slow-migrating lipoprotein B with a migration distance of 14 mm. The high dye optical-density value of 0.238 for the non-migrating protein fraction A in frozen yolk, in contrast to an optical density of 0.151 for the lipoprotein $L_2$ of the native yolk (Table 6), indicates that proteins in lipoprotein $L_2$ complex were altered during freezing and thawing to form non-migrating components.

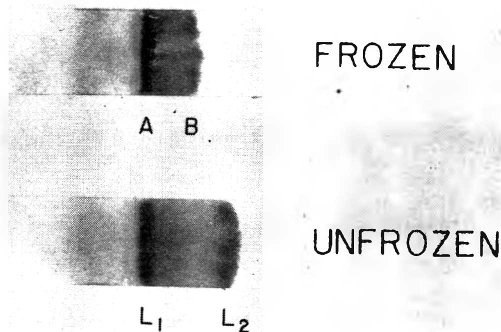


Fig. 3. Electrophoretograms of unfrozen and frozen yolk. $L_1$, lipovitellin; $L_2$, lipovitellenin; A and B, unidentified protein fractions.

Table 5. Effect of sucrose on the viscosity change of yolk in the presence of urea.

| Sucrose conc. (moles/100 g yolk) | Apparent viscosity (poises at 25°C) No urea *A* | Urea added[a] 150 min *B* | Viscosity ratio *B/A* |
|---|---|---|---|
| 0 | 15 | 235 | 15.7 |
| 0.001 | 14 | 245 | 17.5 |
| 0.003 | 14 | 210 | 15.0 |
| 0.006 | 13 | 150 | 11.5 |
| 0.008 | 13 | 125 | 9.6 |
| 0.011 | 11 | 85 | 7.7 |
| 0.014 | 11 | 60 | 5.5 |

[a] Urea concentration was 0.300 moles per 100 g of yolk. The pH of all mixtures was about 6.2 at 25°C.

**Experiments with crude lipovitellin fraction.** The physical change of the lipovitellin complex during yolk gelation could not be demonstrated by the paper electrophoretic techniques. Since Lea and Hawke (1952) observed that their ether-extracted lipovitellin, dispersed in sodium chloride solution, released lipid and became less soluble, experiments were initiated to investigate physical changes of crude lipovitellin (with no ether treatment) from freezing and thawing.

Crude lipovitellin suspensions, containing about 33% total solids with 15–17% ether-extractable lipid, were prepared with distilled water. Freezing below −6°C and thawing of a suspension gave a solid mass that reverted, with shaking, to a suspension whose appearance was similar to that of the unfrozen suspension. Alteration in the structure of the thawed crude lipovitellin was evidenced by a decrease in the transparency of the suspension when $CaCl_2$ solution was added. When 1 ml of 2*M* $CaCl_2$ solution was added to a 5-g portion of unfrozen lipovitellin suspension, a transparent solution resulted, whereas with the same volume of 2*M* $CaCl_2$ solution, the frozen-thawed lipovitellin suspension became opaque. Frozen-thawed lipovitellin suspensions containing either 10% NaCl, sucrose, maltose, raffinose, or arabinose, became as transparent as the unfrozen controls with added $CaCl_2$ solution.

To provide further evidence for the structural alteration of proteins in the crude lipovitellin during freezing and thawing, the viscosity change of a lipovitellin solution containing $CaCl_2$ was determined. The lipovitellin solution, with a pH of 5.7, had 0.1 moles of $CaCl_2$ per L and a total solids content of 30.9%, not including the $CaCl_2$. The solution, after standing 1 hr at 6°C, was frozen at −14°C for 12 hr. Upon thawing, the apparent viscosity of the solution was 200 poises at 25°C. On the other hand, the unfrozen control, having stood 13 hr at 6°C, had an apparent viscosity of 60 poises at 25°C.

Table 6. Migration distances of yolk lipoprotein fractions by paper electrophoresis, and optical densities of solutions containing dye eluted from paper strip sections.

| Yolk sample | Protein fraction | Migration distance[a] (mm) | Band width (mm) | Optical density[b] of eluted-dye solution |
|---|---|---|---|---|
| Unfrozen | lipoprotein, $L_1$ | 0 | 4 | 0.151 |
| | lipoprotein, $L_2$ | 28 | 4 | 0.157 |
| Frozen | fraction A | 0 | 5 | 0.234 |
| | fraction B | 14 | 6 | 0.040 |

[a] Distance between the origin and the front of the specific band.
[b] 590 mμ.

## DISCUSSION

Egg yolk is a complex biological system consisting of about 49% water, 17% proteins, 33% lipids, and 1% inorganic matter (Romanoff and Romanoff, 1949). Subjecting the yolk to high-speed centrifugation sediments granules with a high protein concentration (Schmidt *et al.*, 1956). It has been estimated that yolk granules occupy about 11–13% of the total yolk volume and contain as much as 40% of the total yolk proteins. Burley and Cook (1961) found that proteins in granules consisted of 70% α- and β-lipovitellin, 16% phosvitin, and 12% low-density lipoprotein. Apparently the lipovitellenin resides in the clear supernatant phase.

The results of several investigators indicate that lipoproteins are involved in the gelation mechanism. Feeney *et al.* (1954) found that gelation was less in crotoxin-treated yolk than in untreated yolk. The fact that lipoprotein fractions of yolk were decomposed by crotoxin to lysophospholipoproteins, provides support for the lipoprotein-gelation relationships. According to Fevold and Lausten (1946), lipovitellenin prepared from frozen-thawed yolk, unlike lipovitellenin from native yolk, did not dissolve in an ether-saturated salt solution. These investigators suggested that lipovitellenin from frozen-thawed yolk may be a

mixture of lipovitellenin and lipovitellin that cannot be separated in the usual way. Our study demonstrated the alteration in electrophoretic mobility of the so-called lipoprotein $L_2$ complex. Apparently the mobile lipoprotein B traveled in the lipoprotein $L_2$ band during electrophoresis of native yolk but could be separated electrophoretically from the other lipoproteins of the complex after freezing and thawing the yolk.

No information is yet available on the contribution of the lipovitellin complex (in yolk granules) to the viscosity increment of yolk caused by freezing and thawing. However, our experiments with a suspension of crude lipovitellin indicate that the protein molecules in this fraction are changed to form complexes that were not soluble in a $CaCl_2$ solution. In solution along with $CaCl_2$, the lipovitellin fraction was altered during a freezing-thawing period to the extent that the viscosity was increased. Calcium ions may be closely associated with lipovitellin during the freezing and thawing of yolk since about 87% of the total calcium in yolk is present in the granules (Schmidt *et al.*, 1956).

As shown in Fig. 1, the viscosity change of thawed yolk is dependent on a storage time-temperature (below −6°C) relationship. Under the conditions of constant thawing rate, the results show that the protein structural alterations, which are reflected in the viscosity change of thawed yolk, occur in the frozen state of yolk. Since the rate of viscosity increase was enhanced by a decrease in the storage temperature below −6°C, the possibility must not be overlooked that water plays an important role in the gelation. The lack of gelation in supercooled (−11°C for 7 days) yolk (Moran, 1924) also supports this contention. When a biological material is frozen, pure water is removed from solution to form ice crystals (Meryman, 1956). Consequently, the proteins become less hydrated and the concentration of salts increases. The breakdown of the water shells that, according to the concept of Klotz (1958), surround the protein molecules, could promote rearrangement and aggregation of yolk lipoproteins. Aggregation of yolk proteins during freezing and thawing may be due in part at least to the formation of divalent metal-ion linkages (Lea and Hawke, 1952).

At present, no explanation can be given for the rapid increase in the urea-yolk mixtures during the beginning of the storage period (Fig. 2). Klotz (1958) suggested that, under some circumstances, urea may disrupt the hydration lattice about protein molecules without changing the polypeptide configuration. As pointed out previously, disruption of the hydration lattice of proteins may occur during freezing. According to Bernardi and Cook (1960), lipovitellin in solvents containing 4*M* urea was converted to a slow-sedimenting component in 24 hours. With lower urea concentrations, two components were observed in the sedimentation patterns. Those investigators found that 4*M* urea solutions with lipovitellin in excess of 3% were transformed to a gel.

Evidence for the structural rearrangement of lipoprotein molecules in yolk due to freezing is supported by the experiment of the effect of a low concentration of urea on frozen-thawed yolk (Table 4). When urea at a level insufficient to cause an increase in the viscosity of native yolk was added to frozen (−14°C) and thawed yolk, viscosity increased immediately. The results suggest that the lipoprotein in frozen-thawed yolk had undergone some structural rearrangement and was more susceptible to urea denaturation.

The effects of sugars on the gelation of yolk may be due to inhibition of denaturation or aggregation of yolk proteins. Simpson and Kauzmann (1953) found that the rate of denaturation of albumin in the presence of urea was dependent on sucrose concentration. In our study, the slopes of straight lines from plots of log $B/A$ against sucrose concentration for frozen-thawed and urea-treated yolks (Tables 1 and 5) give a quantitative measure of the effectiveness of sucrose as a viscosity increment inhibitor. Since the slopes for frozen-thawed and urea-treated yolks have similar values, the mechanisms of viscosity increment inhibition for these treated yolks are perhaps alike.

Table 3 indicates that sodium chloride can inhibit viscosity changes of yolk due to freezing and thawing. Simpson and Kauz-

mann (1953) showed that urea denaturation of ovalbumin can be diminished by sodium chloride. Frensdorff *et al.* (1953) suggested that if salt linkages play an important part in the aggregation of protein molecules, electrolytes such as sodium chloride would be expected to inhibit aggregation since they stabilize the charged groups of the proteins.

Since low concentrations of sugars and sodium chloride effectively inhibit viscosity change of yolk upon freezing and thawing, the sulfhydryl-disulfide interchange reaction would not be expected to participate significantly in the gelation mechanism. This reaction should not be overlooked, however, particularly since Husaini and Alm (1955) reported that sulfhydryl groups are liberated during the freezing of egg white and cod muscle. Our studies show that cysteine added to native yolk will partially inhibit the viscosity increment caused by freezing and thawing. The cysteine may rupture the intramolecular S-S bonds in the lipoproteins and consequently bring about a partial uncoiling of the protein molecules.

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# Some Characteristics of a Proteolytic Enzyme System of *Pseudomonas fluorescens* [a]

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**SUMMARY**

**When grown in nutrient broth, *Pseudomonas fluorescens* produced a heat-labile proteolytic enzyme system that was active against blended fresh egg white, spray-dried egg white, and hemoglobin. The proteolytic enzyme system was released into the growth medium when the viable cell population had reached a maximum. The proteolytic activity was greater at the higher (25°C) than at the lower temperatures (15, 5°C) of culture incubation. Some purification was obtained by fractionation with ammonium sulfate and cellulose-column chromatography. The addition of $Fe^{++}$ to the enzyme preparation greatly increased its activity.**

In recent years numerous studies have reported on the types, growth characteristics, and certain other aspects of microorganisms capable of growing at a relatively rapid rate in foods stored at refrigeration temperatures. Information is limited, however, on the characteristics of proteolytic enzymes of psychrophiles. Vanderzant (1957) and Camp and Vanderzant (1957) reported on the endo- and extracellular proteolytic enzymes of *Pseudomonas putrefaciens*. The extracellular system was heat-labile and showed maximum activity against casein, α- and β-casein, and lactalbumin at pH 7.0–7.5. This proteolytic enzyme system was precipitated by 50–75% saturation with ammonium sulfate. A cell-free extract showed the presence of several peptidases with maximum activity at pH 7.0–8.0. With some peptidases, metal-induced activation (Mn, Mg) was observed. Some peptidases retained activity after 8 min at 60°C. Vanderzant and Moore (1955) studied the growth pattern and proteolytic activities of several pseudomonads, including *P. fluorescens* in milk. In a more recent study, Peterson and Gunderson (1960) reported on the proteolytic enzymes of *P. fluorescens* isolated from a frozen chicken pie. The proteolytic activity (with casein as substrate) was present in the growth medium during the early phases of growth. The present report presents data on the activity of a proteolytic enzyme system of *P. fluorescens* against hemoglobin, spray-dried egg white, and blended fresh egg white as substrates.

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## EXPERIMENTAL METHODS

**Cultures.** *P. fluorescens* strains 5, 10, and 17 were selected from a group of pure cultures isolated and identified in a previous study (Hurley *et al.*, 1962). The cultures were carried on slants of tryptone glucose extract (TGE) agar and were transferred daily for two transfers prior to each trial in the growth medium employed in the experiment. Unless indicated otherwise, the cultures of *P. fluorescens* were grown in nutrient broth in 5-gal. Pyrex carboys under slight aeration (1 psi). Incubation was at 25°C for 11 days. The cells were removed by centrifugation in a Sharples super-centrifuge (50,000 rpm). The cell-free growth medium was stored under refrigeration.

**Substrates.** Blended fresh egg white, spray-dried egg white, and hemoglobin were used as substrates to test the proteolytic activity of the cell-free growth medium and enzyme preparations.

The preparation of the blended fresh egg white is described in a previous study (Hurley *et al.*, 1962). Spray-dried egg white substrate (SDEW) consisted of 39 g of spray-dried egg white (Nutritional Biochemicals Corporation) in 1 L of double-distilled water to which 12 ml of 0.1*M* NaOH had been added. This mixture was autoclaved for 10 min at 121°C and 15 lb steam pressure. Following cooling to 21°C, sterile 0.1332*M* phosphate buffer was added to yield a 2% substrate with pH of 8.0.

The hemoglobin substrate was prepared according to a modification of the Northrup procedure as reported by Willms (1960). Five grams of lyophilized salt-free hemoglobin (Mann Research Lab.) was dissolved in 80 ml of distilled water. Following the addition of 80 g of urea, the mixture was incubated 1 hr at 37°C. A solution containing 10 g of urea dissolved in 125 ml of 0.1332*M* phosphate buffer was then added to give a 2% hemoglobin substrate with pH of 8.0.

**Determination of proteolytic activity.** Unless stated otherwise, 1 ml of the cell-free growth medium or enzyme preparation was added to 5 ml of substrate. Following incubation for 10 min at 37°C, 10 ml of 0.3*M* trichloroacetic acid (TCA) was added to terminate the reaction and to precipitate the remaining undigested protein. The tubes remained then in the waterbath for 1 hr at 37°C. The contents of the tubes were filtered through Whatman No. 3 filter paper. Measurement of absorbancy of the TCA filtrates at 280 m$\mu$ in a Beckman DU spectrophotometer was used to indicate extent of protein hydrolysis (Northrup *et al.*, 1948). Blanks were run with each trial by the addition, first, of 10 ml of TCA to 5 ml of substrate, and then the enzyme preparation.

**Bacterial counts.** TGE agar was the plating medium, with incubation of the plates at 25°C for 3 days.

**Fractionation procedure.** Fraction 1 was obtained from the cell-free growth medium by precipitation with ammonium sulfate with 0–50% saturation; fraction 2 was prepared by 50–85% saturation. In each case, the growth medium after addition of ammonium sulfate was allowed to stand 24 hr at 0°C. The precipitated material was collected on paper by filtration. It was then dissolved into 500 ml of cold 0.1332*M* Sorenson's phosphate buffer (pH 8.0). The ammonium sulfate was removed by dialysis against 0.0066*M* phosphate buffer at 2°C. The dialyzed fraction was reduced in volume to 500 ml by evaporation in dialysis bags with the aid of a fan. The material was then stored at 0°C until determinations were made of proteolytic activity and nitrogen.

**Cellulose-column chromatography.** Whatman diethylamino ethyl (DEAE) cellulose and carboxymethyl cellulose (CMC) were used. The DEAE (60 g) was soaked in 1*N* NaOH to ensure complete regeneration. It was washed first with 10 L of double-distilled water to remove the NaOH, and then with 0.002*M* sodium phosphate at pH 7.0. The suspension was allowed to settle for 1 hr. The fine material that did not settle was discarded. The DEAE slurry was then packed in 1.2×70-cm glass columns to a depth of 50 cm. The columns were packed with a vacuum to settle the cellulose and then further compacted with nitrogen (10 psi) until a column height was constant.

The CMC (60 g) was prepared by soaking it in 2 L of double-distilled water to which 10 ml of glacial acetic acid had been added. The CMC then was washed with 10 L of double-distilled water. The suspension was allowed to settle for 1 hr. The fine material that did not settle was discarded. The packing procedure was the same as that described for the DEAE column.

The packed column was then mounted on a Technicon (Technicon Chromatography Co.) fraction collector equipped with a Canalco (Canal Industrial Corp.) ultraviolet analyzer and automatic chart recorder. The column was washed with 1 L of the starting buffer prior to the addition of the sample. A 9-chamber Technicon Autograd, which contained the buffers, was then attached to the column. The fraction collection time per tube was 7 min, during which time 11 ml were collected. The buffer system was composed of the following: chamber 1 of autograd, double-distilled water; chamber 2, 0.002*M* $Na_2HPO_4$; chamber 3, 0.002*M* NaCl; chamber 4, 0.004*M* $Na_2HPO_4$; chamber 5, 0.004*M* NaCl; chamber 6, 0.02*M* $Na_2HPO_4$; chamber 7, 0.02*M* NaCl; chamber 8, 0.2*M* $Na_2HPO_4$, and chamber 9, 0.2*M* NaCl. The fractions separated with the cellulose columns were assayed for proteolytic activity with hemoglobin as substrate.

**Nitrogen determination.** The official micro-Kjeldahl technique (AOAC, 1960) was used to determine the nitrogen content of the samples and enzyme preparations.

## RESULTS

In preliminary experiments, *P. fluorescens* strains 5, 10, and 17 were grown at 25°C and at pH 7.0 in both nutrient and TGE broth. In subsequent experiments the cultures were grown in nutrient broth adjusted to pH 6.0, 7.0, and 8.0, with incubation at 5, 15, and 25°C. The proteolytic activity of the cell-free growth medium was determined by allowing it to react with the hemoglobin substrate for 1 hr. The results of these experiments indicated that proteolytic activity was greatest in the cell-free nutrient broth medium of strain 17 incubated at 25°C. Fig. 1 presents data on the effect of time of incubation at 25°C on the produc-

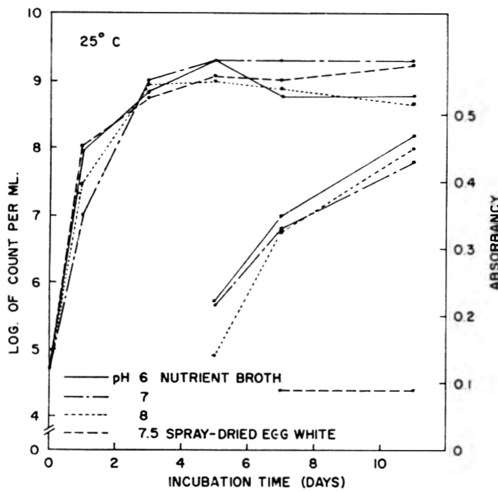



Fig. 1. Effect of age of culture when incubated at 25°C on the bacterial count and on the proteolytic activity (1 ml of enzyme preparation incubated with hemoglobin at pH 8.0 and 37°C for 1 hr) of the cell-free growth medium.

tion of proteolytic enzymes in: a) nutrient broth cultures (strain 17) initially adjusted to pH 6, 7, and 8; and b) a 2% solution of spray-dried egg white (pH 7.5). Little or no proteolytic activity was detected until after 5 days of incubation, when in most cases the level of viable population had reached a maximum. The patterns of growth were similar at 15 and 25°C. The level of population during the first 7 days of incubation was somewhat lower at 5°C than at 15 or 25°C. Following incubation for 7–11 days, levels of population were very similar at 5, 15, and 25°C. In general, after 1 week of incubation the proteolytic activity of the cell-free nutrient broth medium was greatest in the cultures incubated at 25°C, followed by those at 15 and 5°C. The proteolytic activity of the cell-free SDEW medium, however, was greatest when incubated at 5°C for 7–11 days. At culture incubation temperatures of 25 and 15°C, however, proteolytic activity was much greater in the cell-free nutrient broth medium than in the cell-free SDEW medium. Although

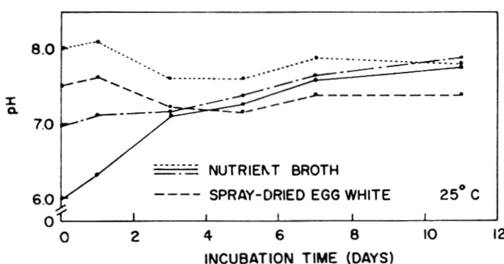
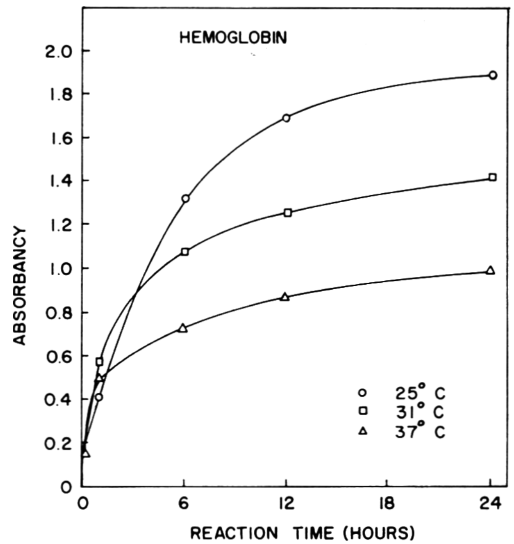



Fig. 2. Effect of age of culture when incubated at 25°C on the pH of the growth medium initially adjusted to pH values ranging from 6.0 to 8.0.

some of the differences in level of viable population and proteolytic activity of the cultures can probably be ascribed to differences in pH value of the cultures, no definite pattern could be established. After incubation at 5 and 15°C for 7–11 days the viable counts of the nutrient broth cultures with initial adjustment to pH 6, 7, and 8 were very close. The same was true for the nutrient broth cultures incubated at 25°C for 5 days. This, however, was followed by a decrease in count in the cultures in which the pH was initially adjusted to 6.0 and 8.0. In most cases little difference in



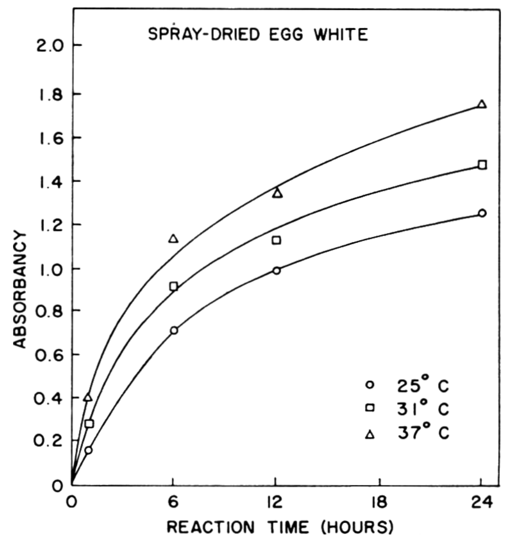




Fig. 3. Effect of time and temperature of reaction on the proteolytic activity of the cell-free growth medium against hemoglobin and spray-dried egg white (1 ml of enzyme preparation incubated with substrate at pH 8.0).

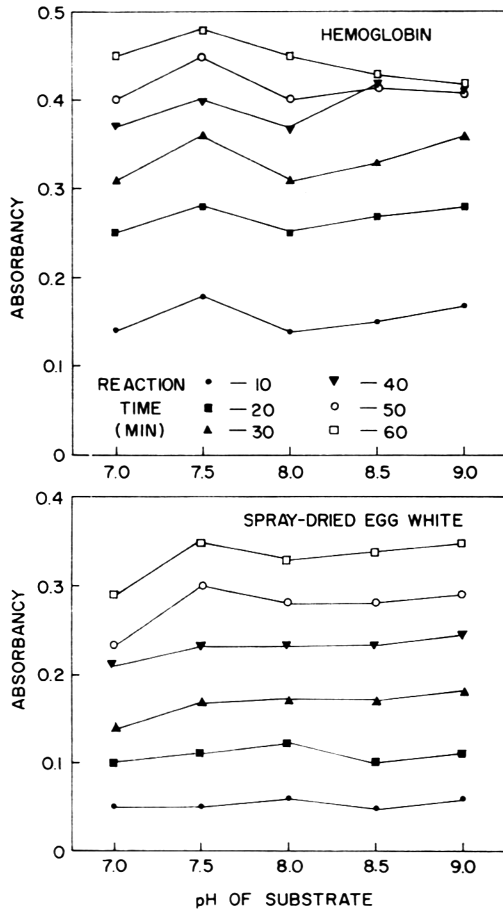



Fig. 4. Effect of pH of substrate and time of reaction on proteolysis (1 ml of enzyme preparation incubated with substrate at 37°C).

proteolytic activity was found. The initial adjustment of the media to pH 6.0, 7.0, and 8.0 had in most cases little influence on the proteolytic activity of the cell-free nutrient broth media after 11 days of incubation. The data in Fig. 2 indicate that after 7–11 days at 25°C the pH values of the cultures initially adjusted to pH 6, 7, and 8 had adjusted themselves to values ranging from 7.3 to 7.9. The changes in pH values at 15 and 5°C were similar to those at 25°C. Fig. 3 indicates that within the temperature limits of this experiment, proteolytic activity of the cell-free growth medium against SDEW was maximum at 37°C, with somewhat lower activities at 31 and 25°C. With hemoglobin as substrate, however, activity after 1 hr of reaction time was maximum at 25°C, with somewhat decreased activities at 31 and 37°C. After reacting for 1 hr, however, the differences in proteolytic activities at the different reaction temperatures were only slight. To study the effects of pH of substrate and reaction time on the extent of proteolysis of hemoglobin and spray-dried egg white solution, the substrates were adjusted to the pH values ranging from 7 to 9 (phosphate buffer), and reacted with the enzyme preparations at 37°C for 0, 10, 20, 30, 40, 50, and 60 min. The results are presented in Fig. 4. The extent of proteolysis was greater with increased reaction time. In most cases, little difference in proteolysis of SDEW was observed at the various pH levels. With hemoglobin as substrate, however, proteolysis was optimum at pH 7.5, and in some cases with a second optimum at pH 9.0. The data of Fig. 4 were also used to check the effect of reaction time on hydrolysis of hemoglobin by the enzyme preparation. Up to 1 hr a linear relation existed between proteolysis and reaction time with spray-dried egg white as substrate at pH values of 7.0–9.0. Subsequent experiments indicated that a similar relation with hemoglobin as substrate existed up to 10–20 min. The data in Fig. 5 indicate a linear relation between quantity of enzyme preparation and proteolysis of hemoglobin or spray-dried egg white solution up to and including 1 ml of cell-free growth medium.

As shown in Fig. 6, the rate of proteolytic activity increased up to a concentration of 1.3–1.4% against hemoglobin as substrate and up to 1.8% against SDEW.

**Heat stability of proteolytic activity.** One-ml quantities of cell-free growth medium were placed in test tubes. The test tubes were placed in constant-temperature water baths at 50, 60, and 70°C. After the contents of the tubes had reached the desired temperature, a sample and a blank were

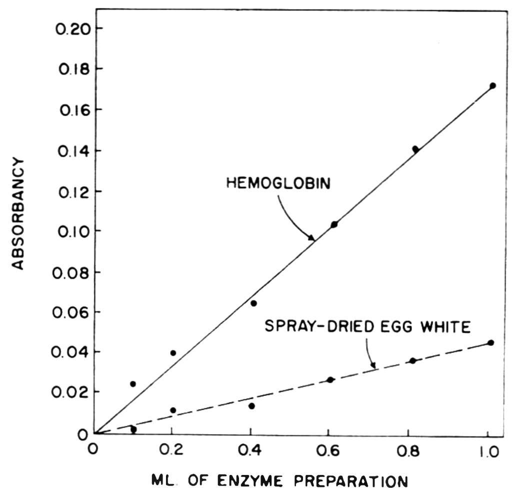



Fig. 5. Effect of quantity of cell-free growth medium on proteolysis (enzyme preparation incubated with substrate at pH 8.0 and 37°C for 10 min).

Table 1. Effect of various compounds on the proteolytic activity[a] of the cell-free growth medium of *P. fluorescens* strain 17.

| Compounds | Hemoglobin 0.01 *M* | Hemoglobin 0.005 *M* | Hemoglobin 0.001 *M* | Spray-dried egg white 0.01 *M* | Spray-dried egg white 0.005 *M* | Spray-dried egg white 0.001 *M* |
|---|---|---|---|---|---|---|
| $MnSO_4$ | I[b] | I | I | 0 | I | I |
| $HgCl_2$ | I | I | I | I | I | I |
| $CoCl_2$ | I | I | A | I | I | I |
| $NiCl_2$ | I | I | I | I | I | I |
| KCN | I | I | I | I | I | I |
| $CaCl_2$ | 0 | I | I | I | I | I |
| $FeCl_3$ | I | I | I | I | I | I |
| $FeCl_2$ | A+++ | A | A | A++ | A | A |
| NaF | I | I | I | I | I | I |
| $MgSO_4$ | 0 | A | A | 0 | I | I |
| $CuSO_4$ | I | 0 | A | I | I | I |
| $ZnSO_4$ | I | 0 | A | I | I | I |
| $Na_2SO_3$ | A+ | I | I | 0 | 0 | I |
| $PbSO_4$ | I | I | I | 0 | I | I |
| Ascorbic acid | A++ | A | I | 0 | I | I |
| Cysteine | I | I | I | 0 | I | I |

[a] 1 ml of enzyme preparation incubated with added compound and then with substrate at pH 8.0 and 37° for 10 min.

[b] A = activated, + = weak, ++ = medium, +++ = strong, 0 = no change from blank, I = inactivated.

removed at desired intervals and quickly immersed in ice water. The proteolytic activity of the sample was then determined. When hemoglobin was used as substrate the time required to inactivate the enzyme preparation was almost 28 min at 50°, 40 sec at 60°, and 20 sec at 70°C. With spray-dried egg white as substrate, activities were lower and inactivation times were smaller.

**Effect of activators and inhibitors.** To test the effect of metal ions and certain other compounds on the proteolytic activity of the enzyme preparation, 1 ml of enzyme preparation was preincubated for 1 hr at 37°C with 1 ml of 0.01, 0.005, and 0.001*M* solutions each of $MnSO_4$, $HgCl_2$, $CoCl_2$, $NiCl_2$, KCN, $CaCl_2$, $FeCl_3$, NaF, $MgSO_4$, $CuSO_4$, $ZnSO_4$, $FeCl_2$, $Na_2SO_3$, $PbSO_4$, ascorbic acid, and cysteine. Following the 1-hr preincubation period, 4 ml of substrate were added. A standard assay for proteolytic activity was then conducted (Table 1). The proteolytic activity of the cell-free growth medium against both hemoglobin and spray-dried egg white greatly increased when preincubated with a 0.01*M* solution of $FeCl_2$. Although some other compounds caused slight increases in proteolytic activity against either hemoglobin or spray-dried egg white, only $FeCl_2$ increased the proteolytic activity against both substrates.

**Fractionation of the cell-free growth medium.**

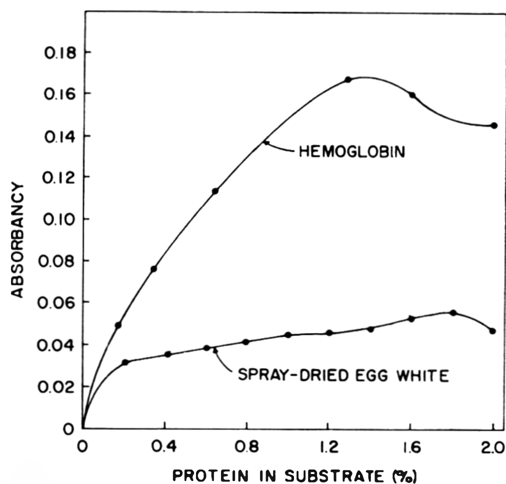



Fig. 6. Effect of substrate concentration on proteolysis (1 ml of enzyme preparation incubated with substrate at pH 8.0 and 37°C for 10 min).

Table 2 presents data on the proteolytic activity of the cell-free growth medium and the fractions prepared from this medium by ammonium sulfate precipitation. In these experiments 1 ml of each preparation was preincubated 15 min at 37°C with 1 ml of 0.01*M* $FeCl_2$. After preincubation, 4 ml of substrate were added and the enzyme and substrate were allowed to react for 10 min at 37°C. Fractions 1 and 2 had greater specific activity than did the cell-free growth medium. Each of the preparations also showed proteolytic activity against fresh egg white.

Table 2. Proteolytic activity[a] of the cell-free growth medium, fraction 1 and fraction 2.

| | Absorbancy of TCA filtrate at 280 mμ | N (mg/ml) | Specific activity (absorbancy/mg N/ml) |
|---|---|---|---|
| Growth medium | | | |
| Hemoglobin | 0.663 | | 0.62 |
| | | 1.07 | |
| Fresh egg white[b] | 0.192 | | 0.18 |
| Fraction 1 | | | |
| Hemoglobin | 0.453 | | 2.53 |
| | | 0.179 | |
| Fresh egg white | 0.066 | | 0.37 |
| Fraction 2 | | | |
| Hemoglobin | 0.716 | | 0.71 |
| | | 1.01 | |
| Fresh egg white | 0.264 | | 0.26 |

[a] 1 ml of enzyme preparation preincubated with $FeCl_2$ and then with substrate at pH 8.0 and 37°C for 10 min.

[b] 10.5% protein substrate.

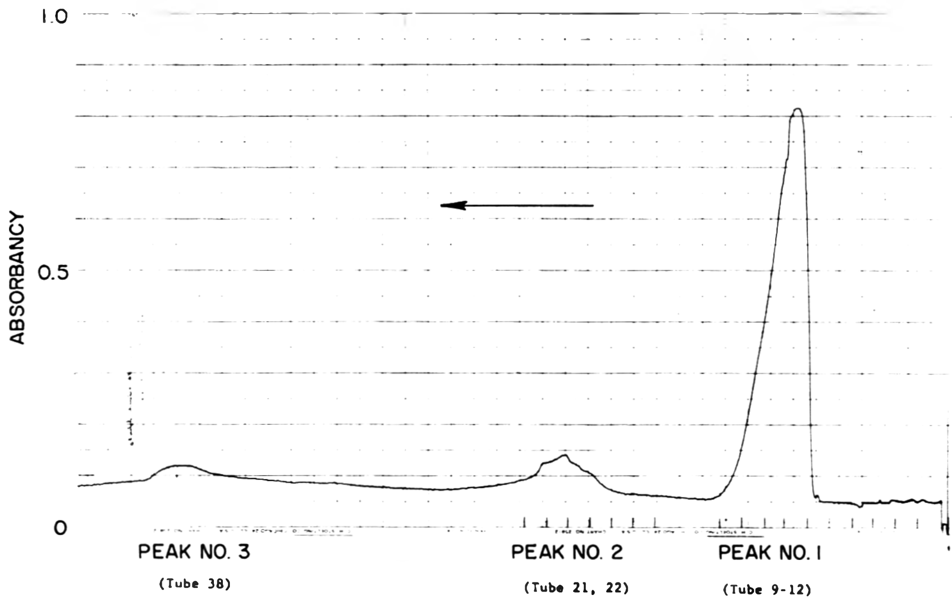



Fig. 7. Changes in absorbancy at 280 mμ of Fraction 2 as the sample passed through a DEAE column.

**Cellulose ion-exchange**. A sample of fraction 2 was diluted 1:5 with double-distilled water and passed through DEAE column. Fig. 7 shows the changes in absorbancy as the fraction passed through the column. Table 3 shows the proteolytic activity (hemoglobin substrate) of the effluents collected during the recording of the peaks. These samples collected from the peak areas had considerably higher specific activities than the fraction had before passing through the column. A sample of fraction 2 was also placed on a CMC column, but passed through this column without separation.

Table 3. Proteolytic activity[a] of effluents collected during the DEAE column study.

| | Absorbancy of TCA filtrate at 280 mμ | N (mg/ml) | Specific activity |
|---|---|---|---|
| Buffer blank | | | |
| Tube #5 | 0 | 0 | 0 |
| Peak 1 | | | |
| Tube #9 | 0.432 | 0.128 | 3.38 |
| Tube #10 | 0.742 | 0.486 | 1.53 |
| Tube #11 | 1.301 | 0.314 | 4.14 |
| Tube #12 | 1.523 | 0.214 | 7.12 |
| Peak 2 | | | |
| Tube #21 | 1.347 | 0.086 | 15.66 |
| Tube #22 | 1.398 | 0.143 | 9.78 |
| Peak 3 | | | |
| Tube #38 | 1.046 | 0.199 | 5.26 |

[a] 1 ml of enzyme preparation preincubated with $FeCl_2$ and then with hemoglobin at pH 8.0 and 37°C for 10 min.

## DISCUSSION

The results indicate that under certain conditions of incubation *P. fluorescens* strain 17 produced a heat-labile proteolytic enzyme that was active against hemoglobin and spray-dried egg white. Proteolysis of fresh egg white was observed only upon activation of the enzyme preparation with $FeCl_2$. Iron also increased the proteolytic activity against hemoglobin and spray-dried egg white. The activity of iron is interesting in view of reports (Garibaldi and Bayne, 1960) that washing of eggs in water with 5–10 ppm of iron caused more spoilage than washing in water containing less than 1 ppm of iron. Furthermore, others (Garibaldi, 1960; Feeney and Nagy, 1952) have reported that the growth of some microorganisms in eggs may be retarded because of the iron-binding properties of conalbumin. Recent studies (Hurley *et al.*, 1962), however, have shown that marked increases in the viable population of various strains of *P. fluorescens* can occur in egg white without added iron and without prior adjustment of pH. The lack of iron available for activation of enzyme activity, however, may explain the lack of

proteolysis in blended egg white at high levels of viable cells even after extensive periods of incubation. The results of these and other studies indicate that the amount of iron present in eggs may affect not only the extent of growth of some microorganisms but also the activity of their enzyme systems.

Information on the production and characteristics of proteolytic enzymes from psychrophiles is limited. Peterson and Gunderson (1960) reported on the proteolytic enzymes of a strain of *P. fluorescens* isolated from a frozen chicken pie. In general the pattern of growth of this strain at the various temperatures of incubation was similar to that of *P. fluorescens* strain 17. In their study, however, liberation of proteolytic enzymes in the growth medium was: a) observed during the early phases of culture incubation, when in most cases the cells were in the logarithmic phase of growth; and b) greatest at the lower incubation temperatures and decreased with increasing temperature. In a study on the growth and proteolytic activity of a strain of *P. fluorescens* in milk, Vanderzant and Moore (1955) reported that proteolytic activity was low until after 10 days, when the level of viable population had almost reached a maximum. The activity was greater at 25 than at 5°C. In the present study, proteolysis was: a) absent or slight until the level of viable population had reached a maximum (after 5 days), and b) greater at the higher temperatures of incubation. Several factors may be directly or indirectly responsible for these differences in results: a) a difference in growth medium (nutrient broth versus tryptone-glucose-meat extract broth); the possibility exists that the protein in the growth medium may have tied up iron so that the amount available during the early stages of growth was insufficient; b) differences in substrates and methods of analysis to detect proteolysis, hemoglobin, spray-dried egg white, and blended fresh egg white versus casein (Peterson and Gunderson, 1960); and c) strain differences (Long and Hammer, 1941; Vanderzant and Nelson, 1953; Vanderzant, 1957; Hurley *et al.*, 1962). With respect to strain differences, numerous studies with many species of bacteria have shown that certain strains may possess high proteolytic activity, others medium, and still others little or none at all. Furthermore, data for characteristics of enzyme systems of psychrophiles are usually reported for one or a few strains only. Other studies, including one by Kereluk *et al.* (1961), have shown that the growth pattern of strains of *P. fluorescens* may differ greatly. It should also be kept in mind that nearly all information available on proteolytic enzymes of psychrophiles is on preparations that are far from pure and that may contain various proteolytic enzyme systems. Choice of a certain set of conditions with respect to growth of culture, enzyme preparation, or type of substrate may be highly selective for one or more systems. Selection of a different set of conditions may favor a different enzyme system(s). Although the results obtained in this investigation furnish answers to a number of questions concerned with the proteolytic enzyme systems of *P. fluorescens*, they also point out the great need for further investigations on the proteolytic enzyme systems of various strains of species of *Pseudomonas*.

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# Peonidin-3-monoglucoside in Vinifera Grapes

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**SUMMARY**

**The band-four pigment in the general eight-band pattern for red vinifera grapes as observed in this laboratory was isolated from Freisa grapes with paper chromatographic techniques. Hydrolysis, partial hydrolysis, and alkaline microdegradation studies on the pure pigment demonstrated that it was peonidin-3-monoglucoside.**

## INTRODUCTION

The development of techniques using paper chromatography has made possible the separation of the many anthocyan pigments of grape skins. Moderate quantities of relatively pure compounds can be isolated for structure determinations by multiple-bar chromatographs on thick papers.

Rankine *et al.* (1958), Albach *et al.* (1959) and Ribéreau-Gayon (1953) have shown that the pigments of the majority of the red wine varieties of *Vitis vinifera* are similar in that eight discrete pigment bands are obtained on paper chromatography. The relative amounts of the different pigments vary and certain of the pigment bands are absent in some varieties. The pigment present in greatest amounts in most of the red wine viniferas is malvidin-3-monoglucoside. This pigment corresponds to band 5 in the nomenclature of Rankine and Albach, in which band 1 is that of highest $R_f$ on an ascending chromatogram on Whatman No. 1 paper using the butanol–acetic acid–water solvent system. The identity of band 4, which has a rose or red-orange color, is the subject of this report. The source of pigment for the investigation was the grape, *Vitis vinifera*, var. Freisa, in which band 4 is present in high relative concentration.

## EXPERIMENTAL

**Chromatographic solvent systems used.**

A-1. Less-dense phase of n-butanol–glacial acetic acid–water (4:1:5) (Bate-Smith, 1950).

A-1c. n-Butanol–glacial acetic acid–water (6:1:2) (Harborne, 1959).

A-2. Forestal solvent (Harborne, 1958).

A-3. More-dense phase of m-cresol–glacial acetic acid–water (50:2:48) (Bate-Smith, 1950).

A-4. Less-dense phase of n-butanol–2N hydrochloric acid mixture (1:1) (Harborne, 1958).

A-5. Water and conc. hydrochloric acid (97:3) (Harborne, 1958).

A-6. 90% aq. formic acid and 3N hydrochloric acid (1:1) (Roux, 1957).

A-7. 90% aq. formic acid–conc. hydrochloric acid–water (5:2:3) (Harborne, 1958).

A-8. Water–glacial acetic acid–conc. hydrochloric acid (82:15:3) (Harborne, 1958).

A-9. 2N hydrochloric acid (Bate-Smith, 1950).

A-10. Less-dense phase of n-butanol–conc. hydrochloric acid–water (5:1:2) (Endo, 1959).

S-1. n-Butanol–pyridine–water (6:3:1) (Endo, 1959).

S-2. Acetone–n-butanol–water (7:2:1) (Heftmann, 1961).

P-1. Less-dense phase of n-butanol and water mixture (Doporto *et al.*, 1955).

P-2. Ethyl acetate–glacial acetic acid–water (9:2:2) (Pridham, 1957).

P-3. Less-dense phase of benzene–glacial acetic acid–water (2:2:1) (Bray and Thorpe, 1954).

**Isolation of band No. 4 pigment.** Clusters of mature Freisa grapes (vines 160:9, 10, Department of Viticulture and Enology vineyards, University of California, Davis) were picked in 1958. The grape skins were separated from the pulp, and the clean skins were washed several times in distilled water. The skins were then dehydrated by placing them in a vacuum desiccator over anhydrous calcium chloride for two days at 25 mm Hg pressure under a nitrogen atmosphere.

[a] Abstracted from an M.S. thesis by R. F. Albach, 1960.

Five grams of desiccated skins were ground with approximately 10 g of fine white sand until the mixture was homogeneous in appearance, 50 ml of 2% methanolic hydrochloric acid were added and the grinding was continued for several minutes. The extract was removed by vacuum filtration and the residue washed several times with 10-ml volumes of the extracting solvent. The combined filtrates were reduced to a volume of 30 ml by vacuum evaporation under a stream of nitrogen.

The pigment mixture was placed on Whatman 3MM chromatographic paper (approximately 3.5 ml of concentrate per sheet) as a bar along the long direction of the paper and developed in a Reco Chromatocab, Model A, by the ascending flow of solvent A-1 for 24 hours. The chromatogram was removed from the chamber, dried, and then rechromatographed three times as in the multiple-development technique described by Smith (1958). After completion of this multiple development of the chromatograms, band 4 was cut out, eluted either with methanol or with 2% methanolic hydrochloric acid, and rechromatographed several times in similar fashion until it was chromatographically pure when checked with several solvent systems. Evaporation of the last methanol eluates to dryness resulted in the isolation of approximately 50 mg of pure band-4 pigment.

**Partial hydrolysis.** With the techniques of Abe and Hayashi (1956) a 2-mg sample of band-4 pigment and 3 ml of an aqueous 10% hydrochloric acid solution were placed in a 12-ml centrifuge tube fitted with a cold finger condenser, and the mixture was brought to a boil by heating in a brine bath. Starting with the onset of boiling, 20 µl samples were removed from the reaction mixture at 5-min intervals for the first ½ hr. Each of the samples was spotted in sequence on a series of Whatman No. 1 chromatographic papers and developed with solvents A-1 to A-10 inclusive. All the chromatograms of partial hydrolysis products were observed under ultraviolet light.

**Comparison of band-four anthocyanidin with known anthocyanidins.** One mg of band-4 pigment was hydrolyzed by boiling 1 hr in 1 ml of 10% hydrochloric acid. The anthocyanidin resulting from hydrolysis was extracted with 2 ml of isoamyl alcohol, spotted on Whatman No. 1 paper along with samples of known anthocyanidins in isoamyl alcohol, and developed by the ascending technique with solvent systems A-1, A-2, and A-7.

**Alkaline microdegradation.** The alkaline degradation scheme reported by Karrer and Widmer (1927) was adapted to a micro scale, and a nitrogen atmosphere was used. One mg of band-4 pigment and 0.4 ml of hot 15% barium hydroxide solution were placed in a 15-ml centrifuge tube fitted with a cold finger condenser and a system to permit maintenance of a nitrogen atmosphere. The mixture was heated 1 hr in a boiling concentrated brine bath, cooled, acidified with a few drops of concentrated hydrochloric acid, and extracted with 0.8 ml of ether. Twenty µl of the ether extract from the alkaline degradation were spotted on Whatman No. 1 paper along with a series of 20-µl samples (4 µg of compound) of ether solutions of possible phenolic products and developed by the ascending technique in solvents

Table 1. Chromatographic comparisons of anthocyanidins.

| Solvent | Anthocyanidin | Rf Value | |
|---|---|---|---|
| | | Observed | Reported [a] |
| A-1 | Band-4 aglycone | 0.71 | 0.71 (Peonidin) |
| | Cyanidin | .67 | .68 |
| | Delphinidin | .34 | .42 |
| | Petunidin | .47 | .52 |
| | Malvidin | .56 | .58 |
| A-2 | Band-4 aglycone | .75 | .63 (Peonidin) |
| | Cyanidin | .58 | .44 |
| | Delphinidin | .36 | .32 |
| | Petunidin | .54 | .46 |
| | Malvidin | .68 | .60 |
| A-7 | Band-4 aglycone | .34 | .30 (Peonidin) |
| | Cyanidin | .25 | .22 |
| | Delphinidin | .15 | .13 |
| | Petunidin | .23 | .20 |
| | Malvidin | .26 | .27 |

[a] Harborne (1958).

Table 2. Chromatographic comparison of alkaline degradation products with phenolic compounds.

| Compound | $R_f$ values | | | | Chromogenic treatment | |
|---|---|---|---|---|---|---|
| | Solvents | | | | | |
| | A-1c | P-1 | P-2 | P-3 | Diazonium reagent | NaOH sol'n and heat |
| Band-4 deg. spot A | 0.78 | 0.68 | 0.87 | 0.00 | Orange-red | Brown |
| Band-4 deg. spot B | .93 | .73 | .97 | .83 | Yellow | Blue |
| Phloroglucinol | .80 | .69 | .86 | .00 | Orange-red | Brown |
| p-Hydroxybenzoic acid | .97 | .82 | .98 | .45 | Pale yellow | Purple |
| Protocatechuic acid | .90 | .54 | .93 | .07 | Brown | Dark brown |
| Gallic acid | .72 | .22 | .76 | .00 | Yellow | Brown |
| Vanillic acid | .95 | .73 | .97 | .83 | Yellow | Blue |
| Syringic acid | .92 | .56 | .95 | .82 | Yellow-orange | Brown |

A-1c, P-1, P-2, and P-3. After chromatography the phenolic spots were visualized by coupling with p-toluene diazonium chloride as described by Swain (1952). The colors of the spots were noted immediately after coupling and after treatment with 0.1*N* NaOH followed by heating for 10 min in an oven at 110°C. Two distinct phenolic spots were present from the alkaline degradation of band 4 and were designated spots A and B.

**Sugar moiety identification.** Approximately 1 mg of band-4 pigment was hydrolyzed by boiling for 1 hr in 1 ml of 10% hydrochloric acid. The mixture was cooled to 0°C and 12 grains of Amberlite IRA 410 ion-exchange resin were added. After storage at 0°C overnight, the ion-exchange resin had become red from adsorption of the anthocyanidin. Twenty-μl portions of the clear supernate were spotted on two Whatman No. 1 chromatographic papers along with a series of spots containing known sugars, and the chromatograms developed using solvent S-1 by the ascending and S-2 by the descending technique. After development the chromatograms were dried and the sugar spots visualized by treatment with the aniline hydrogen phthalate reagent described by Partridge (1949). The positions of the sugars were indicated by the presence of brown or blue spots. The relative positions of the spots were indicated by calculation of the $R_g$ values (Smith, 1958).

## RESULTS AND DISCUSSION

Freisa grapes had been shown by surveys of a large number of vinifera varieties in this laboratory (Rankine *et al.* 1958; Albach *et al.* 1959) to be one of the best sources of band-4 pigment. The amounts of pigments in the various bands were observed to vary widely in the vinifera varieties investigated. Ribéreau-Gayon (1959) observed that the relative amounts of pigment in the various bands also differ considerably within the same variety, depending on whether it is grown in California or in France.

Partial hydrolysis of the band-4 pigment in this investigation gave only two pigment spots in each of ten different solvent systems, the original pigment spot decreasing and the new spot increasing in intensity as the hydrolysis progressed. Neither spot exhibited fluorescence under ultraviolet light. An authentic sample of cyanidin-3,5-diglucoside, submitted to the same partial hydrolysis technique as a check, gave three spots in solvents A-7, A-8, and A-9, and gave the theoretically possible four spots in solvent A-10. The original diglucoside spot and one of the new spots fluoresce brilliantly under ultraviolet light. This is consistent with Harborne's (1958) statement that the fluorescence is characteristic of anthocyanins with the 5-position occupied. Assuming the generality of the fact (Endo, 1959) that attachment of sugar residues is restricted to the 3- and 5-positions in natural anthocyanins, the results with partial hydrolysis indicate that the band-4 pigment is a 3-monoglycoside.

Chromatographic comparison of the anthocyanidin from the band-4 pigment with authentic samples of cyanidin, malvidin, petunidin, and delphinidin in three solvent systems (Table 1) clearly demonstrates that it is different from each of these, and comparison of the observed $R_f$ values with values reported by Harborne (1958) further suggests that the band-4 anthocyanidin is peonidin. This was confirmed by alkaline

Table 3. Chromatographic comparisons of sugars.

| Sugar | Solvent: S-1 | S-2 |
|---|---|---|
| Band-4 sugar | 100 | 97 |
| Glucose | 100 | 100 |
| Fructose | 130 | 123 |
| Galactose | 81 | 87 |
| Rhamnose | 234 | 325 |
| Arabinose | 136 | 167 |
| Xylose | 162 | 200 |

microdegradation studies on the band-4 pigment, which gave phloroglucinol and vanillic acid (Table 2), the expected products from peonidin derivatives (Karrer and Widmer, 1927). The data in Table 3 clearly demonstrate that the sugar in the band-4 pigment is glucose.

The results conclusively identify the band-4 pigment as peonidin-3-monoglucoside. This result is consistent with the proposal by Dupuy and Puisais (1955) that peonidin-3-monoglucoside is present in vinifera grapes, and with Ribéreau-Gayon (1959), who indicated that band D in his two-dimensional pigment pattern from vinifera grapes grown in France was peonidin-3-monoglucoside. It is in contrast, however, with the report (Colagrande and Grandi, 1960) of peonidin-3-monofructoside as a constituent of pigments from vinifera grapes grown in Italy.

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# The Essential Oil of Schinus molle: The Terpene Hydrocarbon Fraction

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**SUMMARY**

**Analysis by gas-liquid chromatography of the terpene hydrocarbon fraction of the essential oil obtained by steam distillation of the fruit of *Schinus molle* L. revealed 11 components. Capillary columns and flame ionization detection increased resolution and permitted tentative identification of 6 additional terpene hydrocarbons not hitherto reported in the oil. The compounds found were: α-pinene, β-pinene, α-phellandrene, β-phellandrene, myrcene, D-limonene, camphene, *p*-cymene, and three unidentified constituents. These components were tentatively identified by calculating relative corrected retention volumes and comparing these with values for known terpene hydrocarbons. The three stationary liquid phases used yielded data that lend credence to the tentative assignment of peak identities.**

In addition to being a handsome ornamental tree, the California Pepper (*Schinus molle* L.) provides an interesting excursion into terpene chemistry. Steam distillation of the berries and leaves yields a volatile oil that has been used as a substitute for black pepper, in flavoring compositions, and in pharmaceutical products (Gonzales and Lombardo, 1946; Ottolino, 1948). In Greece the berries serve for the preparation of certain beverages (Guenther, 1952), and the bark of the tree is sometimes used in leather-tanning preparations (Stather, 1931).

A number of investigators have examined the physicochemical properties and chemical composition of the oil, but to date only some six terpenoids have been tentatively identified (Brückner van der Lingen, 1930; Gonzales, 1931; Ottolino, 1948). Cremonini (1928, 1930) reported the presence of *trans*-terpin, ferrojone, and a sugar with properties similar to glucose. It is quite likely that the presence of this sugar imparts to the oil the ability to reduce Fehling's solution, a useful method for the detection of adulteration of black pepper oil by *Schinus molle* oil (Cremonini, 1930; Nuñez-Samper, 1953; Escalante and Liceaga, 1956). Cremonini (1928) was able to find no thymol, limonene, or piperine in the oils he examined. Numerous researchers (Guenther, 1952) have established the presence of two terpene hydrocarbons, α-pinene, and β-phellandrene, and the terpenoids carvacrol and sobrerol [identification of the latter is quite tentative (Guenther, 1952)].

The physical constants for Pepper Tree oil are as numerous and varied as have been investigations of the oil. Guenther (1952) presents values for densities that range from 0.850 to 0.866 with no mention made as to the temperature during the determinations. Refractive indices, specific rotations, acid numbers, and ester numbers also vary considerably. The range of values for these constants is:

$[\alpha]_D^{17}$ + 26° 55′ to 62° 42′

$n_D^{20}$ 1.47818 to 1.47900

Acid number: 0.4 to 2.5

Ester number: 8.3 to 25.2

Brückner van der Lingen (1930) reports constants for the oil obtained from green leaves and green berries of South African Pepper trees as follows:

Density: 0.8486 (no temperature specified)

$[\alpha]_D$ + 68° 24′

$n_D^{20}$ 1.4732

Acid number: less than 0.1

Ester number: 46.70 (after acetylation 115.80)

It appears quite probable that these differences in constants are due, at least in part, to the wide variety of oils examined, e.g., from Algerian, French, Mexican, and South African sources; and to the fact that many oils were composites prepared from both leaves and berries (some of which were green).

Ottolino (1948) studied the yield of oils from various parts of the tree and was able to show that the leaves contained about 0.2% oil in winter and up to 1% oil in early fall. The flowers contained 2.1% oil, and the fruit 1.6% (state of maturity not specified).

From the above, it is likely that no two investigators examined comparable oils. In addition, most of the studies were conducted before the advent of chromatography and it is conceivable that many constituents present in small amounts were not detected. The present study was undertaken to re-examine previous investigations and to determine if additional terpene hydrocarbons could be detected with one of the more modern methods of analysis, gas-liquid chromatography (GLC).

## EXPERIMENTAL

The berries used were harvested from trees grown for ornamental purposes in William Land Park, Sacramento, California. They were picked in October, 1960; the outer covering (skin) had reached a deep red-brown. Immediately after harvest, the berries were brought to the laboratory, where they were rinsed with cold water and dried with clean cloths. Pedicels and skin were carefully removed by hand from each fruit, and 32.5 g of the reddish-pink berries and 50 ml of distilled water were placed in a high-speed blender and homogenized for 30 min. The homogenate was transferred to a 2-L round-bottom distillation flask, and the contents steam-distilled in the conventional manner at atmospheric pressure through a 30-cm bubble plate column fitted with a Kjeldahl trap and a 50-cm ice-water-cooled condenser. Distillation was discontinued 10 min after the last drop of oil appeared in the receiver and when the latter contained about 500 ml of total distillate. The upper oil phase was separated from the water phase in a separatory funnel, giving a colorless oil with a sharp pepper-like aroma. After drying over anhydrous sodium sulfate for 24 hr, the yield of oil was 1.96 g (6.03% of the total fresh weight).

The density at 29° of this steam-distilled Pepper Tree oil was 0.883. The oil had a specific rotation of −24.87° at 27°, and the refractive index at 24.5° was 1.4732. No determination of ester or acid number was made.

Analyses were made with capillary columns and flame ionization detection. This provides the best resolution currently available by gas chromatographic methods (Bernhard, 1962). The gas chromatographic equipment and procedures for preparation of columns, method of sample introduction, etc., are fully described elsewhere (Bernhard, 1962).

Samples of the whole oil from the steam distillation were introduced into the chromatograph at such temperatures that maximum resolution of the terpene hydrocarbons was achieved (Table 4). From previous experience (Bernhard, 1957, 1961, 1962; Bernhard and Scrubis, 1961; Clark and Bernhard, 1960), these conditions and the retention times for these compounds were known. After the terpenes were eluted, the remaining terpenoids (oxygenated terpene compounds) were removed from the chromatographic column by increasing the column temperature to 150° and maintaining that temperature until no further constituents were eluted. This, in effect, was deterpenation by GLC.

Table 4 presents the exact parameters of operation, i.e., stationary liquid phases, temperatures, flow rates, sample size, etc.

## RESULTS AND DISCUSSION

Examination of the terpene hydrocarbon fraction isolated from the essential oil of *Schinus molle* was carried out with three stationary liquid phases: *n*-butylcyclohexyl phthalate, Tween-20, and LAC-2-R446. Various peak identities were assigned by determination of the corrected retention volumes ($V_R^\circ$) (Ambrose *et al.*, 1958) of known terpene hydrocarbons and comparison with those for the unknown peaks. Peak identity was further confirmed by an enrichment procedure in which the known terpene compounds were added one at a time to fresh portions of the terpene hydrocarbon fraction of the oil and re-examined by gas-liquid chromatography. By these techniques, the majority of the components in this fraction

were tentatively identified. Data are presented in the form of relative corrected retention volumes ($V_R^\circ/V_R^\circ$) (Tables 1–3). It should be noted that it is experimentally impossible to distinguish between two components whose relative corrected retention volumes differ by 2% or less, under the conditions of these analyses, since they will appear on the chromatogram as a single united peak. Differences of the order of 5% are required for complete separation of zones or peaks (Bernhard, 1962).

To be well within the bounds of experimental error, an arbitrary limit of agreement for relative corrected retention volumes not to exceed 1.5% was established. Compounds with values of $V_R^\circ/V_R^\circ$ not agreeing to within 1.5% are enclosed in parentheses (Table 1–3).

When a 0.2-$\mu$l sample of the terpene hydrocarbon fraction was examined by GLC employing a stationary liquid phase consisting of *n*-butylcyclohexyl phthalate, six major peaks were observed (Fig. 1). Table 1 pre-

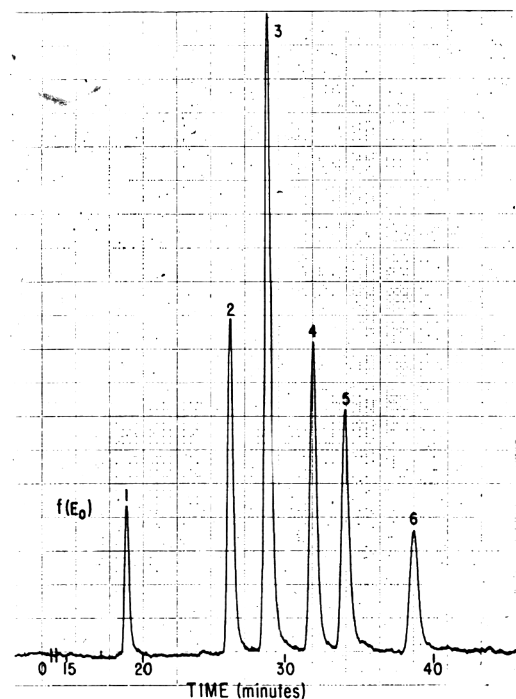


Fig. 1. Gas-liquid chromatogram of the terpene hydrocarbon fraction isolated from the essential oil of *Schinus molle* on *n*-butylcyclohexyl phthalate capillary column. See Table 1 for peak identification and Table 4 for operating conditions. Sample size: 0.2 $\mu$l.

Table 1. Relative corrected retention volumes of the constituents of the terpene fraction of the essential oil of *Schinus molle* L. Stationary phase: *n*-butylcyclohexyl phthalate [a] (D-limonene = 1.00).

| Peak | $V_R^\circ/V_R^\circ$ Unknown | Known | Compound |
|---|---|---|---|
| 1 | 0.612 | 0.626 | (α-Pinene) |
| 1a | 0.683 | 0.685 | Camphene |
| 1b | 0.777 | 0.778 | β-Pinene |
| 2 | 0.826 | 0.836 | Myrcene |
| 3 | 0.906 | 0.907 | α-Phellandrene |
| 4 | 1.00 | 1.00 | D-Limonene |
| 5 | 1.06 | 1.06 | β-Phellandrene |
| 6 | 1.21 | 1.21 | *p*-Cymene |
| 6a | 1.33 | ...... | ........ |
| 6b | 1.37 | ...... | ........ |
| 7 | 2.00 | ...... | ........ |

[a] See Table 4 for operating conditions.

Table 2. Relative corrected retention volumes of the constituents of the terpene fraction of the essential oil of *Schinus molle* L. Stationary phase: Tween20 [a] (D-limonene = 1.00).

| Peak | $V_R^\circ/V_R^\circ$ Unknown | Known | Compound |
|---|---|---|---|
| 1 | 0.541 | 0.559 | (α-Pinene) |
| 1a | 0.607 | 0.613 | Camphene |
| 1b | 0.690 | 0.691 | β-Pinene |
| 2 | 0.862 | 0.856 | α-Phellandrene |
| | | 0.860 | Myrcene |
| 3 | 1.00 | 1.00 | D-Limonene |
| 4 | 1.03 | 1.03 | β-Phellandrene |
| 5 | 1.30 | 1.30 | *p*-Cymene |
| 5a | 1.45 | ...... | ........ |
| 6 | 2.74 | ...... | ........ |

[a] See Table 4 for operating conditions.

Table 3. Relative corrected retention volumes of the constituents of the terpene fraction of the essential oil of *Schinus molle* L. Stationary phase: LAC-2-R446 [a] (D-limonene = 1.00).

| Peak | $V_R^\circ/V_R^\circ$ Unknown | Known | Compound |
|---|---|---|---|
| 1 | 0.488 | 0.491 | α-Pinene |
| 2 | 0.836 | 0.843 | Myrcene |
| 3 | 0.862 | 0.863 | α-Phellandrene |
| 4 | 1.00 | 1.00 | D-Limonene |
| 5 | 1.05 | 1.05 | β-Phellandrene |
| 6 | 1.46 | 1.44 | *p*-Cymene |

[a] See Table 4 for operating conditions.

Table 4. Operating conditions for capillary chromatographic columns (hydrogen-flame detection).

| | n-Butylcyclohexyl phthalate | Tween-20 | LAC-2-R446 |
|---|---|---|---|
| Column length (ft.) | 150 | 100 | 100 |
| Column I.D. (inches) | 0.010 | 0.010 | 0.010 |
| Column temperature (°C) | 100 | 50 | 100 |
| Injection-block temperature (°C) | 90 | 100 | 125 |
| Detector-oven temperature (°C) | 170 | 90 | 90 |
| Hydrogen flow rate (ml/min) | 20 | 20 | 20 |
| Air flow rate (ml/min) | 1500 | 1500 | 1500 |
| Nitrogen velocity (cm/sec) | 10 | 9.8 | 9.9 |
| Injection split ratio | 1:600 | 1:600 | 1:600 |
| Sample size (μl) | 0.2–1.5 | 0.2–0.5 | 0.2 |
| Recorder range (mv) | 10 | 10 | 10 |
| Chart speed (in./hr) | 15 | 15 | 15 |

sents relative corrected retention volume data for these peaks (numbered 1 through 6). Only peak 1 exceeds the 1.5% limit of agreement for relative corrected retention volumes; in this instance agreement is 2.2%. When larger samples were introduced into the chromatograph, e.g., 1.5 μl, five additional peaks appeared on the chromatograms; these are indicated as peaks 1a, 1b, 6a, 6b, and 7 (Table 1). These five peaks are present in the sample in minute amounts, and gross overloading of the chromatographic column is necessary to make them evident.

Relative corrected retention volumes for the components of the sample were determined on a second stationary liquid phase, Tween-20. Once again, six major peaks were found when 0.2-μl samples were examined. Increasing the sample size to 0.5 μl revealed three additional peaks, 1a, 1b, and 5a (Table 2). Peak 1 exceeds the limit of agreement by 1.7%.

As an additional check on identity, relative corrected retention volumes for the components of the terpene fraction of the oil were determined on a third stationary liquid phase, LAC-2-R446 (Table 3). Once again, six major peaks were evident on the chromatograms. In no case did the values for relative corrected retention volumes exceed the 1.5% limit of agreement using this column. No attempt was made to increase sample size to the point of overloading, since the experiments discussed above showed that such procedures destroyed the columns.

Based on these data, it appears that the major terpene hydrocarbon components of the oil of *Schinus molle* are: α-pinene, myrcene, α-phellandrene, D-limonene, β-phellandrene, and the non-terpene *p*-cymene. The minor terpene constituents appear to be: camphene, β-pinene, and three unidentified compounds. There is excellent agreement among the identities determined on each of the three stationary liquid phases (Tables 1–3).

Our findings are also in agreement with those reported by various authors as to the presence of the phellandrenes, and α-pinene.

Examination of Fig. 1 indicates a large amount of α-phellandrene, roughly equal amounts of myrcene and D-limonene, a slightly lesser amount of β-phellandrene, and still smaller amounts of α-pinene and *p*-cymene. Although this latter compound is not a true terpene hydrocarbon, but an aromatic hydrocarbon, its presence has been frequently reported in the terpene fraction of many essential oils (Clark and Bernhard, 1960). Since it appears in this fraction on a chromatogram, it was included under the heading "terpene hydrocarbon" fraction.

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RESEARCH NOTE

# Pectin Content of Raisins

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During an investigation of the methanol content of raisins, information as to the quantity of the pectic substances in raisins was desired. No information could be found on the quantity of pectic materials in raisins; the values reported for water-soluble pectin in grapes range from .11 to .28% on a fresh-weight basis (Kertesz, 1951; p. 302). This note reports the content of pectic substances in Thompson seedless raisins.

Two lots of Thompson seedless raisins from the 1961 California crop were analyzed. One was prepared in the laboratory as follows: Grapes were dried 48 hr at 150°F in a cabinet dryer, and the resulting raisins (moisture content 10–12%) were stored under refrigeration (4°C) without chemical treatment until the analyses were performed. These raisins are referred to herein as "laboratory prep." The second lot of raisins was obtained from a commercial pack prepared by sun-drying and processing by the usual procedures employed in the raisin industry. These raisins are referred to herein as "commercial."

The water-soluble pectin was extracted from 50 g raisins and determined as anhydrouronic acid by the carbazole method of McComb and McCready (1952).

The total pectic substances were extracted by the method of McCready and McComb (1952), and portions of the resulting solution were analyzed for anhydrouronic acid by the method of McComb and McCready (1952).

The values obtained for pectic substances in raisins are shown in Table 1. The two samples of raisins studied contained similar quantities of pectic materials; approximately 1% of the weight of the fruit was pectic substances, 25% of the latter was water-soluble.

Table 1. Pectin content of raisins.

| | Laboratory (mg/g raisins) | Commercial (mg/g raisins) |
|---|---|---|
| Water-soluble pectin | 2.25 | 2.14 |
| | 2.38 | 2.20 |
| Total pectic substances | 10.4 | 9.3 |
| | 9.5 | 9.6 |
| | 10.2 | 9.2 |
| | 9.0 | 8.2 |

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# The Extraction of Pectins from Apple Marc Preparations

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**SUMMARY**

**Methods of preparing alcohol-insoluble solids from apple tissue and different extractives were compared. Marked differences were found in extractability of pectins present in marcs prepared in various ways from the same lot of apples. Enzymatic browning during preparation markedly decreased the solubility of pectins but did not alter the composition of pectins extracted. The solubility of pectins present in fresh or dried commercial apple pomace was markedly lower than that from carefully prepared non-oxidized apple tissue. Pretreatment of browned marc preparations with chlorous acid bleached the marcs, yielded colorless pectins, and increased yields with some preparations. A variety of solvents were used, and the yield of pectins obtained differed markedly. The pectins extracted were precipitated by alcohol and characterized by determination of anhydrogalacturonide content and esterification degree. Some measurements of intrinsic viscosity were made, but without extensive studies of the molecular weight and degree of polymerization.**

The occurrence of pectic substances differing in solubility and ease of extraction has been known since 1848, when Fremy first reported the existence of a water-insoluble precursor of pectin (Fremy, 1848), now designated as protopectin after Tschirch (1909). The pectic substances in plant tissues are known to be composed of water-soluble pectins, of water-insoluble but hot-acid-soluble compounds, and of water-insoluble and hot-acid-insoluble pectic substances that are extracted with hot dilute solutions of alkali or ammonium oxalate. The pectic substances present in the cell wall differ from those in the middle lamella in solubility and histochemical properties. The quantity and composition of pectic substances obtained from a particular tissue sample are known to vary with: the origin of the specimen (the species and variety of plant, the type of tissue, the soil and climatic conditions under which it was grown, the stage of maturity at which it was harvested, etc.); the methods used in the preparation of the tissue (the care taken in preventing post-harvest physiological changes, the degree to which microbial and enzymic activities were inhibited, the method and degree of comminution used, and the nature of the preliminary treatment); the method of extraction (relative proportion and kind of solvent used, the pH, temperature, and time of treatment); the methods used in separating the pectins extracted from other soluble matters; the type and extent of purification followed before analysis; and the analytical procedures used in assay and characterization. Systematic investigations of these and other factors are limited (Joslyn, 1962).

While a considerable amount of data are available on the chemical composition of extracted pectins, investigations of the rate and extent of extraction of pectins from plant tissue preparations are limited. Only rarely have data been reported on the basis of which the completeness of extraction could be estimated. Most investigators have selected conditions for extraction that would yield the highest quantity of the pectin with the particular properties desired. This is particularly true of the data reported, partly in the scientific publications but largely in patent literature on the commercial extraction of pectins (Bender, 1959; Hottenroth, 1951; Kertesz, 1951; Maass, 1951; Ripa, 1951; and Rooker, 1928). The older meth-

ods for extracting and analyzing pectic substances from plant materials have not been entirely free from criticisms (e.g., Kertesz, 1951). In much published data the plant material has not been carefully selected or sufficiently characterized, and post-harvest physiological changes, enzymatic changes, and microbial activity during preparation and extraction have not been eliminated. Pectins similar to those actually occurring in plant tissues have not been obtained, because of the difficulties in avoiding degradation during extraction. Only recently have procedures been introduced for the characterization of pectic substances *in situ* (Gee *et al.*, 1958, 1959).

The recently introduced methods for extraction of pectins are considerably less drastic, and newer methods of analysis better characterize the pectins extracted (Bender, 1959; Berglund, 1950; Deuel, 1943; Doesburg, 1959a; Henglein, 1947; Hills and Speiser, 1946; and Owens *et al.*, 1952).

The use of acids for the extraction of pectins from plant tissues, both for analytical preparation and for commercial production, is now well established, though the chemistry of this transformation is still unknown. The characteristics of the acid-soluble pectins and the nature of the bonds broken by acids are still largely unknown. The historical development and present status of our knowledge of the chemistry and structure of protopectin are presented elsewhere (Joslyn, 1962). The effect of method of preparing apple marc on the extractability of the pectins present, and the yield and properties of alcohol-insoluble pectins obtained from Schöner von Boskoop apple marcs extracted under fairly mild conditions, are presented in this paper. This variety was selected because it was investigated fairly extensively by Eggenberger (1949) and used by Schlubach and Hoffmann-Walbeck (1949). Apples as a fruit have been investigated more thoroughly than other fruits (Hulme, 1958; Smock and Neubert, 1950; Wiley and Stembridge, 1961; and Woodmansee *et al.*, 1959).

The quantity of pectins extracted was estimated by yield of galacturonide found, and the pectins obtained were characterized by measurement of percentage of galacturonides that were esterified. Some measurements of molecular size were obtained by measuring relative and intrinsic viscosity, but not all the extracts obtained were so characterized.

## EXPERIMENTAL

**Preparation of marc samples.** To obtain data on the effect of the method of preparation of marc as well as of ripening in storage on the extractability of pectins, alcohol-insoluble residues were prepared from Schöner von Boskoop apples freshly harvested and after storage at 8°C for various periods. The apples were harvested during the fall of 1959 from standard trees on seedling rootstock, 31 years old, grown in Wädenswil, Switzerland, in sod on a sandy loam. Trees with normal growth and full crop were selected. The trees were in full bloom on April 29, and sound apples of fairly uniform size (170 g, 72.5–80 mm in diameter) were harvested on October 1. At picking, the juice tested pH 3.22, had a total titratable acidity of 8.05 (as g malic per liter when titrated to pH 7.5), a sp. gr. of 1.051, and a solids content of 106 g/L. The solids content of the whole apple was 14.2%, in comparison with 10.1% in the juice. Part of the fruit harvested was used shortly after harvest for the preparation of marc samples, and the rest was stored at 8°C at 90% relative humidity (RH). The pressure test values, determined with a penetrometer of $\frac{7}{16}$-in.-diameter tip, decreased from 21.1 lb on October 1 to 15.0 lb on November 9 and 11.7 lb on December 7. Marcs were prepared from freshly harvested apples and apples stored for 12 days, 40 days, and 113 days. The composition of these marcs is shown in Table 1.

To minimize changes that might occur on drying, the marc preparations were freed of excess of 70% alcohol used in washing by sucking dry under suction while on Buchner funnels and then pressing out the alcohol still present. They were stored wet at room temperature in glass-stoppered bottles. The solids content of these preparations varied from 10.7 to 79.0%, depending on the physical condition of the tissue obtained. The marcs were stored 1–4 months before use. Although Gee *et al.* (1958) reported conversion of esterified carboxyls to free carboxyls in ethanol-washed and air-dried marcs during storage at room temperature in one week, and slow esterification of pectic substances in plant material stored in ethanol for long periods, no significant changes were observed either in extractability of pectin or in degree of esterification in marc preparations stored as above. The data obtained on storage stability, however, were limited, and a more thorough investigation of this is necessary.

Marcs 1 to 4 were prepared from a preliminary sample of Boskoop apples. The apples were peeled

Table 1. Characteristics of Boskoop marcs investigated.

| Marc | Date | Material [a] | Preparation | Yield per 100 g flesh | % dry solids | % N | % ash | % AUA [b] | % esterification [c] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 9/29/59 | FAT | Bl. with 70% alc., not heated | 19.0 | 14.9 | 0.8 | 0.6 | 18.0 | 83.8 |
| 2 | 9/29/59 | FAT | Bl. with alc., heated | 20.8 | 13.5 | 0.8 | 0.6 | 20.0 | 86.0 |
| 3 | 9/29/59 | FAT | Halved, frozen at −20°C, bl. | 16.5 | 18.0 | 0.9 | 0.8 | 20.0 | 88.5 |
| 4 | 9/29/59 | FAT | Sl., heated in alc., bl. | 23.2 | 10.7 | 0.7 | 0.8 | 22.0 | 83.5 |
| 7 | 10/ 2/59 | PAP | Alc.-presvd. pomace | 25.2 | 23.5 | 0.8 | 0.9 | 26.0 | 85.0 |
| 8 | 10/ 2/59 | FAT | Bl. with alc., heated | 20.0 | 14.9 | 0.7 | 1.2 | 18.4 | 86.5 |
| 13 | 10/12/59 | FAT | Bl. with alc., heated | 15.6 | 14.8 | 0.7 | 0.8 | 16.3 | 77.5 |
| 14 | 10/12/59 | FAT | Dehydr. in alc., bl. in acidified alc. | 2.54 | 79.0 | 0.9 | 0.6 | 13.7 | 85.0 |
| 15 | 10/12/59 | FAT | Sl. into alc. at −50°C, bl. after freezing | 7.45 | 28.4 | 0.8 | 1.4 | 29.2 | 85.5 |
| 21A | 11/10/59 | FAT | Bl. with alc., not heated | 10.5 | 21.8 | 0.9 | 0.9 | 35.7 | 87.0 |
| 21B | 11/10/59 | FAT | Bl. with alc., not heated | 14.8 | 14.4 | 1.0 | 0.5 | 37.0 | 87.0 |
| 22 | 11/10/59 | FAT | Bl. with acidified alc. | 7.9 | 24.6 | 0.8 | 0.0 | 35.8 | 74.5 |
| 23 | 1/21/40 | PAP | Alc.-presvd. pomace | 32.3 | ...... | 1.2 | 1.4 | 35.0 | 88.5 |
| 26 | 1/21/40 | FAT | Bl. with water, then with alc. | 8.6 | 31.3 | 0.7 | 0.9 | 28.0 | 87.5 |
| 27 | 1/21/40 | FAT | As in 14 | 14.9 | 18.9 | 1.1 | 0.3 | 30.2 | 80.7 |
| 28 | 1/21/40 | FAT | Bl. with alc., heated | 14.1 | 25.3 | 1.1 | 1.1 | 30.5 | 91.8 |

[a] FAT = fresh apple tissue. PAP = pomace after pressing.
[b] % anhydrogalacturonic acid.
[c] As determined from titration before and after saponification.

and cored, stem and calyx removed, and samples of the apple tissue then prepared. In marc 1, a 250-g portion of apple tissue was cut directly into the glass jar of a Turmix blender containing 600 ml of 95% ethyl alcohol, and blended 5 min at half speed. Two lots of the alcohol-blended mixture were prepared and allowed to stand in alcohol overnight. The mixture was then filtered through 26 G2 sintered-glass filters and sucked dry. It was mixed on the funnel with 500 ml of 70% alcohol, and after this was separated, again treated with another 500 ml of 70% alcohol, and the residue was finally covered with a third portion, which was allowed to percolate through, without suction, overnight. The residue was again washed with a fourth portion of 70% alcohol. The alcohol mixture initially prepared, browned during blending, and was quite brown in color on storage overnight, indicating that under these conditions the polyphenol oxidase was not inactivated.

Marc 2 was prepared by blending cut apple tissue, free of core and skin, with alcohol, but the alcohol mixture was heated to 78°C on an electric hot plate and then boiled 30 min at 78–80°C under a reflux condenser. Initial heating to the boiling point required 1 hr. Subsequently, the alcohol mixtures were heated to boiling on a gas flame within 5–10 min. After heat inactivation, the mixture was allowed to cool overnight, and then filtered and washed with 70% alcohol as above. A white marc was obtained free from any oxidized material.

Marc 3 was prepared by first halving the fruit, separating the core and calyx, and allowing the apples to freeze overnight at −20°C. The next day the fruit was peeled while frozen, and cut into precooled alcohol, blended, and washed with 70% alcohol as in marc 1. This was the procedure that Hulme (1936) recommended to minimize denaturation of proteins and other constituents.

Marc 4 was prepared from fruit tissue cut into alcohol heated on a hot plate to 70°. The mixture was brought to 80° in 15 min instead of 1 hr, as with marc 2, and treated as was marc 2. Owens *et al.* (1952) recommended this procedure, in which enzyme activity is destroyed by slicing the plant tissue with a sharp knife to avoid bruising, dropping the sliced tissue directly into hot alcohol and heating the mixture, whose final alcohol concentration should be not less than 70% (by vol.) at a temperature above 70° for 12–18 min. They actually did not report data on the inactivation of pectic enzymes under these conditions, nor did Schlubach and Hoffmann-Walbeck (1949), who used a similar procedure for inactivation of pectic enzymes in apple slices, but based their choice of conditions on previously reported data on heat inactivation of pectin esterases in aqueous solutions. Marc 3 was grayish-brown and 4 was reddish-brown.

Marc 7 was prepared from pomace after expression of juice from firm apples. This was prepared by crushing 17.55 kg of whole apples and pressing to yield 12.8 L of juice and 3.03 kg

of pomace, which was mixed with an equal weight of alcohol. It was dark reddish-brown. The alcohol-preserved pomace was stored 8 days at 0° before preparation. Two-hundred-gram portions of this pomace were blended with 200 ml of 95% alcohol, allowed to stand overnight, and then filtered and washed as above.

Marc 8 was prepared from a sample of the same lot of Boskoop apples used in preparing the pomace above. This was prepared as was marc 2.

Marc 13 was prepared from apples stored 12 days at 8°. Five-hundred-gram portions of apple tissue were cut into 1 L of 95% ethanol in a large plastic Turmix jar, blended 5 min at half speed, and then transferred, with an additional 240 ml of alcohol, into a 3-L Erlenmeyer flask, heated to 78°C over a gas burner, and then boiled 30 min under reflux. The hot alcoholic mixture was allowed to stand overnight, and then filtered and washed with 70% alcohol as above.

Marc 14 was prepared from the same lot of apples by the procedure of Gee *et al.* (1958). Two thousand one hundred and twenty-five grams of peeled and cored apple tissue were cut into 3 L of 95% alcohol and allowed to stand for 3 hr. Appreciable phenolase-catalyzed enzymatic browning occurred on the surface, and particularly in the interior of the sliced apples. The alcohol was strained through nylon cloth, and the excess of alcohol was pressed out of the tissue, which was swollen and spongy. Three-hundred-and-fifty-gram portions of this tissue were blended with 1 L of acidified alcohol (750 ml 95% alcohol, 50 ml concentrated hydrochloric acid, and 200 ml water) to yield a reddish-brown slurry. The blended mixture was filtered through G-2 sintered-glass filters to yield a cloudy brown filtrate and a spongy brown marc.

Marc 15 was prepared by cutting apple tissue into 95% alcohol precooled to −70° with solid carbon dioxide. This was stored overnight, and then blended, filtered, and washed with 70% alcohol. This preparation yielded a white porous marc.

Marc samples 21 to 28 were prepared, as indicated in Table 1, from stored apples, except marc 23, which was prepared from apple pomace obtained by crushing and pressing 21 kg of stored apples to yield 4 kg of pomace preserved with alcohol and 12.9 L of juice. This was prepared as was marc 7. Marc 26 was prepared by blending 600-g portions of apple flesh, free of skin and core, with 250 ml water at high speed to yield a smooth, thick purée, which was mixed with sufficient alcohol to give a final alcohol concentration of 70% and then treated as was marc 1.

In addition to these marcs, four samples of marc were prepared from *Thurgauer Weinapfeln* obtained from Obipektin A. G., Bischofszell, on October 6. These included preparations from fresh apples used for cider making, factory-pressed apple pomace mixed with an equal weight of 96% isopropanol, and factory-dried pomace. Table 2 shows the composition of these marc preparations.

Table 2. Characteristics of Weinapfel marcs.

| | 9 FAT[a] bl. with alc. heated | 10 FAT bl. with water, then with alc. | 11 FAP presvd. with alc. | 12 Factory-dried apple pomace |
|---|---|---|---|---|
| Yield, g marc per 100 g flesh | 28.6 | 22.8 | 62.5 | 154[b] |
| Solids content, % | 12.7 | 16.1 | 27.3 | 45.0 |
| Nitrogen, % | 1.2 | 0.8 | 1.0 | 1.2 |
| Ash, % | 0.9 | 0.7 | 1.0 | 1.0 |
| % AUA | 18.3 | 20.6 | 10.7 | 14.4 |
| % esterification | 80.0 | 83.5 | 87.0 | 82.5 |

[a] FAT = fresh apple tissue. FAP = fresh apple pomace.
[b] Yield per 100 g dried pomace of marc containing 45% solids.

These preparations were typical of the types of marc that could be obtained. That from the apple pomace, containing skin and core tissue, was representative of the polyphenolase-oxidized brown marc commercially used for pectin extraction. That from the apple flesh represented a marc in which enzymatic oxidation and other enzyme activity, including pectase, was inhibited by heat inactivation. The latter was used in a large series of comparative extractions to determine the relative effectiveness of various reagents. Both preparations contained appreciable quantities of starch; the starch content of the pressed pomace was considerably lower because many of the starch granules present were removed with the juice. In both, considerable quantities of starch were separated by the initial washing with 70% of alcohol, although this was not continued until the filtrate was carbohydrate-negative to anthrone. The starch content was not determined.

Eggenberger (1949) reported, for Boskoop apples harvested on September 28, 1946, averaging 158.0 g in weight and 72.9 mm in diameter, a total solids content of 16.0%, a total titratable acidity of 12.85 meq per 100 g fresh weight, and a starch content of 0.58%. The alcohol-insoluble solids content calculated from his data amounted to 4.8%, the total pectin content 0.71%. The esterification degree of this pectin was 78.5%, and 73.9% of the total pectin was extractable under his conditions (heating 50 min at 92°C at pH 3.1–3.2 in the presence of 1.6 g of Calgon per liter). The extracted pectin had a purity of 64.3%, an

esterification degree of 78.6%, and an equivalent weight of 918.

**Pectins in apple juice.** It has been believed that pectic substances that are more readily soluble and the first to be extracted are lower-molecular-weight more-highly-esterified polygalacturonides (Baker and Woodmansee, 1944; Myers and Baker, 1929; and Rooker, 1928). In commercial practice it was customary to wash the apple pomace with water to remove water-soluble constituents, including the more readily soluble pectins believed to be of lower molecular weight. To obtain some data on the properties of water-soluble pectins, alcohol precipitates were prepared from juice expressed from firm, freshly picked Boskoop apples and from similar apples after cold storage. Since these preparations were found to contain considerable amounts of nitrogenous constituents, the nature of this associated material, believed to be protein, was investigated. The occurrence of proteins in alcoholic precipitates from apple juice was already known by Fremy (1848) and was confirmed by Tromp de Haas and Tollens (1895) and Fellenberg (1914).

The following alcohol precipitates were prepared from apple juice.

*Preparation 6.* This was obtained from the 12.8 L of juice pressed out of 17.55 kg of apples harvested on October 2, 1959. Decanted free of starch that had settled out, 11.1 L of the juice were concentrated at low temperature *in vacuo* to 2.75 L. Attempts failed to clarify the concentrate by centrifuging. The concentrate was stored overnight at 0°C and centrifuged again. Aliquots of concentrate centrifuged at 20,000 rpm were just as cloudy as those centrifuged at 3000 rpm, but the latter removed starch granules and other insoluble matter. The concentrate was mixed with sufficient alcohol to bring the mixture to 70% alcohol and stored overnight at 0°. The granular alcohol precipitate was separated by centrifugation and transferred to 0-2 sintered-glass funnel for filtration and washing. It was filtered dry under suction, washed four times with 70% alcohol and then with 95% alcohol and ether, and sucked dry. From 2.43 L of concentrate were obtained 1.534 g of material, equivalent to 0.1565 g per liter of juice. The material was brown and contained 2.2% N and 7.7% ash on a moisture-free basis.

To purify this preparation partially, 1.00 g was mixed with water, but only a small amount of the material dissolved and the residue turned bluish gray and became tar-like in consistency. Trituration in water gave a dirty bluish-gray dispersion of the whole preparation. When an aliquot of this was mixed with sufficient alcohol to bring the mixture to 70%, a gray tarry sediment and a brownish-gray suspension were obtained. Sufficient alcohol was added to bring the concentration in the material to 64%, but no precipitation occurred. No precipitate formed at 72 or 76% alcohol, but on addition of NaCl a blue-gray rubbery sediment formed. This was centrifuged out and washed with 70% alcohol until $Cl^-$ free, and then with 95% alcohol and ether. The air-dried material obtained weighed 0.579 g (58% of original), was grayish-blue, and on a moisture-free basis contained 2.6% N and 4.2% ash (Preparation 6A).

*Preparation 25.* This was obtained from 12.9 L of juice pressed out of 21 kg of crushed Boskoop apples harvested in October, 1959, and stored until January 21, 1960. The residual pomace weighed 4 kg. The juice, treated with ascorbic acid at the crusher, as before, was light in color but did not have the objectionable grassy flavor found in the ascorbic-acid-treated juice of firm green apples. The juice was concentrated *in vacuo* to 3.5 L and stored overnight at 0°C. The concentrate was centrifuged for 15 min at 3000 rpm, and 10 L of 93% alcohol were added to 3.27 L of concentrate. A small amount of sediment was obtained, and this also was mixed with sufficient alcohol to bring the mixture to 70% alcohol. A voluminous grayish fibrous precipitate separated from the centrifuged concentrate. That from the centrifuged sediment was brown, and more gelatinous in appearance. The alcohol precipitate from the centrifuged concentrate was strained and pressed through cloth, washed with 70% alcohol, then with 95% alcohol, and finally with acetone, and allowed to air-dry. Twenty-one grams of a gray-brown preparation containing 75.5% solids were obtained. This is equivalent to 1.315 g per liter of original juice. This contained, on a moisture-free basis, 2.2% N and 3.7% ash. From the sediment was obtained 0.7484 g of brown precipitate containing 90.4% solids and 3.1% N and 10.4% ash on a moisture-free basis.

Five grams of the alcohol precipitate were redissolved in water and reprecipitated with alcohol, yielding 2.5 g of brown-colored material. This time, no change was observed in color or consistency or solubility in water (Preparation 25A).

**Alcohol precipitation.** Alcohol precipitation of aqueous solutions of pectic substances can be carried out by gradual addition of alcohol to the water extract to a final concentration of alcohol of 50-63% by volume or by addition of the water extract to alcohol. The former procedure is widely used in obtaining alcohol precipitates in pectin analysis because it often yields precipitates that filter and wash easily. The latter procedure has been used in polysaccharide investigations. Under the conditions established, little difference in filtration rate was observed with either procedure, and there was no appreciable increase in yield of alcohol

precipitate in using one or two volumes of ethyl alcohol (50% or 63%). When a final alcohol concentration of 50% was used, hydrochloric acid was added to improve the yield and purity of alcohol precipitate.

**Analyses.** The total pectin content of the marc from apple flesh was estimated by several procedures. Most of the determinations were made by the direct titration procedure of Gee *et al.* (1958) and Owens *et al.* (1952) and the carbazole colorimetric procedure (McCready and McComb, 1952) applied to extracts prepared by the Versene-pectinase procedure of McCready and McComb (1952). In the modification of the carbazole method used, 0.5 ml of pectin solution (prepared by saponifying the pectin extract with alkali and diluting to contain 0–100 $\mu$g of anhydrogalacturonic acid per ml) was mixed with 3 ml of concentrated sulfuric acid while being cooled in ice and water, then heated for 20 min in a boiling-water bath, and cooled in water to room temperature. One-tenth ml of a 0.01% carbazole in absolute methanol was then added, and the color of the pigment was developed for 2 hr and its absorption read at 540 m$\mu$ on a Beckman model B spectrophotometer.

In the direct titration procedure, the acetic acid obtained by hydrolysis of the acetyl groups present is included in the titration after saponification. Gee *et al.* (1958) assumed that under their conditions the hydrolysis of acetyl groups as well as methyl ester groups is complete, and they corrected the titration value after saponification by determining the acetyl content of the marc colorimetrically, by the modified ferric hydroxamate procedure of McComb and McCready (1957). Pippin *et al.* (1950) and Kertesz and Lavin (1954) reported fairly slow deacetylation at room temperature, with complete deacetylation of a 0.1-g portion requiring 3 hr. Since Kertesz and Lavin (1954) reported apple pectin to contain 0.52–0.63% acetyl, with an average of 0.58% in six samples analyzed, the increase in acidity due to the acetyl groups in the apple marc would amount to 8 mg of acetic acid per 1 g of pectin present or 0.153 meq. In comparison, the increase in acidity for a pectin containing 10% esterified methoxyl would be 3.22 meq. The acetyl groups in this case would contribute only 4.13% of total increase in acidity on saponification. For this reason, correction for acetyl content was not applied.

The ash and N content of the samples of marc and of the alcohol precipitates from the juices were determined at the micro-analytical laboratories of CIBA A.G. Basel. N determinations were made by micro Dumas procedure, and ash by incineration at 800°C. The solids content and viscosity measurements were made as recommended by Owens *et al.* (1952). The nonuronide sugars present were detected by chromatographic analysis after hydrolysis with concentrated nitric acid.

**Extraction.** Several methods of extraction were compared. In the first series of tests, successive serial extraction with a variety of solvents at room temperature were planned. It was proposed to extract first with water, to continue this extraction to a negative anthrone test, and then to follow with solvents selected to remove cations (ethylenediaminetetraacetate, EDTA, at pH 6), to break protein-pectin linkages (glutathione, sulfite, etc.), to break hydrogen bonds (urea), and finally acid and alkali to hydrolyze other linkages. Treatment was planned with oxidizing agents (periodate, etc.), with reducing agents (sodium borohydride), with chlorite to remove lignin, and with Schweizer reagent to remove cellulose. This plan was abandoned because, in the first place, starch continued to be extracted with water; even after the fourth serial extraction, starch was present and the extract gave a positive anthrone test. Extraction was tried by percolation of solvent through a sample of the marc in a chromatography column. This was not found suitable, because percolation was too variable in lightly pressed marcs and too slow when these were packed tightly. In preliminary trials, considerable swelling with water occurred in marcs 1 to 4, most pronouncedly in marc 4. Then serial extraction was tried by shaking aliquots of marc with water and several other solvents. This yielded more consistent results but required shaking for 24 hr and was too time-consuming. It was found that extraction was more rapid when aliquots of marc were blended with various solvents and then stored 24 hr at room temperature. Blending comminuted the particles better than shaking alone. Continuous stirring was considered, but was abandoned because not enough stirrers were available. Extraction with hydrochloric acid under various conditions was investigated more completely, but time did not permit completion of the series of tests planned. The data obtained are presented in the following sections along with the details of the method of extraction used.

## RESULTS

**Serial extraction.** Ten-gram portions of marc preparations 1, 2, 3, 4, 13, and 15, and five grams of marc 14 were weighed out into 250-ml polyethylene bottles and extracted serially with 200-ml portions of de-ionized water and other solvents by shaking for 24 hr. The extracts were separated by filtration through a G-2 sintered-glass funnel, and the residue returned to the original bottle for additional extraction. All filtered rapidly except marc 14, which gelled, and this was subsequently

extracted with 500-ml portions of solvent. In the water extracts, starch separated from the filtrates and continued to do so even in the fourth extraction. The filtrates, freed of starch by settling or centrifugation, were poured into 20 volumes of 95% alcohol to precipitate pectins. Alcohol-precipitated pectins were obtained only with marc 14, and the quantity obtained decreased as extraction continued: very little precipitation in the third extract and practically none in the fourth. The alcohol-precipitable pectins from the cold-water extracts of marc 14 were combined, filtered, washed with 70% alcohol and then 95% alcohol and acetone, and dried to yield 0.90 g of pectin from 16 g of original marc preparation, representing a recovery, on a dry-solids basis, of 11.4%. The alcohol precipitate obtained contained 0.9% N and 3.7% ash. Its anhydrogalacturonide content, determined by titration, was 61.5%, and the degree of esterification was 88.5% in comparison with 77.0% in the marc. The yield of pectin, expressed as anhydrouronic acid (AUA), was 52.0% of that present.

The water-extracted marcs were then shaken with 0.5% EDTA adjusted to pH 6.0. The EDTA extracts gave only a slight amount of precipitate with alcohol, mostly in marcs 1, 13, and 15. The second extract with EDTA also contained a small amount of alcohol-precipitable matter. The precipitate in all cases appeared to be crystalline, and was found to contain appreciable quantities of EDTA salts. The preparation, on a moisture-free basis, contained 7.3–7.6% N and 17.5–17.7% ash. The preparation analyzed by titration contained 52.5% anhydrogalacturonides of 12.3% esterification, but the presence of EDTA in the alcohol precipitate probably affected both the direct titration with sodium hydroxide and the titration after saponification and acidification. The presence of EDTA salts in alcohol precipitates was confirmed subsequently with other EDTA extractions.

The marcs were then extracted with EDTA containing 1% of added thioglycolic acid. No alcohol-precipitable pectins were observed, although Ginsburg (1958) had reported that addition of thioglycolic acid increased rate and extent of cell separation in presence of EDTA. This treatment was followed by extraction with 0.5% EDTA with 5% butanol at pH 6 and with 0.5% EDTA with 7.5 g of urea per liter. Neither treatment extracted any alcohol-precipitable matter.

The final residue was mixed with 0.1$N$ NaOH, precooled to 0°, and allowed to stand overnight. The extracts were filtered, and, on acidification, pectic acid gel formed in all. When poured into 95% alcohol, the gel dehydrated and only a small amount of pectic acid precipitate was obtained. The combined precipitate from all seven marcs, after washing with 70% alcohol until free of $Cl^-$, and then with alcohol and acetone, and drying, yielded 1.70 g of material containing 0.5% N and 4.3% ash. It had an AUA content of 28.5% with an esterification degree of 16.5%.

The residue after treatment with cold NaOH was then treated with 200 ml of boiling water, and, after standing 30 min at an average of 70°C, was filtered. The filtrates were not found to contain any alcohol-precipitable matter. The final residues were washed with 70% alcohol, then 95% alcohol and acetone, and dried *in vacuo* at 90°C. The weight and composition of the marc residues is shown in Table 3. The residual anhydrouronide content was quite low; the titrations of 200- to 500-mg portions of dried residue required only 1 drop or less of 0.1$N$ NaOH to neutralize the mixture to the Hinton indicator end point, and only 0.1–0.25 ml for titration after saponification and acidification. The esterification values of this residual pectin were high, and are likely to be in error because of the low titration values. The marcs lost 53–74% in weight during the serial extraction, and 88–99% total of pectin was extracted.

**Comparison of extractives.** Five-gram portions of marc 8, representative of the marcs tested above, were blended with 250 ml of various solvents for 10 min at half speed in a Turmix blender, and the mixture was transferred, with an additional 250 ml of the same solvent, into beakers and stored 24–48 hr at room temperature with occa-

Table 3. Weight and composition of final residues of serial extraction.

| Marc | Weight g | % N | % ash | % AUA | % esterification | Decrease in weight (%) | Uronide extracted (%) |
|---|---|---|---|---|---|---|---|
| 1 | 0.7 | 0.7 | 2.2 | 0.61 | 66.8 | 53.0 | 97.0 |
| 2 | 0.6 | 1.1 | 2.0 | 0.89 | 73.3 | 55.5 | 96.0 |
| 3 | 0.7 | 0.5 | 1.9 | 1.07 | 100.0 | 61.0 | 97.0 |
| 4 | 0.5 | 0.5 | 3.6 | 1.06 | 75.0 | 62.5 | 95.0 |
| 13 | 0.4 | Traces | 2.1 | 1.86 | 108.0 | 73.0 | 88.0 |
| 14 | 2.1 | 0.1 | 1.6 | 0.89 | 86.0 | 73.5 | 90.0 |
| 15 | 1.3 | 0.5 | 5.8 | 0.46 | 84.8 | 54.5 | 91.0 |

Table 4. Residual uronide content of marc 8 after extraction with various reagents.[a]

| | Reagent solution | pH of reagent solution | pH of mixture | Residual uronide (mg) % | % esteri-fication | % uronide extracted |
|---|---|---|---|---|---|---|
| 1 | Water | 5.9 | 5.3 | 159 | 91.0 | 0.0 |
| 2 | 0.1*M* NaCl | 6.5 | 4.0 | 138 | 98.0 | 13.6 |
| 3 | 0.1*M* $NH_4Cl$ | 5.3 | 4.1 | 147 | 96.5 | 7.3 |
| 4 | 0.1*M* $NaHSO_3$ | 4.4 | 4.1 | 159 | 88.0 | 0.0 |
| 5 | 0.1*M* $Na_2HPO_4$ | 9.1 | 8.9 | 91 | 81.5 | 43.0 |
| 6 | 0.1*M* $Na_2SO_3$ | 10.1 | 8.9 | 55 | 69.0 | 65.5 |
| 7 | 1*M* urea | 7.2 | 6.6 | 141 | 91.5 | 11.3 |
| 8 | 0.001*M* EDTA | 6.0 | 6.0 | 159 | 91.0 | 0.0 |
| 9 | 0.01*M* EDTA | 5.9 | 5.9 | 131 | 91.0 | 18.7 |
| 10 | 0.01*M* EDTA + 0.1*M* urea | 6.0 | 5.8 | 129 | 96.5 | 18.1 |
| 11 | 0.1*M* EDTA + 1.0M urea | 6.1 | 6.0 | 129 | 94.0 | 19.2 |
| 12 | 0.01*M* EDTA + 4.0*M* urea | 6.3 | 6.4 | 117 | 97.0 | 26.0 |
| 13 | 0.01*M* EDTA + butanol | 6.0 | 6.0 | 123 | 98.0 | 22.6 |
| 14 | 0.01*M* EDTA + thioglycol | 4.2 | 4.2 | 129 | 94.0 | 19.2 |
| 15 | 0.1*M* $Na_2HPO_4$ | 9.1 | 8.7 | 87 | 82.5 | 53.2 |
| 16 | 0.1*M* $Na_2B_4O_7$ | ...... | 9.1 | 29 | 75.0 | 82.0 |
| 17 | 0.1*M* $NH_4OH$ | ...... | 10.3 | 45 | 88.0 | 71.8 |
| 18 | 0.1*M* amm. oxalate | ...... | 6.2 | 131 | 98.5 | 17.51 |
| 19 | 0.1*M* oxalic acid | ...... | 1.5 | 116 | 80.5 | 20.1 |
| 20 | 0.1*M* hydrochloric acid | ...... | 1.3 | 110 | 79.5 | 19.2 |
| 21 | 0.1*M* NaOH | ...... | 11.7 | 27 | ...... | 83.0 |
| 22 | 0.1*M* Na acetate | 7.8 | 9.0 | 28 | 90.0 | 82.5 |
| 23 | 0.1*M* Ca acetate | 6.8 | 9.2 | 65 | ...... | 40.6 |
| 24 | 0.1*M* acetic acid | 3.0 | 3.0 | 143 | 84.0 | 10.2 |
| 25 | 0.02*M* cupri amm. hydr. | 11.6 | 11.3 | 53 | 35.6 | 66.5 |
| 26 | 0.02*M* cupri amm. hydr. + $Na_2SO_3$ | 10.6 | 10.6 | 53 | 40.6 | 66.5 |
| 27 | Schweizer reagent air | ...... | ...... | 0 | ...... | 100.0 |
| 28 | Schweizer reagent $N_2$ | ...... | ...... | 111 | 27.7 | 30.5 |
| 29 | Conc. ammonia | ...... | ...... | 23 | 88.0 | 86.0 |
| 30 | Schweizer reagent air | ...... | ...... | 20 | 54.5 | 87.5 |

[a] 5-g portions.

sional stirring. The extracts were then filtered through 11 G-2 funnels, and the residue washed with the same solvent. The filtrate was centrifuged to separate starch, and then added to an equal volume of 95% alcohol acidified with 15 ml of concentrated hydrochloric acid per 500 ml of alcohol. As shown in Table 4, the pH of the extraction mixtures before filtration varied from 1.5 to 11.7 and higher. The marc samples treated with extractives 1 to 14 were stored for 48 hr at room temperature before filtration. Only a slight amount of gelatinous precipitate was observed in extracts 1–4, but more was present in 6–8. In extracts 8 and 9, a white granular material separated out, markedly in 9. It was suspected to be EDTA. The addition of 1 vol of the original extracting solutions 1–14 to 1 vol of acidified alcohol resulted in precipitation only with 9 (0.01*M* EDTA). In the presence of even a slight amount of calcium chloride, a more copious precipitate formed. In preparing reagent 14, 9 ml of an 80% pure thioglycolic acid were added to EDTA and the solution was then neutralized with NaOH. It was planned to bring the pH to 6.0, but even after addition of 140 ml of 0.5*N* NaOH the solution remained acid. Extracts 15–30 were stored for 24 hr but, as comparison of 5 and 15 shows, extraction was as complete in 24 hr as in 48 hr. Extractions 25 and 26 were made with reagents as prepared by Kolthoff and Stricks (1951). Cupriammonium hydroxide with sodium sulfite was proposed as a protein solvent by Swan (1957) and used in extracting proteins of wheat by McDermott and Pace (1959). Extractions 27 and 28 were made with freshly prepared Schweizer reagent. The marc blended with Schweizer reagent in air and stored for 24 hr dissolved completely, leaving only a few hard particles. In the experiment with Schweizer reagent in nitrogen, the reagent was deaerated and added to 5-g por-

tions of marc under an atmosphere of nitrogen and the mixture was stirred with nitrogen. In comparison with this, another portion was treated with Schweizer reagent added without blending. The alcohol precipitates with reagents 15–30 were appreciable except with 18, 20, and 24. Copper was present in residues from extractions 25–28 and 30. The residue from 25 was blue and gelatinous; 26 was brown and gelatinous; 27 was too small in amount; 28 was light-blue and gelatinous; and 30 was deep-blue and granular.

The residues of the extracted marc samples were washed with 70% alcohol and then with 50 ml water. Residues from extractions 25, 26, 28, and 30 were heated first with 200 ml of alcoholic hydrochloride to remove cupric salts present. It was difficult to remove all copper from 28, and a large excess of alcoholic HCl had to be used. Then these residues were washed with 70% alcohol until free of $Cl^-$, and finally with 50 ml water as above. The residual uronide content was determined by titration before and after saponification. The results are shown in Table 4.

The alcohol precipitates were separated from the acidified alcohol by centrifugation, and this was tested to determine whether additional matter precipitated on increasing the alcohol content from 50 to 75% by volume. Additional precipitate was obtained only with extracts 25, 26, 29, and 30. No weights were obtained for alcohol precipitates from extracts 5, 6, 9, 16. 16, 17, 19, 25, 26, 27, and 30. The remainder of the samples were washed with 70% alcohol until chloride-free. The chloride-free alcohol precipitates obtained from various extracts were analyzed by titration, with the results shown in Table 5.

There is a considerable discrepancy between the values for uronide extracted, calculated from titration of marc residues and those found by titration of the alcohol precipitates. Since neither the residues nor the precipitates were finely ground before titration, some error due to slow diffusion of alkali into the material was expected, but this should have been greater with the marc residues and greater with the initial titration than with the back titration. The marc residues may not have been washed sufficiently and undoubtedly contained more cations. Titration of ammonia-treated materials is subject to error, as pointed out by Joseph *et al.* (1949). Joseph *et al.* (1949) reported that the esterification degree of ammonia-demethylated pectinic acids by titration was too high because both $NH_4^+$ bound carboxyls and acid amide groups are included. The data clearly show, however, that neutral sodium salts such as NaCl extract more pectin than ammonium oxalate, even at room temperature, as was also found at 100° by Shioiri and Haginuma (1954), and that hydrochloric acid extracts more pectins than acetic acid and possibly oxalic acid. With alkaline salts and alkalis, the anion has more effect than pH since sodium borate at pH 9.1 extracts almost as much pectin as sodium hydroxide at pH 11.7, and more than ammonia at pH 10.3. Sodium sulfite at pH 8.9 extracted more pectin than sodium phosphate at pH 8.7.

The Schweizer reagent was found to extract apple starch as well as pectin, cellulose, protein, and other constituents. The starch was present in solubilized form in the alcohol precipitate and was removed only by prolonged leaching with hot water, in which the copper pectinates were not soluble. The pectins extracted with Schweizer reagent after precipitation with alcoholic HCl, treatment with hot water to remove starch, and washing with 70% alcohol until chloride free, were found to have an esterification degree of 92.5%. This value is probably too high, because of errors due to ammonium ion and acid amide groups, but it does indicate that demethylation is less than in sodium hydroxide extracts. Pectin swells readily in Schweizer reagent and forms a cupric pectinate gel. Pectic acid, on the other hand, does not swell, and is not dissolved by Schweizer reagent.

Sucharipa (1924) reported that, after prolonged extraction with Schweizer reagent in air at 0°C, the residual pectins present in a dried lemon peel preparation, when extracted with hot water at 0.5 atmosphere, 50% sucrose solution and 0.5% ammonium oxalate, yielded pectins of increasingly lower methoxyl content. It is likely that, under his conditions, de-esterification occurred both during treatment with Schweizer reagent and during

Table 5. Composition and yield of alcohol precipitates obtained from extracts (compare with Table 4).

| Extract | Uronide content (mg) | Degree of esterification (%) | Uronide extracted (%) |
|---|---|---|---|
| 1 | 2.7 | | 1.7 |
| 2 | 65.5 | 93.5 | 41.4 |
| 3 | 5.3 | ... | 3.4 |
| 4 | 83.5 | 93.5 | 25.0 |
| 7 | 31.5 | 86.5 | 25.0 |
| 8 | 108.5 | 90.5 | 68.6 |
| 10 | 40.4 | 78.0 | 25.6 |
| 11 | 45.8 | 82.5 | 29.0 |
| 12 | 61.7 | 84.5 | 39.0 |
| 13 | 48.7 | 78.0 | 30.8 |
| 14 | 28.9 | 83.5 | 18.3 |
| 18 | 30.5 | 81.0 | 19.3 |
| 20 | 71.0 | 78.5 | 44.9 |
| 22 | 51.7 | 79.5 | 32.7 |
| 23 | 20.8 | 80.0 | 13.2 |
| 24 | 25.1 | 78.5 | 15.9 |

pectin extraction. Branfoot (1929) reported practically complete solution of apple solids on treatment with Schweizer reagent in air, just as we observed.

At room temperature, 0.1*M* sodium hydroxide extracts at least 83% of the pectin present in the marc prepared from firm apples, but at 0°C it extracts less than half this quantity, and the sodium pectate obtained still contains appreciable quantities of nonuronide sugars.

Marc preparation 8 (149.2 g) treated with 800 ml of 1*N* NaOH for 65 hr at 0° and filtered gave a filtrate that, after acid hydrolysis, contained no detectable galacturonic acid, and a residue that washed with water to pH 12 also yielded a filtrate that contained only traces of galacturonic acid. The washed residue suspended in 0.1*M* NaCl and adjusted to pH 7.8 with HCl also gave a filtrate free of galacturonic acid. The final residue, heated with 1100 ml of water at 100°, gave a filtrate that contained galacturonic acid, galactose, and arabinose. This filtrate was poured into two volumes of alcoholic HCl (900 ml ethanol and 100 ml of 10*N* HCl). The alcoholic precipitate, after washing with alcohol and drying at 40°C, gave 2.758 g of material. This had a galacturonide content of 54.4%, representing a yield of 12.0% on a dry solids basis and a yield of 30.2% on the basis of uronide present in the marc. In a previous test, Overnet (1959) found that under similar conditions 47.72% of the pectin present in apple pomace and 32.09% of the pectin present in pear pomace could be recovered. These pomaces were treated with alcoholic hydrochloride for two days to remove cations, washed chloride-free with 70% alcohol, dried at 45°C *in vacuo*, and ground to fine powder before treatment with alkali.

**Extraction with hydrochloric acid at room temperature.** To obtain data on the effect of method of preparation of marc on the solubility of pectins, samples of marc preparations 1 to 14, previously dried *in vacuo*, weighing 0.50–2.00 g, were mixed with 20 ml 1*N* hydrochloric acid and allowed to stand 5 days at room temperature. Then they were mixed with 200 ml water, pH was adjusted to 3.5 by addition of 43–45 ml of 0.5*M* NaOH, and the mixtures were allowed to stand for 1 day to solubilize the pectins. The extracts were then filtered, and the total pectin extracted was determined by carbazole assay. The data obtained are shown in Table 6. In the Boskoop marcs, more pectin was extracted from marcs 13 and 14 than the others. Marc 14, prepared by treating cut apple tissue with 70% alcohol and then blending with alcohol acidified with HCl, by the Gee *et al.* (1958) procedure, yielded the most, and marc 4, prepared by first heating cut flesh in alcohol before blending, the least. The marc prepared from apple pomace, 7, yielded about 50% less pectin than 8, prepared by heating the alcoholic mixture of the fruit flesh after blending. The Weinapfel marcs, 9–12, yielded appreciably more pectin than similar Boskoop marcs. The factory-dried pomace, 12, yielded less pectin than fresh apple pomace preserved with alcohol, 11; and the marc prepared by allowing apple flesh to oxidize before treatment with alcohol, 10, yielded about 50% less pectin than that prepared from heat-inactivated flesh, 9.

During treatment with 1*N* HCl, marcs changed in color: the originally white marcs became red, and the brown marcs became reddish-brown. The particles swelled and became gelatinous in appearance.

**Effect of acid concentration.** To obtain data on the effect of concentration of hydrochloric acid at room temperature on the extractability of pectins, 10-g portions of marcs 7 and 8 were mixed with 20 ml of hydrochloric acid varying from 0.1*N* to concentrated, and the mixture was allowed to stand 16 hr at room temperature. Then 250 ml water were added to each preparation, and sufficient 0.5*N* NaOH to adjust the pH to 3.2. Sufficient NaCl was added to the more dilute acid treatments to adjust them to approximately the same sodium content, and the mixtures were allowed to stand for 24 hr with occasional stirring. They were then filtered, and the filtrate poured into two volumes of alcohol to precipitate the pectins. The precipitated pectins were washed with 70% alcohol until chloride-free, dried, and weighed, and their uronide content was determined. An aliquot of the pectins obtained was used to measure relative viscosity by dissolving 25 mg in 25 ml of solution containing 0.2 g NaCl, and 0.05 g EDTA adjusted to pH

Table 6. Extraction of pectins with 1*N* hydrochloric acid.

| Marc | Weight of marc (g) | AUA present in marc (mg) | AUA extracted (mg) | % extracted |
|---|---|---|---|---|
| 1 | 0.48 | 86.5 | 16.3 | 18.9 |
| 2 | 0.64 | 128.0 | 24.8 | 19.4 |
| 3 | 0.90 | 180.0 | 61.0 | 33.9 |
| 4 | 0.57 | 125.4 | 15.6 | 12.4 |
| 7 | 1.29 | 345.4 | 72.5 | 21.0 |
| 8 | 1.65 | 304.0 | 100.0 | 32.9 |
| 9 | 0.54 | 99.0 | 36.7 | 37.2 |
| 10 | 0.68 | 140.0 | 33.6 | 24.0 |
| 11 | 1.41 | 151.0 | 46.2 | 30.6 |
| 12 | 2.26 | 326.0 | 78.0 | 23.9 |
| 13 | 0.69 | 112.8 | 60.5 | 53.6 |
| 14 | 0.89 | 122.0 | 79.2 | 65.0 |
| 15 | 1.14 | 333.0 | 90.5 | 27.1 |

Table 7. Acid concentrations used in extraction of marcs 7 and 8.

| Sample [a] | Weight (g) | Conc. of HCl | pH of extract | Vol. 0.5N NaOH added to adjust pH to 3.2 | pH after addition of NaOH | NaCl added (g) |
|---|---|---|---|---|---|---|
| 7–1 | 10.00 | 0.1N | 2.4 | 0.35 | 3.2 | 12.5 |
| 7–2 | 10.00 | 1.0N | 2.25 | 21.0 | 3.15 | 11.5 |
| 7–3 | 10.00 | 6.0N | 1.75 | 129 | 3.15 | 5.0 |
| 7–4 | 10.00 | 12.0N | 1.50 | 213 | 3.15 | 0.0 |
| 8–1 | 10.02 | 0.1N | 3.15 | 0 | 3.15 | 12.5 |
| 8–2 | 10.01 | 1.0N | 2.20 | 20 | 3.15 | 11.5 |
| 8–3 | 10.02 | 6.0N | 1.65 | 130 | 3.15 | 5.0 |
| 8–4 | 10.03 | 12.0N | 1.40 | 225 | 3.15 | 0.0 |

[a] Number identifies marc used (7 or 8) and concentration of acid added.

Table 8. Composition of alcohol precipitate obtained.

| Sample | Weight (g) | AUA (%) | % esterification | Relative viscosity |
|---|---|---|---|---|
| 7–1 | 0.1396 | 37.0 | 97.0 | 1.34 |
| 7–2 | 0.1560 | 31.6 | 96.0 | 1.33 |
| 7–3 | 0.3005 | 38.3 | 93.5 | 1.13 |
| 7–4 | 0.4382 | 31.4 | 92.5 | 1.09 |
| 8–1 | 0.2040 | 21.8 | 97.5 | 1.33 |
| 8–2 | 0.2202 | 50.0 | 97.0 | 1.81 |
| 8–3 | 0.3382 | 38.6 | 94.0 | 1.27 |
| 8–4 | 0.4576 | 59.2 | 71.5 | 1.08 |

6 as recommended by Owens *et al.* (1952). The alcoholic filtrate was saved, and the content of lower-molecular-weight uronides was determined by carbazole assay. The residue after extraction was washed with 70% alcohol until chloride-free, and the residual uronide determined by titration.

The conditions of extraction are shown in Table 7, which gives the pH after addition of water and after addition of alkali. The marcs did not change in color or texture on treatment with 0.1N HCl or 1.0N HCl, but on treatment with 6N HCl they browned and became gelatinous, and on treatment with concentrated HCl the marc from apple pomace liquefied and became dark-red, and that from fresh apples became red-brown and liquefied. As the data in Table 8 indicate, depolymerization of pectins was appreciable in the presence of 6N and 12N hydrochloric acid. This is shown not only by a decrease in relative viscosity of a 0.10% solution but also by an increase in the concentration of alcohol-soluble uronides. The degree of esterification also decreased with increase in acid concentration, but this was more marked with the unoxidized marc 8 than with the brown pomace marc. The recovery of pectins was considerably higher from the white marc than from the browned marc, as shown in Tables 9 and 10. In the latter cases the residues from marc 7 were too dark in color to be titrated except for the final residue.

## RATE OF EXTRACTION DURING ACID TREATMENT

It was planned to obtain data on rate of extraction of pectins from several marcs during treatment with hydrochloric acid at room temperature and at 40°C, but time permitted only one run with marc 14. One-gram portions of this were mixed with 5 ml of 0.5N HCl at 40°C, and the mixture was held 0–48 hr at 40°C. At various intervals the samples were diluted with 200 ml of water, adjusted to pH 3.5, and allowed to stand at room

Table 9. Recovery of pectic substances in extracts.

| Sample | AUA in alcohol (mg) | AUA in alcohol-pptd. (mg) | AUA recovered (%) |
|---|---|---|---|
| 7–1 | 1.9 | 51.8 | 8.9 |
| 7–2 | 3.7 | 49.2 | 8.8 |
| 7–3 | 5.2 | 115.0 | 19.8 |
| 7–4 | 53.0 | 137.5 | 31.4 |
| 8–1 | 0.4 | 44.6 | 15.9 |
| 8–2 | 3.7 | 110.0 | 36.2 |
| 8–3 | 5.7 | 131.0 | 43.5 |
| 8–4 | 28.2 | 270.0 | 94.5 |

Table 10. Composition of marc residues.

| Sample | % esterification | AUA (mg) | % of original AUA |
|---|---|---|---|
| 7–1 | ...... | ...... | ...... |
| 7–2 | ...... | ...... | ...... |
| 7–3 | ...... | ...... | ...... |
| 7–4 | 80.0 | 113.5 | 18.7 |
| 8–1 | 87.5 | 204 | 64.8 |
| 8–2 | 83.3 | 149 | 47.5 |
| 8–3 | 61.5 | 112 | 35.6 |
| 8–4 | 60.0 | 23 | 7.2 |

Table 11. Yield and composition of pectins obtained during extraction with 0.5N HCl.

| Time (hr) | Alcohol precipitate (mg) | AUA (mg) | % esterification | Viscosity | Alcohol solution AUA content (mg) | Marc AUA content (mg) | Residue weight (mg) |
|---|---|---|---|---|---|---|---|
| 0 | 70 | 52.2 | 73 | 2.15 | 1 | 160 | 780 |
| 2 | 90 | 64.5 | 73 | 1.95 | 1 | 130 | 750 |
| 4 | 110 | 67.2 | 71 | 1.95 | 3 | 124 | 650 |
| 8 | 130 | 88.2 | 76 | 1.92 | 2 | 124 | 670 |
| 16 | 140 | 64.5 | 58 | 1.67 | 2 | 109 | 620 |
| 24 | 100 | 60.8 | 58 | 1.67 | 6 | 119 | 600 |
| 32 | 90 | 55.2 | 55 | 1.47 | 3 | 130 | 650 |
| 48 | | 6.5 | ..... | ..... | 10 | 143 | 650 |

temperature for 24 hr. The pectins extracted were then precipitated with alcohol and analyzed. As the data in Table 11 indicate, the weight of the alcohol precipitate recovered increased up to 16 hr and then decreased until after 48 hr; no alcohol-precipitable matter was obtained. The esterification degree of pectins extracted was constant up to 48 hr and then decreased. The marc initially contained 137 mg of AUA, so that maximum recovery occurred at 8 hr. The data, however, are incomplete because losses of uronide during washing with 70% alcohol to remove chloride were not included. The chief changes in nonuronide sugars during extraction were found to be in the glucose and galactose sugars; the former decreased and the latter increased in amount as extraction time increased. The pentose sugars appeared to be unchanged.

The change in degree of polymerization of the pectins extracted at longer periods was surprising. Weber (1944) found that pectins did not change appreciably in degree of esterification or degree of polymerization at 40°C in 0.5N HCl, and the commercial jelly grade of low-methoxyl pectins obtained from apple pomace heated under similar condition is high. It is possible that the type of marc used may have affected the results. Marc 14, an alcoholic hydrochloric-acid-heated marc was selected because it contained more readily soluble pectins.

**Effect of chlorite treatment.** During extraction with hydrochloric acid the marcs reddened, particularly in the presence of more concentrated acid. Commercial apple pectins, on treatment with concentrated hydrochloric acid, changed from greenish-yellow to dark-brown to red-brown. Commercial citrus pectin changed from light-yellow to gray-green, but citrus polygalacturonic acid did not change in color. Pear pectin also became reddish-brown. This color change was suspected to be due to the presence of leuco-anthocyanins and other flavonoids. They were recently shown to be present in commercial apple pectins in appreciable amounts (Kalab and Zitko, 1959). Sugar-beet pectins and pectates did not change in color on treatment with excess of concentrated hydrochloric acid. A sample of highly purified white apple pectin, which had been decolorized with chlorous acid, also remained white. Pallmann and Deuel (1945) reported that chlorine dioxide was without effect on viscosity of pectins but had a decolorizing action.

Preliminary tests indicated that pretreatment of unoxodized white marcs with chlorite in acetic acid solution had no effect on recovery of pectins, but the possible effect of this on recovery of pectins from browned marcs was not investigated. The effect of treatment with an excess of sodium chlorite was investigated as follows:

Ten-gram samples of marc preparations 2, 7, 9, 10, 11, 12, 13, 21A, 26, and 28 were weighed out in duplicate, transferred to Erlenmeyer flasks with 500 ml 1*N* acetic acid, and to one set (designated *a*) 10 mg of Mathison impure $NaClO_2$ were added. They were allowed to stand at room temperature, exposed to light, for three days, and were then

Table 12. Yield and composition of alcohol precipitate and extract from chlorite-treated marcs.

| Marc | Dry weight (g) | Alcohol precepitate: Cold HCl extract (g) | Alcohol precepitate: Hot HCl extract (g) | Residue (g) | HAc extract (mg AUA) |
|---|---|---|---|---|---|
| 2 | 1.35 | 0.07 | 0.27 | 0.55 | 79.7 |
| 7 | 2.35 | 0.09 | 0.30 | 0.60 | 60.5 |
| 9 | 1.27 | 0.11 | 0.19 | 0.48 | 70.5 |
| 10 | 1.61 | 0.11 | 0.33 | 0.62 | 70.5 |
| 11 | 2.73 | 0.12 | 0.46 | 1.52 | 40.6 |
| 12 | 4.50 | 0.14 | 0.75 | 3.53 | 68.8 |
| 13 | 1.48 | 0.06 | 0.24 | 0.72 | 95.5 |
| 21a | 2.19 | 0.25 | 0.15 | 1.33 | 108 |
| 26 | 3.13 | 0.11 | 0.21 | 1.36 | 225 |
| 28 | 3.53 | 0.15 | 0.27 | 1.51 | 97.2 |

filtered and the residue washed with 200 ml water and with 500 ml 0.1*N* HCl. After storage for 2–3 days, the cold HCl extract was separated and the residue again extracted with 0.1*N* HCl at 80°C for 1 hr. The original acetic acid extract, the alcohol solution from precipitated pectins of cold and hot HCl extraction, was analyzed for uronides by carbazole assay. The alcohol precipitates from hot and cold HCl extracts, after washing free of $Cl^-$ with 70% alcohol, were washed with 93% alcohol and acetone, allowed to air dry, and then dried for 6 hr at 50°C. The uronide content of residue and alcohol precipitates was determined by titration before and after saponification to Hinton indicator end point, with the results shown in Tables 12 and 13.

The uronide content in the original marc preparations and in the alcohol precipitates obtained is shown in Table 14, and of the uronide content of the residues in Table 15. The characteristics of the pectin in marc, alcohol precipitates, and residue are shown in Table 16.

The data indicate that the recovery of pectins was increased in marcs 2, 7, 10, 11, 12, 13, 21, and 26, but not in 9 or 28. It was previously found that treatment of marc 8 with chlorite in 0.1*N* acetic acid solution at lower levels had no effect on pectin recovery.

The lower extractability of pectins from oxidized marcs may be due to the formation of

Table 13. Analyses of original acetic acid extracts and alcohol extracts from chlorine-treated marcs.

| Sample | HAc extract | mg of AUA HCl (cold) | HCl (hot) | Total |
|---|---|---|---|---|
| 2 | 79.7 | 4.6 | 8.3 | 92.6 |
| 2a | | 3.2 | 0.9 | (83.8)[a] |
| 7 | 60.5 | 3.2 | 6.2 | 69.9 |
| 7a | | 2.5 | 8.8 | (71.8) |
| 9 | 70.5 | 2.8 | 10.8 | 84.1 |
| 9a | | 1.2 | 5.0 | (76.2) |
| 10 | 70.5 | 2.7 | 7.1 | 80.3 |
| 10a | | 2.5 | 7.5 | (80.5) |
| 11 | 40.6 | 3.2 | 11.6 | 55.4 |
| 11a | | 2.7 | 6.2 | (49.5) |
| 12 | 68.8 | 3.7 | 8.8 | 81.3 |
| 12a | | 3.2 | 11.6 | (83.6) |
| 13 | 95.5 | 1.2 | 1.0 | 97.7 |
| 13a | | 1.2 | 2.2 | (98.9) |
| 21a | 108 | 2.5 | 1.0 | 111.5 |
| 21aa | | 1.2 | 0.8 | (110.0) |
| 26 | 225 | 3.2 | 3.5 | 231.7 |
| 26a | | 0.9 | 1.0 | (226.9) |
| 28 | 97.2 | 1.2 | 7.5 | 105.9 |
| 28a | | 0.9 | | (106 ) |

[a] (  ) = estimated.

Table 14. Uronide extracted from chlorite-treated marcs.

| Sample | Original uronide (mg) | Uronide in alcohol precipitate | | Total uronide recovered (mg) | % extracted |
|---|---|---|---|---|---|
| | | Cold HCl (mg) | Hot HCl (mg) | | |
| 2 | 0.272 | 24 | 9.2 | 0.126 | 46.8 |
| 2a | | 91 | 32.2 | 0.217 | 80.5 |
| 7 | 0.605 | 52.5 | 88.3 | 0.211 | 35.0 |
| 7a | | 234 | 74.5 | 0.380 | 63.0 |
| 9 | 0.232 | 37.7 | 28.5 | 0.150 | 64.5 |
| 9a | | 39.0 | 30.4 | 0.146 | 63.0 |
| 10 | 0.330 | 45.11 | 57.0 | 0.182 | 55.0 |
| 10a | | 151 | 17.5 | 0.249 | 75.5 |
| 11 | 0.470 | 46.0 | 93.8 | 0.195 | 41.5 |
| 11a | | 76.3 | 109.5 | 0.235 | 50.0 |
| 12 | 0.645 | 93.1 | 123.2 | 0.298 | 46.5 |
| 12a | | 261 | 47.0 | 0.392 | 61.0 |
| 13 | 0.238 | 38.6 | 44.2 | 0.181 | 71.0 |
| 13a | | 98.3 | 35.0 | 0.232 | 97.0 |
| 21a | 0.513 | 89.3 | 35.0 | 0.236 | 46.0 |
| 21aa | | 147 | 90.2 | 0.347 | 67.5 |
| 26 | 0.875 | 89.3 | 48.8 | 0.370 | 42.2 |
| 26a | | 186.5 | 5.5 | 0.419 | 48.0 |
| 28 | 0.775 | 100.5 | 127.8 | 0.334 | 43.2 |
| 28a | | 202.0 | | 0.308[a] | 40.0[a] |

polymerized oxidation products of the naturally occurring phenolic substrates, which would decrease diffusion of solvent into the tissue or the outward diffusion of solubilized pectins. It is also possible that combinations of pectins with the products of oxidation might occur in a manner similar to the recently reported inclusion of sugars in lignins produced by oxidation of their precursors in the presence of sugars (Freudenberg and Grien, 1959). These hypotheses were tested by oxidizing catechol, chlorogenic acid, or apple tannin extracts in the presence of pectins, by polyphenolase preparations, in the presence of chlorite-bleached purified pectins, and in presence of commercial apple pectin. Oxidations of catechol and apple tannins occurred readily at pH 6, but oxidation of chlorogenic acid was much less. Oxidative browning was more pronounced with commercial apple pectin than with purified pectin. An increase of viscosity was observed in solutions containing pectin, phenolase, and catechol or apple tannins, but precipitation of pectins did not occur under the conditions used.

In the industry it has been observed that the extractability of pectins from apple pomace decreases in drying, and that the decrease is greater when drying is delayed. This would indicate that oxidation of apple pomace constituents decreases the solubility of pectins. The pectins extracted from fresh pomace, pomace dried shortly after pressing, and pomace stored for 1 or 2 days before drying are similar in degree of esterification and jelly grade.

Table 15. Residual uronide in chlorite-treated marcs.

| Sample | Residual uronide (mg) | % | Residue, dry wt (mg) | % of original marc |
|---|---|---|---|---|
| 2 | 12.9 | 4.8 | 0.55 | 40.8 |
| 2a | 2.8 | 1.0 | 0.61 | 45.0 |
| 7 | 163.5 | 27.0 | 0.60 | 25.4 |
| 7a | 95.7 | 14.8 | 0.90 | 38.0 |
| 9 | 35.8 | 15.5 | 0.48 | 37.7 |
| 9a | 42.3 | 18.2 | 0.50 | 39.4 |
| 10 | 71.7 | 3.5 | 0.62 | 38.5 |
| 10a | 6.5 | 2.0 | 0.46 | 28.6 |
| 11 | 143.5 | 30.5 | 1.52 | 55.5 |
| 11a | 202 | 43.0 | 2.55 | 93.5 |
| 12 | 195 | 82.5 | 3.53 | 78.2 |
| 12a | 93.8 | 39.5 | 0.95 | 21.2 |
| 13 | 15.6 | 6.5 | 0.72 | 51.0 |
| 13a | 24.8 | 10.2 | 0.51 | 34.4 |
| 21a | 15.8 | 3.0 | 1.33 | 61.0 |
| 21aa | 99.3 | 19.3 | 1.02 | 46.7 |
| 26 | 152.8 | 17.5 | 1.36 | 43.5 |
| 26a | 166.5 | 19.0 | 1.12 | 36.0 |
| 28 | 139.8 | 18.0 | 1.51 | 60.0 |
| 28a | ...... | ...... | ...... | |

**Association of proteins with pectins in apple tissue.** Marc preparations 2, 7, and 28, described above, and two preparations of alcohol precipitate

Table 16. Characteristics of pectins in marc, alcohol precipitates, and residue.

| | Original marc | | Cold HCl extract | | Hot HCl extract | | Marc residue | |
|---|---|---|---|---|---|---|---|---|
| | % uronide | % esterification | % uronide | % esterification | % uronide | % esterification | % uronide | % esterification |
| 2 | 27.0 | 83.8 | 34.4 | 57.5 | 3.2 | 90.0 | 2.4 | 64.5 |
| 2a | | | 60.5 | 50.5 | 12.4 | 74.5 | 0.1 | 0.0 |
| 7 | 25.8 | 85.5 | 58.5 | 47.5 | 29.4 | 82.5 | 4.5 | 85 |
| 7a | | | 67.0 | 45.5 | 24.1 | 84.0 | 1.8 | 81 |
| 9 | 18.2 | 80.0 | 34.4 | 56.0 | 15.0 | 90 | 3.1 | 89.5 |
| 9a | | | 39.5 | 65.0 | 16.0 | 91 | 3.6 | 89 |
| 10 | 20.5 | 83.5 | 41.8 | 51.0 | 17.3 | 97 | 3.5 | 92.5 |
| 10a | | | 49.5 | 56.0 | 5.3 | 84.5 | 0.4 | 57 |
| 11 | 17.2 | 87.0 | 38.2 | 46.0 | 20.4 | 80.5 | 2.0 | 87 |
| 11a | | | 40.0 | 50.0 | 17.4 | 83 | 1.7 | 85 |
| 12 | 14.3 | 82.5 | 66.5 | 50.0 | 16.4 | 77 | 0.8 | 90.5 |
| 12a | | | 67.0 | 49.0 | 12.3 | 92 | 1.5 | 87.5 |
| 13 | 16.1 | 77.0 | 64.5 | 47.5 | 18.4 | | 0.9 | 41.0 |
| 13a | | | 89.5 | 68.5 | 35.0 | 89.5 | 2.0 | 81.5 |
| 21a | 35.6 | 87.5 | 70.5 | 50.0 | 23.4 | 84 | 1.5 | 88.5 |
| 21aa | | | 59.0 | 51.0 | 39.2 | 87 | 1.1 | 87.0 |
| 26 | 28.0 | 87.5 | 81.5 | 49.5 | 23.3 | 94.5 | 1.7 | 44.5 |
| 26a | | | 78.0 | 58.0 | 2.6 | 66.5 | 1.7 | 44.5 |
| 28 | 30.6 | 77.5 | 67.0 | 56.0 | 47.5 | 61.5 | 1.2 | 88.5 |
| 28a | | | 74.5 | 52.5 | | | | |

from apple juice were investigated for the presence of proteins in association with pectins. The total N content of alcohol precipitates was determined, and the effect of added proteases on extractability of pectins was investigated.

The treatment with proteases was conducted by addition of 0.20 g of pepsin purum Ph.H.V. from B. Siegfried to 5.00-g portions of marc suspended in 200 ml of 0.1*N* HCl, or by addition of 0.25 g of Pineapple Stem Bromelain (containing pectin esterase as well as other enzymes) preparation No. 62, Hawaiian Pineapple Co., to 5.00-g portions of marc suspended in 200 ml of 0.1*M* phosphate buffer at pH 5.0. The marc-protease mixtures were allowed to stand, with occasional stirring, for 4 days, and then filtered, and the residue washed with 200 ml of water. The washed residue was then suspended in 250 ml of 0.1*N* HCl, heated to 70°C in an oven (1 hr), and held 1 hr at 70°C. The acid extract was then separated by filtration, and the pectins present were precipitated by addition of 1 volume of alcohol. The alcohol precipitate was separated by centrifuging, then filtered, and washed $Cl^-$ free with 70% alcohol. The final residue was washed free of $Cl^-$. The dry weight of alcohol precipitate and residue was obtained by drying *in vacuo at* 70°C, and the anhydrogalacturonic acid content by titration as above.

**Pectins in apple juice.** The alcohol-precipitable material present in juice expressed from freshly harvested and from storage-ripened apples was found to contain a surprisingly high amount of nitrogenous matter, chiefly protein. It is curious that the material present in low concentrations in the juice of firm apples (0.157 g per L) contained as much nitrogen (2.2%) as that present in much higher concentrations (1.315 g per L) in juice from storage-ripened apples. On the basis of the results reported by Koch *et al.* (1959), only traces of protein would be expected in the former. The protein content of the juice from firm apples amounted to 21.6 mg/L, in comparison with 181 mg (8.38 times as much) in ripe apple juice.

As shown in Table 17, the pectin content of the alcohol precipitates, calculated as anhydrogalacturonic acid, is lower in preparation 6 than in preparation 25, and the intrinsic viscosity (a measure of the viscosity average molecular weight) of the pectin from green apple juice (from Fig. 1 of Owens *et al.*, 1952) is about 2.5, whereas that for riper apple juice is about 5.0. The pectins in the latter, far from being degraded, are much more highly polymerized. It is interesting too that the degree of esterification is higher in the latter case. Doesburg (1959b) and Woodmansee *et al.* (1959) recently reported that, contrary to previously published observations, neither the degree of polymerization nor the degree of esterification of apple pectins changes materially during ripening and storage.

Table 17. Characteristics of apple juice pectin preparations.

| Preparation | AUA (mg/g) | % esterification | Relative viscosity: Conc. (g/100 ml) | Relative viscosity: $\eta$ |
|---|---|---|---|---|
| 6 | 184 | 75.0 | 0.0510 | 1.10 |
| 6A | 147 | 77.0 | 0.1050 | 1.26 |
| 25 | 466 | 94.5 | 0.1002 | 1.60 |
| 25A | 474 | 97.0 | 0.0995 | 1.57 |

The nonuronide content of the alcohol precipitate from the juice of firm apples amounted to 81.6%, of which ash accounted for 7.7% and protein for 13.75%, and the balance was hemicelluloses. The hydrolysate of this contained arabinose, glucose, galactose, and xylose, as well as several additional components visible in the ultraviolet between the galactose-glucose and arabinose-xylose bands.

In comparison, the nonuronide content of the alcohol precipitate from stored apple juice amounted to 53.3%, of which ash accounted for 3.7% and protein 13.75%. Arabinose, glucose, galactose, and xylose were present, as well as other constituents visible in uv. The chromatogram was similar in appearance to that obtained above, but with more intense galactose and xylose bands.

The N content of these preparations was much higher than that found in the apple flesh itself (0.3% N and 0.8% ash), in the marcs prepared (these contained 0.7%-1.2% N, depending on ripeness of apple or method of preparing the marc), or in pectins extracted from these marcs. The cold-water-soluble pectin contained 0.9% N and 3.7% ash, and the cold-dilute-alkali-extracted pectin contained 0.5% N and 4.3% ash, and the cold-dilute-hydrochloric-acid-extracted pectin as precipitated contained 0.5% N and 0.2% ash. Several commercial pectin preparations were analyzed for nitrogen and ash content, with the following results:

| | % N | % ash |
|---|---|---|
| Exchange citrus pectin NF | 1.5 | 1.2 |
| Exchange citrus polygalacturonic acid | 0.0 | 2.4 |
| Marwel spray-dried apple pectin | 0.0 | 12.3 |
| Marwel apple pectin | 0.1 | traces |

The occurrence of proteins in the concentrations found above requires re-evaluation of the chemistry of clarification of apple juice with fungal pectinase preparations. Joslyn *et al.* (1952) did not find that the rate or extent of clarification of apple juice by several commercial pectin enzyme preparations was related either to their pectin esterase or pectin polygalacturonidase activity. This was recently confirmed by Neubeck (1959). The nonuronide

constituents present may act as protective colloids, and after their hydrolysis flocculation may occur. Since fungal pectinase preparations are likely to contain proteases as well as pectinases and hemicelluloses, it may be that clarification is the result of changes in both pectin and nonpectin constituents.

It is unlikely that the pectins in the juice are combined with proteins. Fremy (1848) reported earlier that pure pectins could be obtained from juice by selecting ripe fruit, filtering the expressed juice, and precipitating the proteins present by addition of tannic acid. The pectins remaining in the juice could then be precipitated with alcohol to yield protein-free alcoholic precipitates.

With regard to the apparently high viscosity of the pectin precipitated from the juice of stored apples, Saverborn (1945) reported that the molecular weight of pectin obtained from the juice of fresh ripe Cox orange apples was 201,000, in comparison with 58,000 for that obtained from the same lot of fruit boiled for four successive times (20 min each) with 96% alcohol and then in water for 1 hr. A commercial apple pectin had a molecular weight of 67,000 under the same conditions. Schlubach and Hoffmann-Walbeck (1949) reported that the molecular weight of pectins precipitated from the juice of Roter Eisapfel was as high as 238,000, in comparison with 165,000 for nitropectins obtained by direct nitration of apple shreds. They obtained nitropectins of highest molecular weight (342,000) by extracting pectins from alcohol-treated heat-inactivated apple shreds with phosphate buffer at pH 4.6 for 72 hr at 35°C.

**Effect of treatment of marc with protease.** On the assumption that proteins form water-insoluble compounds with pectins it should be possible to increase the solubility of the pectins present by treatment with suitable proteases, provided that the proteases selected will be able to act on the proteins present. To test this hypothesis, three marc preparations were treated with two protease preparations as described above. The 5 g of marc preparation 2 contained 136 mg of anhydrouronic acid with an esterification degree of 86.0%; marc preparation 7 contained 302.5 mg of anhydrouronic acid with an esterification degree of 86.0%, and marc 28 contained 387.5 mg AUA with an esterification degree of 94.5%.

After treatment with protease for 4 days, the marc suspension were filtered, and the filtrate diluted to 500 ml. Table 18 shows the pH value of the diluted filtrate, its relative viscosity, and the anhydrogalacturonic acid content as determined by carbazole assay. The data indicate that viscosity as measured by a Brookfield viscosimeter was not appreciably different in phosphate buffer with or without bromelain, and, although it was somewhat higher in the HCl solution, there was no detectable difference between marcs treated with pepsin or untreated. On the other hand, the quantity of pectins extracted by the phosphate buffer from marcs 2 and 7 was appreciably higher in the presence of bromelain, but no change occurred with marc 28. Hydrochloric acid extracted considerably more pectin than did phosphate buffer, but only with marc 2 did pepsin have an appreciable effect; with marc 7 the increase was very small, and with 28 a decrease was found.

Table 19 shows the effect of protease treatment on extractability of pectins in hot dilute hydrochloric acid. Treatment with bromelain decreased the weight of alcohol-precipitable matter obtained from marcs 2 and 28, and increased the weight from marc 7. The uronide content of the precipitate was low, and, if anything, treatment with bromelain tended to decrease this. The total uronide recovered by acid extraction decreased after treatment with bromelain, the decrease being more marked with marc 2. Pepsin had little effect on the yield of alcohol-precipitable matter, but tended to increase its purity. The total uronide recovered by acid extraction increased after treatment with pepsin except for marc 2, and this increase was more appreciable with marc 7.

Table 20 compares the total anhydrogalacturonide content extracted, initially and after treatment with hot acid, as calculated from the weight of final residue and its uronide content, with that calculated from data on uronide content of the original extract, the alcohol-precipitable matter extracted with hot acid, and alcohol-soluble uronide content. There are appreciable differences between these two values, largely from titration errors. The residual marc was difficult to suspend,

Table 18. pH, viscosity, and pectin content of extracts with and without added protease.

| Treatment [a] | pH | Relative viscosity | mg AUA in extract | % of total AUA extracted |
|---|---|---|---|---|
| 2B1 | 5.05 | 11.5 | 6.7 | 4,8 |
| 2B2 | 4.45 | 11.0 | 25.4 | 18.7 |
| 7B1 | 4.70 | 10.5 | 7.5 | 2.5 |
| 7B2 | 4.10 | 10.5 | 26.2 | 8.7 |
| 28B1 | 4.65 | 12.5 | 106.1 | 27.3 |
| 28B2 | 4.05 | 11.5 | 107.8 | 27.8 |
| 2P1 | 1.85 | 12.0 | 40.2 | 29.5 |
| 2P2 | 1.85 | 12.0 | 45.2 | 33.2 |
| 7P1 | 1.85 | ...... | 88.0 | 29.1 |
| 7P2 | 1.85 | ...... | 86.5 | 28.6 |
| 28P1 | 1.85 | 13.5 | 105.9 | 27.4 |
| 28P2 | 1.85 | 13.5 | 70.5 | 18.3 |

[a] The first number indicates marc preparation; B, bromelain; P, pepsin treatment; 1, control; 2, enzyme-treated.

Table 19. Extractability of pectins in hydrochloric acid, with and without protease.

| Extract | Alcohol precipitate Weight (mg) | Alcohol precipitate AUA (mg) | % esterification | % uronide | Alcohol solution (mg AUA) | Total uronide extracted mg | Total uronide extracted % |
|---|---|---|---|---|---|---|---|
| 2B1 | 250 | 45.1 | 94 | 18.0 | 3.5 | 48.6 | 35.7 |
| 2B2 | 200 | 26.6 | 80 | 13.3 | 4.6 | 31.2 | 23.0 |
| 7B1 | 250 | 87.3 | 88 | 35.0 | 5.6 | 92.9 | 30.7 |
| 7B2 | 300 | 69.0 | 95 | 23.0 | 5.4 | 74.4 | 24.7 |
| 28B1 | 230 | 69.0 | 95 | 30.0 | 3.5 | 72.5 | 18.7 |
| 28B2 | 200 | 59.8 | 93 | 30.0 | 2.2 | 62.0 | 16.0 |
| 2P1 | 140 | 30.4 | 73 | 21.9 | 2.8 | 33.2 | 24.4 |
| 2P2 | 130 | 27.6 | 87 | 21.2 | 3.5 | 31.1 | 22.9 |
| 7P1 | 210 | 29.8 | 97 | 14.2 | 5.4 | 35.2 | 11.6 |
| 7P2 | 240 | 56.1 | 92 | 23.3 | 5.4 | 61.5 | 20.3 |
| 28P1 | 210 | 64.3 | 95 | 30.7 | 2.3 | 66.9 | 17.2 |
| 28P2 | 190 | 73.6 | 78 | 38.7 | 2.9 | 75.9 | 19.6 |

and the end point faded because of slow diffusion into the interior. The results would have been more consistent if the marc had been ground to a fine powder and blended with water before titration.

On the basis of residual uronide analyses, bromelain treatment increased the solubility of pectins appreciably in marcs 2 and 7 and only slightly in marc 28. Pepsin treatment increased the solubility of pectins in all three marcs, but its effect was greater in marc 7. In no case, however, was solubility complete.

It is likely that the conditions selected were not such as to allow free diffusion of protease into the particles of the marcs used, but it is surprising that the effects observed were as appreciable with marc 7, an oxidized brown marc containing apple skin particles, as with marc 2, a white, more porous marc. Optimum conditions for the proteolysis of apple tissue proteins are not known, but the results obtained would have been more significant had they been conducted under optimum conditions.

The generally small effect of protease treatment on the solubility of pectins present in apple marc similar to 2 is similar to the effects at pH 6 of 0.5% EDTA solution with and without added urea, butanol, and thioglycolic acid. None of these had an appreciable effect on the solubility of pectins although Ginsburg (1958) found them to have an appreciable effect on cell separation. The addition of sodium sulfite to a dilute cuprammonium hydroxide solution, introduced by Kolthoff and Stricks (1951) for the polarographic assay of cystine, and used by Swan (1957) and McDermott and Pace (1959) for solubilizing sulfur-bound proteins, had no effect on the solubility of apple marc pectins. Protein S—S bonds, thus, are not involved in the insolubility of apple pectins, and protein-pectin combinations may not be limiting factors, if present.

## CONCLUSIONS

The differences explain the differences in the solubility of pectins reported in the literature. Maturity and variety of apples also affect rate and extent of extraction of pectins.

Successive serial extraction with a variety of solvents at room temperature with several marc preparations indicated that marc prepared by blending apple tissue with alcoholic hydrochloric acid solutions contained pectins that were more readily extractable with water than marc treated only with alcohol.

Extraction of a selected sample of marc with a variety of solvents indicated that neutral salts such as sodium chloride extracted appreciably more pectins than water alone. Ethylenediaminetetraacetate at pH 6 did not extract more pectins than water

Table 20. Pectin recovered with and without protease on basis of residue.

| Extract | Residue mg | Residue AUA (mg) | Loss in weight (%) | Loss in AUA (%) | AUA recovered (%) |
|---|---|---|---|---|---|
| 2B1 | 210 | 39.5 | 57.5 | 61.0 | 69.8 |
| 2B2 | 210 | 21.2 | 71.2 | 84.4 | 57.3 |
| 7B1 | 790 | 120.5 | 33.0 | 60.4 | 73.1 |
| 7B2 | 700 | 83.6 | 41.0 | 72.4 | 60.1 |
| 28B1 | 770 | 86.4 | 39.5 | 71.4 | 68.4 |
| 28B2 | 650 | 79.1 | 49.0 | 73.2 | 64.3 |
| 2P1 | 330 | 33.1 | 54.5 | 75.6 | 78.4 |
| 2P2 | 270 | 39.6 | 63.0 | 80.2 | 85.3 |
| 7P1 | 780 | 72.6 | 34.0 | 76.1 | 64.8 |
| 7P2 | 760 | 103.8 | 36.0 | 65.5 | 83.5 |
| 28P1 | 730 | 80.8 | 42.5 | 79.2 | 65.5 |
| 28P2 | 630 | 56.0 | 50.5 | 85.6 | 52.5 |

alone, and the addition of glutathione, butanol, or urea to it did not increase the extraction of pectins. Reagents used for the extraction of proteins bound in plant tissues by S—S bonds had no effect on the extractability of pectins. Schweizer reagent in air extracted practically all the constituents of apple marc, including starch that was solubilized. In nitrogen, Schweizer reagent extracted much less pectin.

Comparison of the extractability of pectin from various marc preparation with hydrochloric acid solutions under various conditions indicated that oxidation of apple tissue constituents and drying of apple pomace reduced the solubility of pectins.

Pretreatment of marcs with chlorous acid bleached the marcs and yielded white pectins. This treatment increased the solubility of pectins in some marc preparations, particularly the browned marcs, and eliminated constituents responsible for the reddening of apple pectins during treatment with hydrochloric acid of higher concentrations or for longer times.

Preliminary data on the rate of extraction of pectins from dried marcs treated with hydrochloric acid at 40°C indicated that degree of esterification, degree of polymerization, and nonuronide content of extracted pectins changed appreciably during treatment.

A surprisingly high amount of protein was found associated with pectin in apple juice. The effect of protease treatment on extractability of pectins from apple marcs was investigated. It was found that the type of marc used and the pH of the extracting solution had a greater effect on the recovery of pectin than did protease treatment. This, however, did increase recovery with marc prepared from unstored apples.

The data obtained do not support the hypotheses that polyvalent cations such as $Ca^{++}$ are responsible for the insolubility of apple pectins. Chemical combinations between pectins and proteins, lignin, or cellulose also are not likely involved.

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# Direct Chromatographic Analysis of Milk [a]

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(Manuscript received June 17, 1962)

**SUMMARY**

**Some organic chemical compounds were detected in milk by a simple, direct chromatographic analysis of head-space vapors. After modifying the head-space gas-sampling technique by saturating the aqueous solution with sodium sulfate and increasing the amplification of the chromatographic hydrogen flame detector electrometer, some organic compounds were detected at less than 0.1 ppm concentration in milk. Samples of fresh and stored raw milk, with and without off-flavors, were analyzed successfully. Some volatile chemical compounds developed during storage at 2°C. Off-flavors were characterized by the development of certain chromatographic peaks. Chromatograms were recorded for analyses of rancid, oxidized, sunlight-oxidized, and high-acid milks. A number of chromatographic peaks were characterized as carbonyl from their reaction with hydroxylamine. Acetaldehyde, propionaldehyde, acetone, and 2-butanone were identified. An indication of the concentration of some of these peaks was obtained by analyzing solutions of 0.1 ppm acetaldehyde, propionaldehyde, and 2-hexanone added to water and to milk.**

## INTRODUCTION

Gas chromatographic analysis has been used extensively for flavor analysis. Ionization detectors have improved this basic method. However, with chromatographic techniques, direct injection of liquid proteinaceous, lipoidal, or biological fluids creates a problem. Such liquids contaminate the chromatographic column. The problem has been overcome in some instances by using head-space gas-sampling. Studies of pronounced aromas of raspberries by Weurman (1961), cabbage by Bailey *et al.* (1961), and a wide spectrum of food flavors by Mackey *et al.* (1961) have been conducted with this procedure. Similar techniques applied in this laboratory to milk were found to be ineffective because of the extremely low concentration of volatiles present.

Teranishi *et al.* (1962) made direct vapor analyses of some vegetables and fruits with temperature programming. Dual columns and dual flame ionization detectors were employed to balance out column bleeding and environmental changes that accompanied temperature programming. Some innovations with the electrometer were incorporated to reduce electronic noise.

Jennings *et al.* (1962) employed a dual flame ionization detector in isothermal analyses of milk vapors. Gas samples for these analyses were obtained from milk that had been heated one hr. at 80°C in a stoppered Erlenmeyer flask. Chromatographic peaks were demonstrated in normal as well as off-flavored milk. Peaks were shown to develop with an oxidized flavor from copper (undisclosed concentration) added to milk. Fluorescent and germicidal light activation of milk from 1 to 6 hrs also resulted in differences in the chromatographic patterns of the milk. These workers also illustrated differences in the chromatogram of milk with an alfalfa feed flavor as compared with raw mixed herd milk. Evidence for identification of various components was limited to a comparison of retention times of previously identified compounds.

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The present study was undertaken because it was felt that modifications of the previously employed head-space gas methods offered possibilities for studying off-flavors of milk.

## MATERIALS AND METHODS

Two modifications were used to obtain the sensitivity necessary to analyze milk volatiles. The head-space gas technique was modified by saturating the liquid sample with sodium sulfate (to enrich head-space vapors in the volatile constituents) and the hydrogen flame electrometer was altered (see Apparatus) to increase instrument sensitivity 16 times. A combination of these adaptations to head-space gas techniques permitted direct chromatographic analysis of volatiles in milk.

**Apparatus.** *Chromatographic unit.* A Model A-90-C Aerograph, with a hydrogen flame-detector kit and 1-mv Brown Honeywell recorder, was used to obtain the chromatograms. The electrometer was modified according to manufacturer's instructions (Aerograph Research Notes, 1961) to increase sensitivity 16 times.

Operating Conditions:

| | |
|---|---|
| Column temperature (°C) | 100 |
| Nitrogen outflow (ml/min) | 20 |
| Nitrogen input (psig) | 18.7 |
| Hydrogen outflow (ml/min) | 20 |

*Column.* Ten-foot, commercially prepared, 1/8-in. stainless-steel column packed with 20% Carbowax 20 M on 60–80-mesh Chromosorb W.

*Sampling bottles.* Serum vials, 15 mm diam × 52 mm, of "5-ml capacity" with self-sealing rubber caps.

*Syringe.* 1-ml gas-tight syringe, Hamilton No. 1001.

**Reagents.** Acidic hydroxylamine. A solution containing 0.4g $NH_2OH \cdot H_2SO_4$ treated with 3 ml 1*N* NaOH and diluted to 10-ml volume with distilled water. Sodium sulfate, anhydrous.

**Samples.** Aliquots of a 1-gal. sample of milk obtained from the pipeline at milking time were treated to produce off-flavors (Table 1). This table also shows average flavor scores of the samples when scored by experienced judges.

After treatment, each aliquot was stored in a refrigerator at 2°C until the chromatograms were made at daily intervals. The oldest samples were also chromatographed after being reacted with hydroxylamine to indicate the presence of carbonyl compounds.

**Head-space gas sampling for chromatographic analyses.** The head-space gas from each sample of milk analyzed was prepared according to the procedure of Bassette *et al.* (1962): 1.2 g of sodium sulfate was transferred into a serum vial followed by 2 ml of the sample to be analyzed. After the vial was sealed with a serum cap, the solution was mixed on a mechanical shaker for 5 min. The vial was then placed for 3 min in a 60°C water bath at a depth slightly above the level of the liquid in the vial. A 1-ml sample of the head-space gas was obtained by inserting the needle of the gas-tight syringe through the rubber serum cap and drawing the vapors into the syringe. The syringe was evacuated and refilled three times. After the syringe was removed from the vial the volume was adjusted to 1 ml and the gas sample injected into the chromatograph.

**Treatment with hydroxylamine to eliminate carbonyl compound peaks.** One-tenth ml of the acidic hydroxylamine reagent was added to 2.0 ml of milk that had been saturated previously with sodium sulfate in a serum vial. With the rubber

Table 1. Off-flavored milk samples, and methods used to produce them.[a]

| | Flavor | | | |
|---|---|---|---|---|
| No. | Expected | Criticisms | Score [b] | Method of producing flavor |
| 1 | Control | Unclean, feedy | 36.8 | |
| 2 | Rancid | Rancid | 31.0 | Add 10% homogenized milk |
| 3A | Oxidized | Oxidized | 35.2 | Add 2 ppm $Cu^{++}$ |
| 3B | Oxidized | Oxidized, cardboard | 31.8 | Add 8 ppm $Cu^{++}$ |
| 3C | Oxidized | Oxidized, unclean | 31.0 | Add 20 ppm $Cu^{++}$ |
| 4 | Sunlight | Oxidized, unclean | 36.5 | Expose to sunlight 1 hr |
| 5 | Oxidized | Oxidized, unclean | 33.8 | Both No. 3A and No. 4 |
| 6 | High-acid | High acid, (0.17–0.25%)[c] acetone, unclean | 30.7 | Incubate 24 hr at 25°C |

[a] All samples were produced from the same raw milk.
[b] Samples were 3 days old when organoleptic analyses were made. Scores were averages of three judgments.
[c] Initial and final titratable acidity expressed as lactic acid.

serum cap in place, the mixture was shaken 1 hr on a mechanical shaker.

The serum vial was then placed in a 60°C bath for 3 min and the sample analyzed as head-space gas as previously described. The peaks obtained from hydroxylamine and water were subtracted from results of hydroxylamine and milk analyses. These peaks obtained from acid hydroxylamine and water were found to be quite reproducible. Although the identity of these peaks was not established, it can be assumed that they were not carbonyl.

## RESULTS AND DISCUSSION

The results of all of the analyses are included in Figs. 1–4. Chromatograms 1 and 1a (Fig. 1) show fresh milk before and after hydroxylamine treatment. In addition, chromatograms of each of the off-flavored milks after four days of storage, before and after treatment with hydroxylamine, are reproduced in Figs. 1–3.

**Control milk.** Several of the recorded volatile components in control milk increased in concentration during storage. A comparison of the peak heights of the fresh (0 days) and aged (4 days) milks in chromatograms 1 and 2 (Fig. 1) illustrates these changes. The components emerging from the column at 4.3 and 6.2 min represented relatively large increases in concentrations of volatiles during storage, perhaps even 1 ppm. In terms of proportionate increases, however, some of the other volatiles showed greater increases (peak heights) after storage, although these increases may represent little more than 0.01 ppm.

Treating these samples with hydroxylamine (chromatograms 1a and 2a, Fig. 1), eliminated or reduced peaks occurring at [2.8], 3.4, [3.8], 4.3, 6.2, 8.3, 9.0, [9.6] min. The peaks shown in brackets were eliminated from either the fresh or the aged sample, but not from both. Because of the agreement of retention times and the low molecular weights of some of the early peaks, there was not much doubt of their identity. The 2.8-min peak was acetaldehyde; 4.3, acetone; and the 6.2-min peak undoubtedly was, at least in part, 2-butanone. As retention time increased and the corresponding molecular weights became larger, there was less certainty of the identity of the compounds. Many possible isomers or closely related compounds could elute at a given retention time.

Untreated raw milk stored under refrigeration released more volatiles, thus suggesting possible deterioration. Mechanism for this decomposition was not investigated. However, since raw milk was used, bacterial or enzymatic activity could have occurred during storage even at 2°C.

**Rancid milk.** The volatile chemical compounds detected by this technique in rancid milk were not very different from those found in untreated raw milk. As can be seen in chromatogram 3 (Fig. 1), peaks did develop at 2.0 and 2.8 min but these components were not identified. It was presumed that neither was carbonyl since neither was affected by hydroxylamine (see chromatograms 3a and 3, Fig. 1).

The lack of any significant difference between the rancid and control milk chromatograms was not unexpected. Fatty acids, which are considered the major source of this off-flavor, are not very volatile. Also, polarity of the acids prevents their being drawn into the head-space vapor. These acids with boiling points at least 162°C (butyric) if drawn into the head space would not be likely to pass through the column.

**Copper-induced oxidized flavors.** Four chromatographic peaks developed from an analysis of oxidized flavored milk as a function of the concentration of copper added to milk and the time of its exposure. In addition to peaks that developed in control milk on storage, the data in chromatograms 1, 2, and 3 in Fig. 2 show that peaks from oxidized milk developed at 2.8, 3.8, 9.0, and 15.0 min. Treating the oxidized milk with hydroxylamine reagent eliminated or markedly reduced all of those peaks (Fig. 2). If it is presumed that these constituents are carbonyl, the 2.8-min peak should be acetaldehyde, and the 3.8 min peak propionaldehyde.

The material responsible for the 9.0-min peak, assuming it to be carbonyl, must be similar to 2-pentanone or pentanal. This does not eliminate the possibility that it may be an unsaturated carbonyl, a related carbonyl, or a mixture of these compounds.

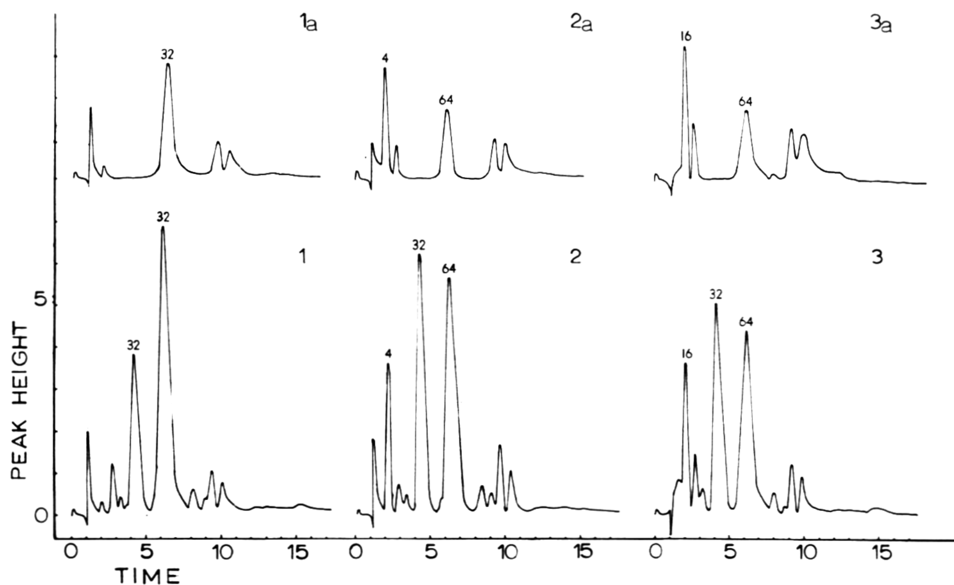


Fig. 1. Chromatographic analyses of volatiles in untreated and rancid milk. 1) untreated fresh raw milk; 2) after stored 90 hr; 3) rancid milk 90 hr old. Subscript *a*, same samples after treatment with hydroxylamine; peaks contributed by hydroxylamine are not shown. Numbers above the peaks are attenuation factors; peaks with no numbers have an attenuation factor of 2.

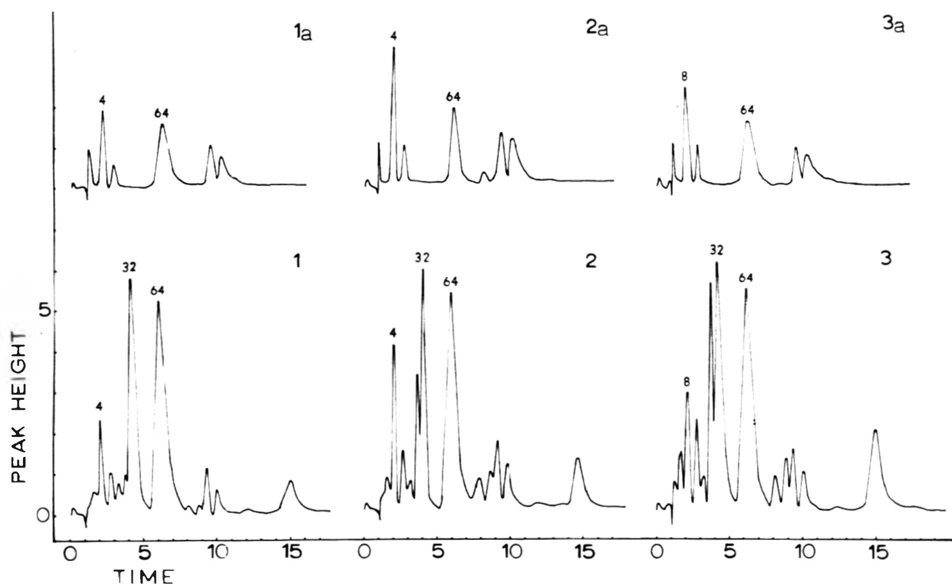


Fig. 2. Chromatographic analyses of volatiles in copper-treated milk. 1) 2 ppm; 2) 8 ppm; 3) 20 ppm. Subscript *a*, same samples after treatment with hydroxylamine; peaks contributed by hydroxylamine are not shown. Numbers above the peaks are attenuation factors; peaks with no numbers have an attenuation factor of 2.

The peak that emerges at 15.0 min represents a material with a retention time identical with that of 2-hexanone. Although no attempt was made to identify these peaks, an idea of the characteristics of these materials may be useful to other workers.

Copper-induced oxidized milk illustrated the value of direct chromatographic analysis for flavor studies. The chromatographic peaks that appeared to be associated with this defect increased with the age of the copper-treated samples and with the level of copper. Comparison of chromatogram 1 (Fig. 2) and chromatogram 2 (Fig. 4) confirms that 2.8 and 3.8 min peaks were acetaldehyde and propionaldehyde and that their concentration in the 2-ppm-treated sample stored 4 days was below 0.1 ppm. The 15.0-min peak may have been hexanal, as El Negoumy *et al.* (1961) found in a study of oxidized butterfat, but it could represent some other similar or related compound. Focus of this study was on typical patterns exhibited by chromatograms of specific off-flavors; however, using the same or similar techniques to identify peaks was suggested.

**Sunlight.** Milk exposed to sunlight was judged to have a very slight oxidized flavor (score 36.5). This milk produced a chromatogram similar to those of copper-induced oxidized milk (Fig. 3). Characteristic peaks developed on storage at 2.8, 3.8, 9.0, and 15.0 min. All peaks were shown to be carbonyl by their response to hydroxylamine. The identity of these components was discussed under the oxidized flavor study.

**Sunlight and copper.** Adding copper to a milk sample before exposing it to sunlight increased heights of most of the peaks already discussed. As can be seen in Fig. 3, the peaks at 2.8, 3.8, 9.0, and 15.0 min also developed with sunlight and copper. The 3.8- and 15.0-min peaks were particularly larger when copper was added and the milk exposed to sunlight.

Sunlight and sunlight-and-copper-induced flavors produced chromatographic patterns quite similar to those of samples oxidized by copper alone. The effects of copper and sunlight together caused some of the peaks to increase markedly. It appeared that most of the peaks associated with the oxidized flavor were the same whether induced by copper, sunlight, or copper and sunlight.

**High acid.** The high-acid sample (chromatogram 3, Fig. 3) shows that many volatiles developed as the sample aged. Many of the peaks that increased in size had the same retention time as those that appeared in the control milk, but were much larger. One of the peaks with a retention time of 12.5 min was peculiar to the high-acid sample. Fewer of the peaks were removed by treating with acid hydroxylamine than peaks of other samples (compare chromatogram 3a, Fig. 3, with other $NH_2OH$-treated samples). Since the acidity of this sample was only 0.25%, it was not badly decomposed. Probably the peaks that developed were dependent upon the particular bacterial flora in the milk.

**Quantitative study.** Chromatograms 1, 2, and 3 (Fig. 4) show the results of an analysis of a second sample of fresh raw milk, the same milk after 0.1 ppm of each of acetaldehyde, propionaldehyde, and 2-hexanone were added, and the same concentration of the same carbonyl compounds in water, respectively. These three carbonyl compounds at 0.1 ppm concentration added to milk were easily detected (compare chromatograms 1 and 2, Fig. 4). Peak heights of acetaldehyde and propionaldehyde in milk differed only slightly from those obtained from an aqueous solution of the same concentration (compare chromatograms 2 and 3). 2-Hexanone analyzed in water produced a peak more than 7 times as high as the one resulting from the same analysis in milk. Apparently milk fat acts as a solvent and thus limits the amount of hexanone that escapes as head-space vapor. Other factors may also account for some of the difference.

The sample of milk employed in the quantitative study showed a different chromatographic pattern from milk used in the flavor study. The peak occurring at 6.2 min in the quantitative study apparently was entirely 2-butanone since it was completely removed by hydroxylamine (chromatograms 1 and 1a, Fig. 4). Both the acetone (4.3) and 2-butanone (6.2) peaks were considerably smaller in this sample than in the previous

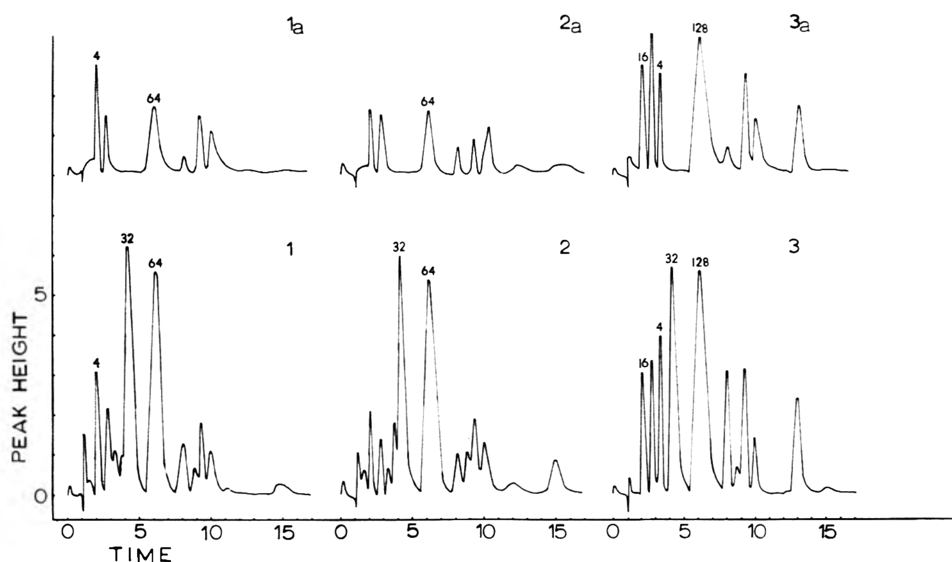


Fig. 3. Chromatographic analyses of volatiles in sunlight-treated and high-acid milk. 1) exposed to sunlight 1 hr; 2) treated with 2 ppm copper and exposed to sunlight 1 hr; 3) high-acid. Subscript *a*, same samples after treatment with hydroxylamine; peaks contributed by hydroxylamine are not show. Numbers above the peaks are attenuation factors; peaks with no numbers have an attenuation factor of 2.

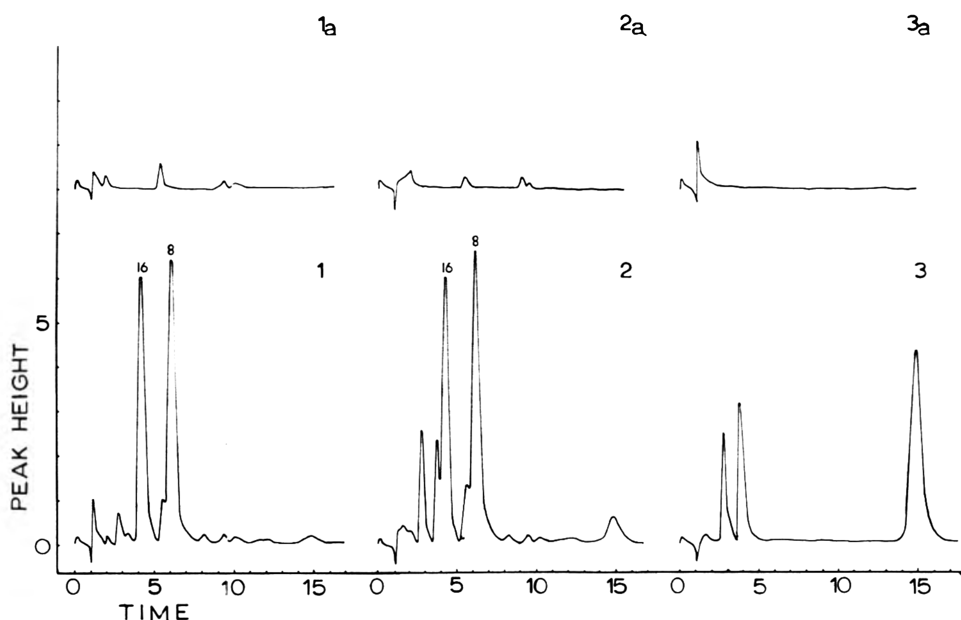


Fig. 4. Chromatographic analyses of carbonyls in milk and water: 1) untreated fresh raw milk; 2) after addition of 0.1 ppm acetaldehyde, propionaldehyde, and 2-hexanone; 3) water with identical additions. Subscript *a*, same samples after treatment with hydroxylamine; peaks contributed by hydroxylamine are not shown. Numbers above peaks are attenuation factors; peaks with no numbers have an attenuation factor of 2.

one, probably from differences in rations consumed by the cows.

Heights of peaks from measured amounts of known carbonyls added to milk or to water were quite similar except the 2-hexanone peak, which was many times higher in water than in milk. This was probably due to the solubility of this carbonyl in milk-fat. It is possible to get an idea of the concentration of some volatiles that developed with off-flavors by comparing the height of peaks from 0.1 ppm concentration of some carbonyls in milk with those from milks with off-flavors.

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# Volatile Esters of Bartlett Pear. II.[a]

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**SUMMARY**

**On the basis of gas chromatographic studies, infrared and ultraviolet spectroscopy, and carbon-hydrogen balance, the C-10 unsaturated acid previously reported to be a major constituent of pear essence hydrolysates was identified as 2,4-decadienoic acid. An alcohol previously isolated was found to be a contaminant (2,-ethylhexanol-1) that could occur frequently in this type of work. Analyses of freshly prepared essence hydrolysates indicate the presence of ethyl, *n*-propyl, and probably *n*-amyl, esters.**

In an earlier publication, Jennings (1961) reported isolating several alcohols and acids from a mixture of the hydrolysis products of Bartlett pear esters. Characterization studies of the components of this mixture identified *n*-butyl alcohol, *n*-hexyl alcohol, and acetic, butyric, capronic, caprylic, pelargonic, and capric acids. The major pear ester acid was reported to be an unsaturated C-10 acid that hydrogenated to give *n*-capric acid. This paper reports further characterization of components of this mixture.

## METHODS

**Gas chromatography.** The chromatography studies utilized a commercial gas chromatograph, with a four-filament thermal-conductivity cell and helium as carrier gas. Substrates were used at the indicated levels on 40–60-mesh C-22 acid-washed firebrick in ¼-in. × 10-ft packed stainless-steel columns. All runs were isothermal. Collections were made by passing the emergent gas stream through a short length of 2-mm Pyrex tubing to a 2-ml conical test tube supported in an ice bath.

**Infrared spectroscopy.** Spectra were determined on thin films, sandwiched between two NaCl plates, on a Beckman IR-5 infrared spectrophotometer. The sandwich was supported in a jeweler's poising vise as far from the source compartment as possible. This minimized the possibility that the sample might warm up during a run, decreasing in viscosity and presenting films of various thicknesses. This technique permitted several repeat runs with identical[b] results.

**Ultraviolet spectroscopy.** A Beckman DB spectrophotometer was used on preliminary scans and to establish absorption maxima. A Beckman DU

[a] This study was supported in part by funds from Pear Zone 1.

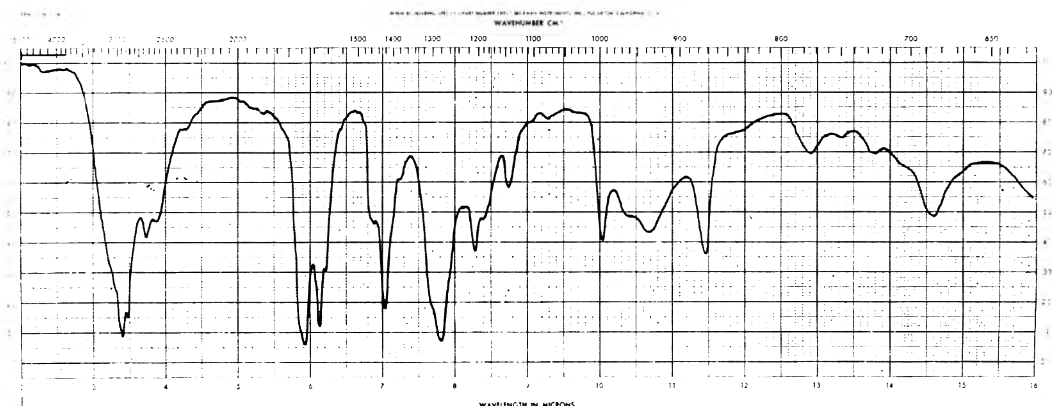


Fig. 1. Infrared spectrum of pear ester acid. Thin-film technique.

spectrophotometer was used to establish extinction coefficients.

## RESULTS AND DISCUSSION

It had been established (Jennings, 1961) that one of the major acids occurring in pear essence hydrolysates was a C-10 unsaturated acid that hydrogenated to yield *n*-capric acid. The infrared spectrum of this acid is shown in Fig. 1.

The sharp absorption at 990 $cm^{-1}$ could be assigned to a trans configuration, the double bond conjugated with that of the carbonyl. Of even greater significance is the fact that the primary acid absorptions occurred at 1690–1700 $cm^{-1}$ instead of at 1705–1725 $cm^{-1}$, the region where they are normally encountered. According to Bellamy (1958), this frequency fall can most probably be attributed to olefinic conjugation.

Retention of the free acid on an Empol dimer acid column (Fig. 2) is 2.0 at 200°C (*n*-capric acid = 1.0). Under these same conditions the retention of a synthetic 2-decenoic acid is approximately 1.6. Altering the position of the double bond would most probably have some effect on the retention, but it is doubtful that it would approach this magnitude (Lipsky *et al.*, 1959). It seems more probable that this is a di-unsaturated acid.

This raises the question of double-bond position. As stated above, the infrared spectrum gives some reason to expect conjugation, but it remains to be explored whether this involves conjugation of two carbon-carbon double bonds, one carbon-carbon double bond and the carbonyl oxygen, or both of these in a triconjugate system. A conjugate system would be expected to possess a characteristic ultraviolet absorption spectrum, and in the event of a triconjugate system it would be reasonable to expect a large extinction coefficient.

Fig. 3 illustrates an ultraviolet absorption curve for this acid in isooctane. Crossley and Hilditch (1949) reported a very similar curve for deca-2,4-dienoic acid. Neither curve possesses fine structure, but consists of a single broad peak with a maximum at 260 mμ. Crossley and Hilditch reported an

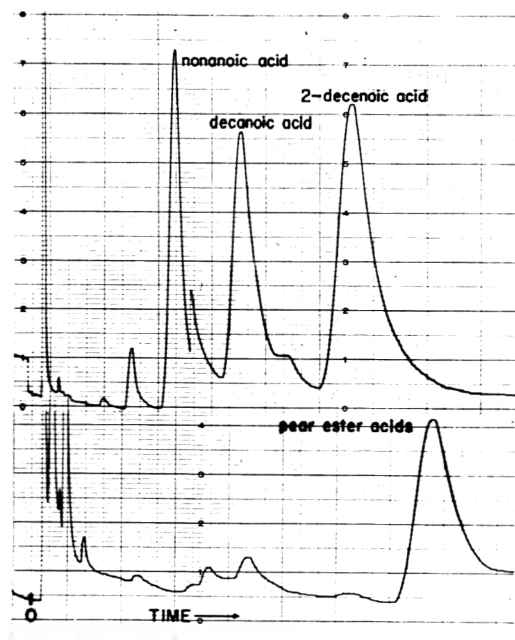


Fig. 2. Chromatogram of a series of known acids compared with pear ester acid. 15% empol dimer acid at 200°C, flow rate 85 cc/minute. Injections, *ca.* 2 μl ethereal solution.

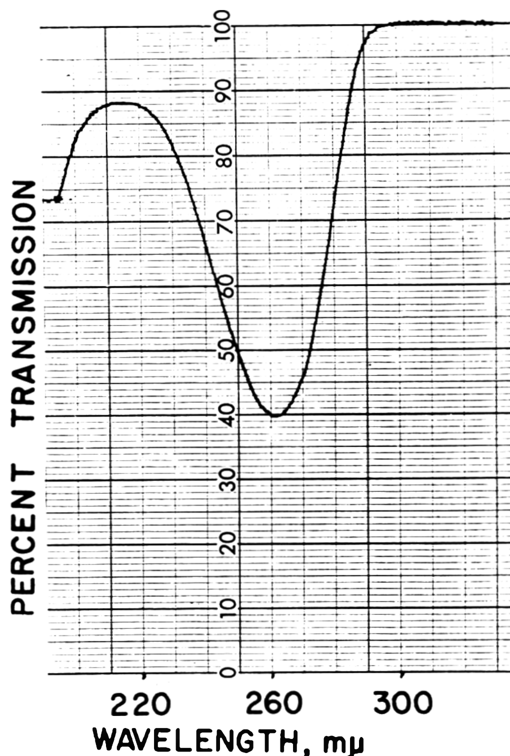


Fig. 3. Ultraviolet absorption spectrum of pear ester acid.

extinction coefficient of 24,000. Devine (1950) reported an extinction coefficient of 25,800. The extinction coefficient of the chromatographically pure acid isolated in the present study was 26,000.

Results of the elemental analyses (Table 1) compare well with values predicted for a di-unsaturated C-10 acid.

Table 1. Elemental analyses of pear acid.

| | C | H |
|---|---|---|
| Calculated for $C_{10}H_{14}O_2$ | 71.39 | 9.59 |
| Found | 71.47 | 9.41 |

Earlier work (Jennings, 1961) established the presence of an alcohol, designated pear alcohol 3, with a relative retention of 1.5 (*n*-hexyl alcohol = 1.0) on diethyleneglycol-succinate (DEGS) at 175°C. On the basis of retention agreement on DEGS, Apiezon L, and diglycerol columns, precise matching of infrared spectra, and good agreement with combustion analyses, this has been identified as 2-ethylhexanol-1. Teranishi *et al.* (1962) kindly advised us that they had found this alcohol to result from hydrolysis of 2-ethylhexyl phthalate, used as a plasticizer in Tygon, and suggested that the possibility of contamination be checked. Although these samples had not been in contact with plastics, the unexpected occurrence of this branched-chain alcohol made a recheck seem advisable.

Accordingly, several 10-lb lots of fresh Bartlett pears were steam distilled under reduced pressure, scrupulously avoiding all contact with plastics. Gas chromatography of the essence hydrolysates showed no evidence of 2-ethylhexanol-1, but did indicate the presence of ethyl, *n*-propyl, and probably *n*-amyl, as well as *n*-butyl and *n*-hexyl alcohols (Fig. 4). The source of 2-ethylhexanol-1 was evidently a line, composed of alternate short lengths of copper and Tygon tubing that supplied vacuum to the rotary flash evaporator used in making the earlier concentrations. Back-diffusion of vapor carried a considerable quantity of 2-ethylhexanol-1 to the concentration flask.

Previous attempts failed to demonstrate the presence of ethyl alcohol (Jennings, 1961). The amounts isolated in this investigation were too small to permit infrared verification, but retention agreed closely with those of known alcohols on DEGS, Apiezon L, and diglycerol substrates. The particularly high resolution of diglycerol for alcohols makes this evidence considerably stronger than mere agreement on any three dissimilar columns.

Fig. 5 is a plot of carbon atoms vs. log of retention for a series of known primary *n*-alcohols. Curve *a* was constructed from data reported by Webb and Kepner (1961);

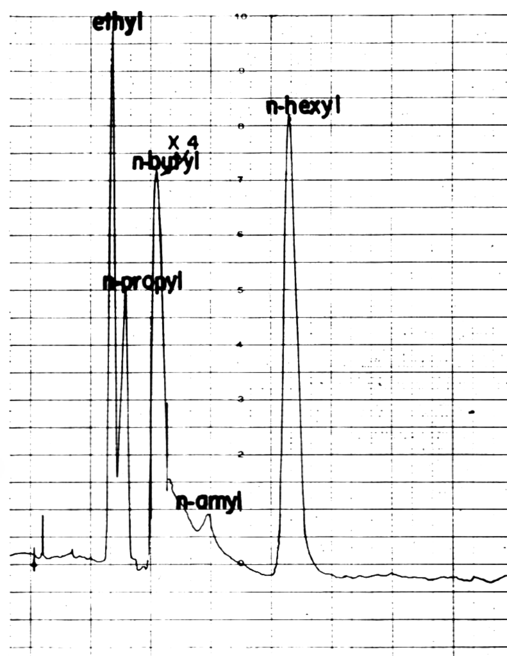


Fig. 4. Chromatogram of alkaline extract of pear essence hydrolysate. 12% diglycerol at 75°C, 150 cc/minute.

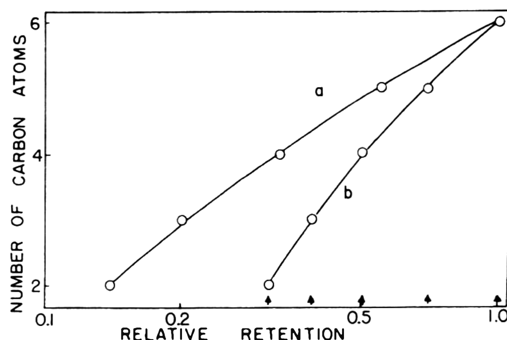


Fig. 5. Retentions of primary *n*-alcohols on diglycerol at 75°C. a, data of Webb and Kepner (1961); b, data from this study. Arrows at bottom depict retentions of peaks in Fig. 4.

the value for *n*-hexyl alcohol was calculated from their figures. Curve *b* represents information from this study. There are differences not attributable to the use of different alcohols as reference materials; these can probably be ascribed to the higher substrate concentration used in this study, to differences between lots of diglycerol, and to differences between lots and methods of preparing the C-22 firebrick.

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# Color Studies on Processed Dried Fruits [a]

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(Manuscript received August 25, 1962)

**SUMMARY**

**A procedure for color measurement that can be used in quality determination of dried fruits is described and evaluated for accuracy and reproducibility. Rates of darkening, based on this analytical procedure, are shown for raisins and golden raisins at 50, 70, and 90°F. The apparent activation energies for the browning reaction in these fruits are calculated.**

## INTRODUCTION

It is well known that color deterioration takes place in dried fruits when they are exposed to unfavorable conditions for sufficient time. Stadtman (1948) and Stadtman *et al.* (1946b) provided important knowledge about darkening of apricots under various conditions of storage and noted the dearth of information on the stability of dried fruits other than apricots. Hodge (1953) compiled important contributions on the chemistry of browning in dehydrated foods. Maier and Schiller (1960, 1961a,b) described chemical changes associated with the deterioration of domestic dates and reported rate measurements of several of these changes. Nury *et al.* (1960a,b,c) described quality changes in retail packages of dried fruits under a variety of conditions.

Although regular raisins (not bleached) are naturally dark, and further darkening is difficult to observe visually, it is possible to measure these changes by chemical analyses. On the other hand, visual color grading of golden raisins by USDA inspectors is a routine practice and is a factor in determining the value of the product. In either case, an objective study of color changes provides an excellent criterion of quality and a very good indicator of time-temperature experience of raisin products; it provides information that is sought by many packers and handlers of these products, and can be used in quality control and evaluation.

A color extraction procedure and studies of rates of change in color and of apparent activation energies for darkening or browning of processed raisins are reported here. This information may be useful in predicting the shelf-life of commercially processed and packaged golden bleached raisins and natural (sun-dried) raisins.

## ALCOHOL-SOLUBLE COLOR METHOD

Apple rings, Calimyrna figs, French prunes, apricots, and raisins (sun-dried) were used in the study of the alcohol-extractable color method. Freshly packed 10- to 16-oz packages (Saran-cellophane) of commercially processed dried fruits were obtained from packing houses in California. To provide samples at various stages of browning or color deterioration, the fruits were held at 110°F, and three bags of each fruit were withdrawn from storage periodically.

After several solvent systems were tested, 50% ethanol (one volume of 95% ethanol mixed with one volume of water) was selected for the extraction. This is the solvent used by Stadtman *et al.* (1946a) in studies of the stability of dried apricots.

Absorbance at 440 mμ of alcoholic extracts of dried fruits was chosen as a measure of the amount of extractable brown pigment.

The contents of each bag of fruit were separately ground to a paste in a food chopper. Duplicate 15-g samples were transferred to flasks and covered with 200 ml of 50% ethanol; likewise, duplicate 30-g samples were transferred to flasks and covered with 400 ml of 50% ethanol. The flasks were covered and held about 23 hr at room temperature (70°F). They were shaken occasionally. The color extraction system comes to equilibrium in about 10 hr (Fig. 1); however, this is an inconvenient time interval, so 23- to 24-hr extractions were used. The colored solution was filtered

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[a] Presented at Institute of Food Technology meeting in Miami Beach, Florida, June 10–14, 1962.

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

Table 1. Analyses of variance of alcohol-extractable color measurements on dried fruit (analyses performed on logarithms of the absorbance readings).

| Source of variation | Degrees of freedom | Mean squares | | | | |
|---|---|---|---|---|---|---|
| | | Apples | Figs | Raisins | Apricots | Prunes |
| Periods (P) | 3 | 5.191,853 | 0.658,834 | 1.076,397 | 3.873,487 | 0.202,971 |
| Bags (B) within | 8 | 0.279,244** | 0.045,022** | 0.014,226** | 0.008,727** | 0.011,704** |
| Sizes (S) | 1 | 0.000,819* | 0.000,076 | 0.000,091 | 0.000,619 | 0.002,497 |
| S × P | 3 | 0.000,096 | 0.004,483 | 0.000,974 | 0.001,015* | 0.009,615 |
| S × B within | 8 | 0.000,132 | 0.001,813 | 0.001,056 | 0.000,234 | 0.007,711 |
| Samples within | 24 | 0.000,199 | 0.001,752** | 0.000,506** | 0.000,264** | 0.001,360** |
| Duplicates | 48 | 0.000,228 | 0.000,015 | 0.000,015 | 0.000,054 | 0.000,010 |

* Significant at $P<0.05$.
** Significant at $P<0.01$.

through Whatman No. 2 paper, and the absorbance (water = 0) was measured at 440 mμ with a Bausch & Lomb Spectronic 20 (½-in. cuvette). Two readings were taken on each solution.

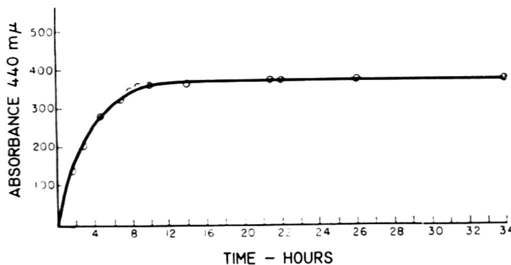


Fig. 1. Effect of time of extraction on absorbance of alcohol extract of raisins.

## SAMPLING STUDIES

Statistical analyses of the data (Table 1) showed that only with apples did the size of the sample (15 or 30 g) taken from a bag of fruit have a significant effect on the results. The average difference in absorbance readings between 30-g samples and 15-g samples was 0.0026.

The greatest source of error was attributable to differences between bags. The ratios between variance of bags and variance of samples within bags ranged from 8.6 for prunes to 1403 for apples.

Because of the highly significant difference between bags, color analyses were run on regular raisins and golden bleached raisins to determine how many 1-lb bags should be drawn from a lot for color extraction analyses. Samples consisting of 1, 2, and 4 bags were tested; four samples of each size were prepared. Each sample was prepared for analysis by mixing the raisins well and grinding them to a paste with an electrically powered food chopper. Duplicate 15-g subsamples were transferred to flasks, and the alcohol-extractable color was determined as noted above.

Increasing the number of bags used per sample beyond two did not reduce error variance (Table 2).

## STABILITY STUDY

Commercially packed 1-lb bags of regular and golden bleached (sulfured) raisins were held at 50, 70, and 90°F for periodic sampling. Proximate analyses of the raisins appear in Table 3. The storage atmosphere was kept at 60% RH, approximately the equilibrium value for raisins of 16% moisture content. The samples showed no significant changes in weight during storage. Sampling continued for about 5–15 months, depending on the temperatures maintained.

Absorbance measurements on alcohol extracts of single samples from composites of 4 bags of each of the dried fruits were made periodically by the method described above. Figures 2 and 3 show the optical density of the alcohol extracts of raisins and golden raisins held at different temperatures. At 50°F, little or no color change occurred even after 15 months of storage. At 90°F, however, excessive darkening occurred in 3–4 months.

Based on this procedure an extracted color absorbance value of 0.3 for golden raisins and about 0.9 for regular raisins (moisture-free basis)

Table 2. Effect of sample size on error variance.

| Source of variation | Degrees of freedom | Mean squares for sample size: | | |
|---|---|---|---|---|
| | | 1-bag | 2-bag | 4-bag |
| Golden raisins | | | | |
| Total | 7 | 125 | 19 | 12 |
| Samples | 3 | 286 | 20 | 16 |
| Duplicate | 4 | 4 | 19 | 8 |
| Regular raisins | | | | |
| Total | 7 | 1250 | 303 | 277 |
| Samples | 3 | 2392 | 253 | 471 |
| Duplicate | 4 | 394 | 341 | 131 |

Table 3. Analyses of raisins used in the stability study.

| | Raisins | Golden raisins |
|---|---|---|
| Moisture | 16.08 | 16.05 |
| Crude fiber, % | .98 | .82 |
| Nitrogen, % | 0.54 | 0.46 |
| Ash, % | 1.89 | 1.77 |
| Sugar content: % total | 70.6 | 71.2 |
| % reducing | 70.2 | 70.7 |
| % fructose | 39.2 | 40.0 |
| % glucose | 31.0 | 30.7 |
| % sucrose | .4 | .5 |
| $SO_2$, ppm (as-is basis) | ...... | 1450. |

represent darkening to the point at which acceptability is considered doubtful.

Apparent activation energies for the darkening of the raisins can readily be calculated by the Arrhenius equation:

$$\Delta Ha = \frac{2.3\, RT_1T_2}{T_2 - T_1} \log \frac{k_2}{k_1}$$

where $k_2$ and $k_1$ are the rates for increase in optical density at temperatures $T_2$ and $T_1$, respectively, and $R$ is the gas constant. In the temperature range of 50–90°F, apparent activation energies, in kilocalories/mole, were 26 for regular raisins and 24 for golden bleached raisins; the corresponding $Q_{10}$ values are 4.2 and 3.9.

Reasonable estimates of the quality or shelf life of raisins at a given temperature or integrated series of temperatures can be made by measuring the alcohol-soluble color and applying the indicated rate factors (see Figs. 1 and 2 legends). Assuming

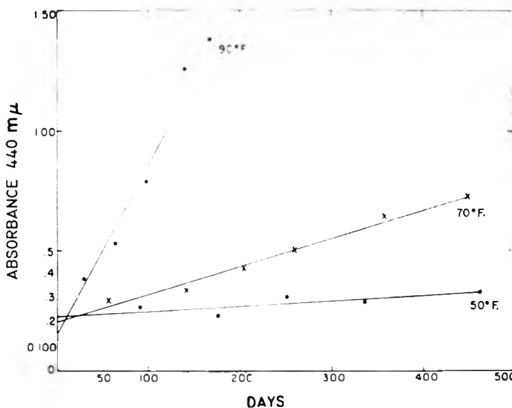


Fig. 2. Alcohol-extractable color of regular raisins held for various periods at 50, 70, and 90°F. Increase in absorbance per day at 90°F was 0.00730; at 70°F, 0.00117; at 50°F, 0.00025. Average initial absorbance = 0.212 moisture-free basis.

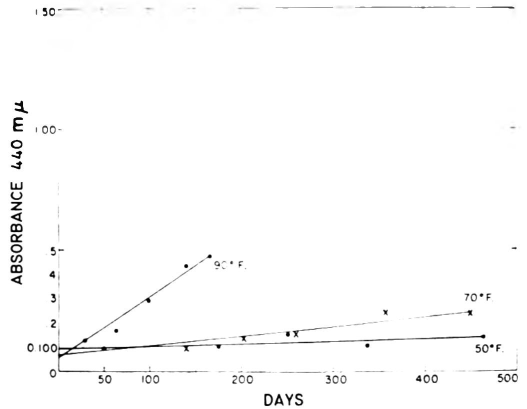


Fig. 3. Alcohol-extractable color of golden bleached raisins held for various periods at 50, 70, and 90°F. Increase in absorbance per day at 90°F was 0.00251; at 70°F, 0.00041; at 50°F, 0.00012. Average initial absorbance = 0.087 moisture-free basis.

the end-points noted above, the shelf life of the regular raisins in these experiments would be about 95 days at 90°F and about 600 days at 70°F; for the golden raisins studied here, the shelf life would be about 80 days at 90°F and about 500 days at 70°F. The additive effects of other deteriorative changes would tend to reduce the estimated shelf life.

## ACKNOWLEDGMENTS

The authors gratefully acknowledge the advice and counsel of Mrs. E. Taylor and Miss M. Sandomire. Thanks are also due Mr. E. C. Marshall and Mr. Howard Charman for technical assistance.

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

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# A Comparison of the Press Method with Taste-Panel and Shear Measurements of Tenderness in Beef and Lamb Muscles[a]

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(Manuscript received August 21, 1962)

**SUMMARY**

**The longissimus dorsi muscle was taken from 7 beef ribs, 15 beef shortloins, 129 lamb loins, and the semitendinosus muscle from 51 beef rounds and tested for tenderness by panel, press, and shear methods. Results showed that the press and shear methods used on cooked meat gave comparable relations with sensory-panel scores. When raw samples were tested by the press, little relation with panel scores of the cooked meat was found.**

## INTRODUCTION

Tenderness is a very important characteristic of meat. Many methods have been developed to measure this characteristic in cooked meat. These methods, when applied to raw meat, give results that lack accuracy in predicting the tenderness of the cooked product. A method (press) was developed (Sperring *et al.*, 1959) that indicated accuracy for predicting the tenderness of cooked meat from raw-meat observations. The same authors reviewed the various measures of tenderness used by previous workers. The present study was undertaken to provide more data on the relations between press readings from various raw beef and lamb muscles and shear and panel tenderness evaluation of the cooked muscles.

## MATERIALS AND METHODS

The longissimus dorsi (LD) muscles of 129 U.S.D.A. Choice or Prime wholesale trimmed lamb loins were used. Similarly, the same muscle from 7 wholesale beef ribs and 15 wholesale beef shortloins of like grade were tested for tenderness. In addition, 51 excised beef semitendinosus (ST) muscles were utilized. Both sides of the lamb loins were used, whereas the beef cuts were from one side only. All cuts were individually wrapped, frozen, and stored at −20°F for periods from 8 to 24 months.

In preparing the muscles for testing, the wholesale beef cuts were removed from frozen storage, defrosted 24 hr at room temperature, and then held at 38°F for another 24 hr. The lamb loins and beef ST muscles were defrosted 24 hr at 38°F. All bone and external fat was removed from each cut. The entire LD from the beef shortloins was used, but only the LD from the 9–11 rib portion of the beef ribs was utilized.

Cooking was done in deep fat maintained at 225 ± 1°F to an internal temperature of 165°F. These conditions were selected to give uniform doneness without excessive crusting of the outer surfaces. The muscles were allowed to cool for 15 min before samples were removed for testing.

A Carver laboratory press was equipped with a motor and gear-reduction box to duplicate the apparatus described by Sperring *et al.* (1959). Raw and cooked samples 1 inch in diameter were used with this machine. A Warner-Bratzler shear was used for testing the 1-inch cores of cooked beef and ½-inch cores of cooked lamb. A panel of 18 judges scored samples for tenderness only, on a scale of 1 (extremely tough) to 9 (extremely tender). Panel personnel varied to some extent from test to test. The judges were given ½-inch-long 1-inch cored beef samples and ¾-inch-long ½-inch cored lamb samples. Sampling positions within each muscle were randomized for the tenderness determinations.

## RESULTS AND DISCUSSION

Six raw-press readings were taken on each cut and averaged as the tenderness

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Table 1. Means and standard deviations of tenderness measurements.

| | No. | Press raw | | Press cooked | | Shear cooked | | Panel cooked | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean | Std. dev. | Mean | Std. dev. | Mean | Std. dev. | Mean | Std. dev. |
| LD beef ribs | 7 | 178 | 17 | 292 | 85 | 9.73[a] | 1.31 | 7.51 | 0.53 |
| LD beef shortloins | 15 | 175 | 29 | 282 | 72 | 9.94[a] | 1.53 | 6.97 | 0.81 |
| ST beef rounds | 51 | 402 | 55 | 489 | 70 | 11.29[a] | .19 | 5.60 | 0.64 |
| LD lamb loins | 129 | 155 | 31 | 250 | 63 | 4.79[b] | 2.17 | 7.14 | 0.90 |

[a] lb to shear 1-in. core.
[b] lb to shear ½-in. core.

index. The press and shear readings of the cooked cuts were the average of three tests on each muscle. Analysis of the means shown in Table 1 indicated that, for each method of tenderness evaluation, there were significant differences among the carcasses that provided the cuts. The only exception noted was in the raw-press readings of the 7 beef rib LD muscles. When the average raw and cooked press values are compared, it is seen that cooking markedly increased the values. This effect of cooking meat has also been noted when the shear apparatus was used (Ramsbottom *et al.*, 1945).

Sperring *et al.* (1959) recorded raw LD (rib) values of 329 and cooked values of 396. Their cooking endpoint was 140°F. In the present study, raw and cooked press values of the LD were respectively 178 and 291. It should be emphasized that all of these meat cuts had a minimum frozen storage period of 8 months, whereas those of Sperring *et al.* were fresh, and aged a maximum of 10 days.

Correlation coefficients (Table 2) show that with cooked meat there is very little difference between the press or the shear apparatus as a predictor of tenderness as determined by a sensory panel. The lack of significant correlations between raw-press readings and panel scores for cooked meat indicates that the press has limited application when used with raw samples. This is in contrast to the results of Sperring *et al.* (1959), who reported coefficients of .899, −.017, and .199 between raw-press values and panel scores on fresh LD muscles aged for 3, 7, and 10 days, respectively. The shear apparatus when used with raw pork also does not agree with panel scores of cooked pork (Murphy and Carlin, 1961).

It was noted during preliminary work that shutting off the motor did not eliminate one or two pumping strokes of the press, which produced greatly increased readings. To counteract this effect, the fluid release valve was opened manually when the first extrusion of meat was observed. A more critical definition of the endpoint and cessation of pressing may increase the accuracy of the press tenderness measure.

Table 2. Correlation coefficients of tenderness measurements.

| | LD beef ribs $n = 7$ | LD beef shortloins $n = 15$ | ST beef rounds $n = 51$ | LD lamb loins $n = 129$ |
|---|---|---|---|---|
| Press (raw) and panel scores | −.22 | −.47 | −.12 | −.05 |
| Press (cooked) and panel scores | −.85* | −.95** | −.34* | −.51** |
| Press (raw) and press (cooked) | .24 | .47 | .13 | .10 |
| Press (raw) and shear (cooked) | −.32 | .62* | −.09 | .14 |
| Press (cooked) and shear (cooked) | .81* | .75** | .11 | .47** |
| Shear (cooked) and panel scores | −.67 | −.75** | −.38** | −.57** |

* $P<.05$.
** $P<.01$.

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# Sensory Examination of Four Organic Acids Added to Wine

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(Manuscript received July 22, 1962)

**SUMMARY**

**Difference thresholds for 4 organic acids, determined at a reference concentration of 0.615 (as g of tartaric acid per 100 ml), were found to be ±0.13 g per 100 ml for tartaric, ±0.18 g per 100 ml for citric, ±0.10 g per 100 ml for fumaric, and ±0.13 g per 100 ml for adipic. The response reaction of the panel to these acids appeared to be to molar concentration rather than to pH or normality when dealing with near-threshold amounts added to a highly buffered medium.**

**When the acids were compared directly by addition of equal molar amounts of each to a wine (equal to 0.20 g tartaric acid per 100 ml on a molar basis), citric acid was judged most sour, fumaric and tartaric about equal, and adipic the least sour. Preference data indicated trends in favor of citric and tartaric acids over fumaric and adipic acids.**

## INTRODUCTION

The acid taste is an important sensory property of many foods and beverages. In dry wines acidity is one of the major factors determining the flavor balance and general taste impressions. Legally and commercially, citric acid or tartaric acid may be added to wines in amounts sufficient, generally, for adjusting to a desired acid level.

It was recently reported (Anon., 1962) that fumaric acid might be more advantageous than citric acid for adjusting acid levels. Fumaric acid is a stronger acid than citric, and would be expected to affect pH to a greater extent on a weight-per-volume basis because of its lower molecular weight. Amerine *et al.* (1959) stated, however, that relative sourness is a function not only of pH but also of titratable acidity. Beatty and Cragg (1935) summarized work on the sourness of acids, and indicated that relative sourness, in unbuffered solutions, is not a function of molarity. They indicated that relative sourness in unbuffered solutions is proportional to the amount of phosphate buffer necessary to bring the solutions to a pH of 4.4.

A study was made to compare four organic acids (tartaric, citric, fumaric, and adipic) on a direct molar basis in dry white wine. The first two acids are found in or added to wine, and the last two have been suggested as additive acids for wine. In addition, difference thresholds were determined for the four acids.

## METHODS AND MATERIALS

Food-grade or CP anhydrous acids (minimum purity 99.5%) were used. Table 1 gives the dissociation constants (Hodgman, 1943; Karrer, 1946) of the acids and the molar ratio of each acid compared to tartaric acid. Calculated amounts of the acids were weighed out each day and added to the wines. Difference levels for the constant-stimuli comparisons were prepared by blending the unacidified wine with the wine containing the highest amount of added acid. The wine used, a standard white wine from grapes grown in the Davis vineyard, would be typical of a commercial wine acidified during cellar treatments.

Difference thresholds for each acid were determined by the constant-stimuli method, and solutions for detectable difference were made by the maximum-likelihood method described by Jones (1957). This method is a slightly modified version of probit analysis (Finney, 1952). Direct paired comparison of the various acids was evaluated by Fisher rank-score analysis as described by Jones (1957).

For evaluation of the difference threshold (50% correct above chance), 7–10 panel members tasted six pairs of one acid per day, at three evenly

Table 1. Physical constants of four acids.

| Acid | Equal molar ratios (comparison by weight) | Dissociation constants | | |
|---|---|---|---|---|
| | | 1st hydrogen | 2nd hydrogen | 3rd hydrogen |
| Tartaric | 1.0 | $1.1 \times 10^{-3}$ | $6.9 \times 10^{-5}$ | ................ |
| Citric | 1.28 | $8.4 \times 10^{-4}$ | $1.8 \times 10^{-5}$ | $4.0 \times 10^{-6}$ |
| Fumaric | 0.77 | $1.0 \times 10^{-3}$ | $3.0 \times 10^{-5}$ | ................ |
| Adipic | 0.97 | $3.9 \times 10^{-5}$ | $5.3 \times 10^{-6}$ | |

spaced molar concentration levels above, and three below, the constant stimuli (Table 2). The four acids were alternated by days, and orders were randomized within and between pairs. Each acid was tasted for a total of 6 days, allowing 48 or more individual determinations on each comparison to be made with the constant stimuli. Earlier work by Ough and Stone (1961) showed that this number gives sufficient stability to the variances.

Direct-comparison tests were made on the four acids by giving each of 10 tasters 6 pairs daily (all possible combinations of the four acids) in randomized orders. This test was replicated once on each of four days. Acids were added in equal molar amounts equal to 0.20 g of tartaric acid per 100 ml.

In the difference tests, panel members were asked to state which was the most acid. In the comparison tests they were asked to state which sample tasted more sour and also to state a preference. The instructions indicated that they were not to base the preference decision solely on their acid decision but on the general taste impression. Dual questions asked on the same samples can sometimes lead to non-independent answers, but in this case, with a highly experienced panel, it was felt to be legitimate.

To determine the effects of the added acid on the hydrogen ion concentration, pH's were determined on a series of each of the acids.

Tests on data for independence were determined by the normal $\chi^2$ methods. Homogeneity of variances were tested by Bartlett's (1947) method. Test for differences in slope were made using the standard Student's t-comparison.

## RESULTS AND DISCUSSION

The differences in pH between tartaric, citric, and fumaric are negligible. Adipic, because of its low dissociation constant, had very little effect on pH (Table 3).

Figs. 1 and 2 show the results of the difference tests for the four acids. Table 4 gives the analyses of variance. Residual terms of the analyses were all insignificant, indicating no deviation from the normal-response law. The linear regression equations in the figures indicate the slopes of

Table 2. Acids added in the constant method.

| Acid | Base wine as g/100 ml $H_2Ta$ | Grams per 100 ml Levels (as coded molar differences) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | −3 | −2 | −1 | 0[a] | +1 | +2 | +3 |
| Tartaric | 0.540 | 0.000 | 0.025 | 0.050 | 0.075 | 0.100 | 0.125 | 0.150 |
| Citric | 0.540 | 0.000 | 0.032 | 0.064 | 0.096 | 0.128 | 0.160 | 0.192 |
| Fumaric | 0.540 | 0.000 | 0.019 | 0.039 | 0.058 | 0.077 | 0.096 | 0.116 |
| Adipic | 0.540 | 0.000 | 0.024 | 0.049 | 0.073 | 0.097 | 0.121 | 0.146 |

[a] Reference.

Table 3. Measured pH values of acid levels.

| Acid | Constant-stimuli method | | | | | | | Direct comparison |
|---|---|---|---|---|---|---|---|---|
| | Levels (as coded molar differences) | | | | | | | Acids added in equal molar amounts equal to 0.2 g $H_2Ta$/100 ml |
| | −3 | −2 | −1 | 0[a] | +1 | +2 | +3 | |
| Tartaric | 3.68 | 3.64 | 3.61 | 3.58 | 3.54 | 3.50 | 3.46 | 3.41 |
| Citric | 3.71 | 3.67 | 3.62 | 3.59 | 3.57 | 3.52 | 3.49 | 3.46 |
| Fumaric | 3.71 | 3.68 | 3.63 | 3.60 | 3.57 | 3.51 | 3.48 | 3.42 |
| Adipic | 3.71 | 3.71 | 3.71 | 3.70 | 3.69 | 3.69 | 3.70 | 3.70 |
| Control | ...... | ...... | ...... | | ...... | | | 3.74 |

[a] Reference.

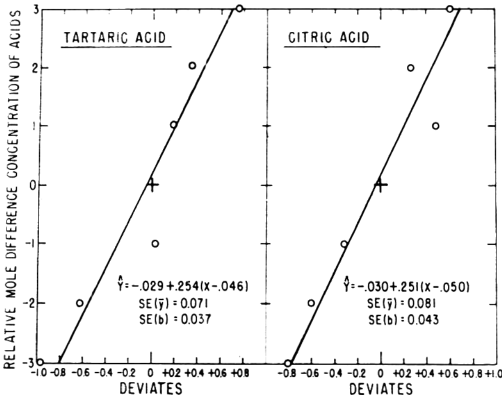


Fig. 1. Constant-stimuli comparison of equal molar difference concentrations of tartaric and citric acids.

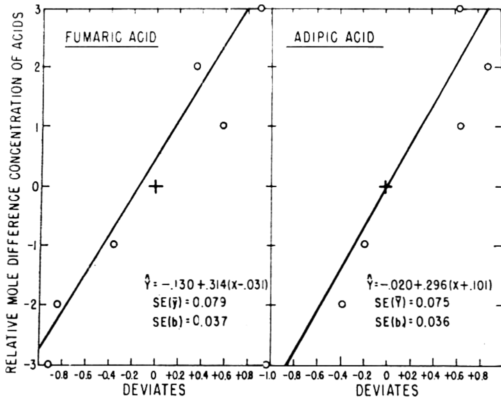


Fig. 2. Constant-stimuli comparison of equal molar difference concentrations of fumaric and adipic acids.

the lines. These slopes were compared to each other, and no significant differences could be shown, indicating that at the relatively small differences needed for difference detection, the panel did not react any differently to one acid than to another. It thus appears that responses to the four acids were primarily to molar concentration. This was surprising considering the relatively large pH effects of tartaric, citric, and fumaric in comparison to the insignificant changes caused by the adipic and that the titratable acidity of the citric acid is greater than that of the other acids. The total number of correct decisions for each acid was tested for independence and found to be homogeneous. Testing for independence of replicates also indicated that the data were homogeneous. However, inspection of the raw data indicated quite wide variations in the tasters. The test for independence for tasters gave a very highly significant $\chi^2$ value of 42.39 with 9df. Further inspection indicated that the panel could be divided into two nearly uniform groups—one (I) with high sensitivity to the acid differences, and one (II) with low sensitivity. The test for independence on the two groups indicated homogeneity. Group I consisted of tasters 6, 8, 9, and 10, and Group II of tasters 1, 2, 3, 4, 5, and 7.

Fig. 3 plots the total data for each group. The analysis of variance of the data for each group showed insignificant residual terms. The slopes of the regression lines were significantly different ($t = 5.86$ with 8 df). This large and significant difference in group response is not unusual, as similar response differences have been reported by Baker *et al.* (1958) and Mrak *et al.* (1959). There were insufficient replications to determine individual regression lines for each acid for each group, but it was possible to determine the independence of the number of correct responses to each acid for each group. Analysis indicated the responses for each acid by groups to be homogeneous (Group I, $\chi^2 = 1.48$ 3 df, and Group II, $\chi^2 = 6.46$ 3 df). For Group II the slight trend indicated was associated with fewer correct answers for tartaric acid comparisons.

Hinreiner *et al.* (1955), with a 12–16-member panel using triangular taste tests, found a difference threshold of $\pm 0.15$ g of

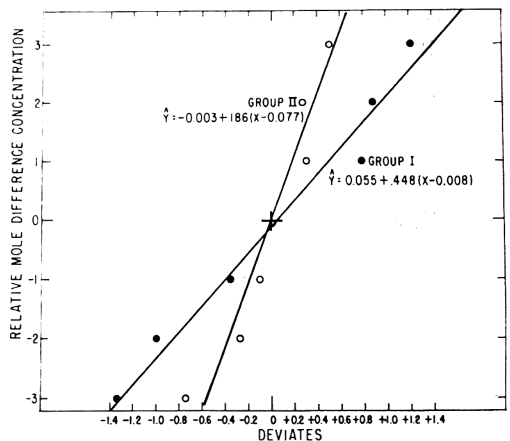


Fig. 3. Comparison of two taster groups using results of the combined acid tests.

tartaric acid per 100 ml with a reference wine of 0.74 g of tartaric acid per 100 ml. In the present investigation, difference thresholds for the four acids were tartaric, ±0.13 g per 100 ml; citric, ±0.18 g per 100 ml; fumaric ±0.10 g per 100 ml; and adipic, ±0.13 g per 100 ml. Considering that the reference concentration was a lower value (0.615 as g of tartaric per 100 ml) and that a more sensitive test (paired) was used, the results for the tartaric acid are reasonably close to the reported difference threshold.

The difference threshold (on pooled results and calculated as response to tartaric acid) was ±0.112 g per 100 ml for Group I and ± 0.165 g per 100 ml for Group II.

The use of Fisher's rank scores for the analysis of paired comparisons applies to tests where object differences are suspected but no criteria for a difference are available. The added molar amount of the four acids were equal and based on 0.20 g of tartaric per 100 ml. The pH values (Table 3) conform reasonably well to previous measurements and indicate little difference between tartaric, citric, and fumaric. Again, a large difference is shown by the adipic.

Citric acid was considered most sour, adipic least sour, and tartaric and fumaric about equal. Table 5 gives the mean ranks and the analysis of variance. This reinforces the conclusion of Amerine *et al.* (1959) that the apparent sourness is dependent on pH and titratable acidity. Despite the fact that the base wine contained mainly tartaric acid, and also that the buffer capacity of any wine is reasonably high, the results tend to agree with those of Beatty and Cragg (1935).

It is of interest to see the number of agreeing judgments involved in the direct comparison study (Table 6). In 40 trials per comparison only citric vs. adipic could be differentiated decisively when the panel was asked for an acid judgment. Despite the large amount of added acid, no differences could be found in 40 trials between direct comparison of citric, fumaric, and tartaric, or comparison of fumaric or tartaric to adipic.

Separate analysis of each panel member's responses (for the four replicates) indicated that four subjects could significantly rank the acids. This group (I) contained tasters 3, 8, 9, and 10. Group II contained tasters 1, 2, 4, 5, 6, and 7. It appeared that the acid × replicate interaction variance might separate into two groups. Using Bartlett's test for homogeneity among variances yielded a $\chi^2$ value of 9.58 for 9 df, indicating no real differences in individual variances. However, the pooled variances of the groups did differ significantly. It is of

Table 4. Constant-stimuli test of equal molar acid differences.

| Source of $\chi^2$ | Analysis of variance | | | |
|---|---|---|---|---|
| | df | ss | $\chi^2$ | Probability |
| Tartaric acid | | | | |
| Regression | 1 | 0.953 | 46.70 | $p < .001$ |
| Residual | 4 | 0.095 | 4.66 | $.40 > p > .30$ |
| Total | 5 | 1.048 | | |
| Citric | | | | |
| Regression | 1 | 0.057 | 45.94 | $p < .001$ |
| Residual | 4 | 0.059 | 2.83 | $.70 > p > .50$ |
| Total | 5 | 1.016 | | |
| Fumaric | | | | |
| Regression | 1 | 1.373 | 68.65 | $p < .001$ |
| Residual | 4 | .105 | 5.23 | $.30 > p > .20$ |
| Total | 5 | 1.478 | | |
| Adipic | | | | |
| Regression | 1 | 1.199 | 64.75 | $p < .001$ |
| Residual | 4 | .165 | 8.92 | $.10 > p > .05$ |
| Total | 5 | 1.3642 | | |

Table 5. Panel comparison of equal molar amounts of acids.

| Source | Analysis of variance df | ss | ms |
|---|---|---|---|
| Total | 120 | 74.737 | |
| Acids | 3 | 14.473 | 4.824** |
| Tastings × acids | 117 | 60.264 | 0.515 |

Mean rank scores

| Tartaric | Citric | Fumaric | Adipic |
|---|---|---|---|
| 0.049 | 0.359 | 0.055 | −0.463 |

** Significant at 1% level.

Table 6. Direct comparison of the acids.

| | Tartaric vs. citric | | Tartaric vs. fumaric | | Tartaric vs. adipic | | Citric vs. fumaric | | Citric vs. adipic | | Fumaric vs. adipic | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Higher acid | 22 | 18 | 19 | 21 | 26 | 14 | 21 | 19 | 37*** | 3 | 25 | 15 |
| Preference | 16 | 24 | 24 | 16 | 27* | 13 | 20 | 20 | 21 | 19 | 21 | 19 |

Number of agreeing judgments

* Significant at 5% level (two-tail test).
*** Significant at 0.1% level (two-tail test).

Table 7. Group comparisons of equal molar amounts of the acids.

| Source | Analyses of variance df | ss | ms |
|---|---|---|---|
| Group I | | | |
| Total | 48 | 28.109 | |
| Acid | 3 | 16.027 | 5.342** |
| Acid × tastings | 45 | 12.082 | 0.268 |
| Group II | | | |
| Total | 72 | 46.628 | |
| Acid | 3 | 2.853 | 0.951 |
| Acid × tastings | 69 | 43.775 | 0.634 |

Mean rank scores

| Groups | Tartaric | Citric | Fumaric | Adipic |
|---|---|---|---|---|
| I | 0.086 | 0.682 | 0.044 | −0.726 |
| II | 0.025 | 0.143 | 0.121 | −0.288 |

** Significant at 1% level.

Table 8. Preference evaluation.

| Tasters | Replicates | Mean rank scores: Tartaric | Citric | Fumaric | Adipic | Analyses: Taster × acid df | Taster × acid Variance | Acid (3 df) Variance |
|---|---|---|---|---|---|---|---|---|
| All | 1,2,3,4 | 0.174 | 0.123 | −0.091 | −0.206 | 117 | 0.584 | 1.284 |
| 4,9 | 1,2,3,4 | 0.040 | 0.805 | −0.548 | −0.296 | 21 | 0.223 | 2.766** |
| 1,2,3,5,6,7,8,10 | 1,2,3,4 | 0,208 | −0.048 | 0.023 | −0.184 | 93 | 0.610 | 0.853 |
| All | 1,2,4 | 0.212 | 0.187 | −0.123 | −0.277 | 87 | 0.540 | 1.719* |
| 4,9 | 1,2,4 | 0.047 | 0.735 | −0.502 | −0.288 | 15 | 0.264 | 1.755** |
| 1,2,3,5,6,7,8,10 | 1,2,4 | 0.254 | 0.050 | −0.028 | −0.275 | 69 | 0.573 | 1.146 |

* Significant at the 5% level.
** Significant at the 1% level.

interest that 3 of the 4 tasters in Group I were also in the prior Group I. Analyzing the two groups separately indicated that most of the acid variance was associated with Group I (Table 7).

Analysis of the preference choices for the group showed that the group as a whole did not have a significant preference for any one acid. Two tasters (4 and 9) did have significant preferences. A test of the replicate × acid interaction variances of the group showed no heterogeneity. The pooled variance of tasters 4 and 9 did differ significantly from the rest of the group. Further inspection of data showed an unusually low acid variance for the 3rd replicate. If this replicate is removed, the analyses indicate a significant ranking for the group as a whole. The mean rank scores and the analyses are shown in Table 8 for the various groupings of the data. Tasters 4 and 9 preferred citric acid and disliked fumaric to a significant degree. The rest of the tasters (with or without the 3rd replicate removed) showed no significant preference. The group as a whole (with the third replicate removed), however, showed about equal preference for tartaric and citric and did not like the adipic and to a lesser degree the fumaric. (Reference to Table 6 indicates the direct-comparison, agreeing-preference judgments for 40 trials.)

The preference data are rather inconclusive for the group as a whole, but the trend was toward preference of citric and tartaric over fumaric and adipic. It should be remembered that this is a small panel, only 10 individuals, and that the preference of these individuals does not necessarily apply to the population as a whole. It should also be noted that the level of acid in itself could be affecting preference decisions in that the impression of citric acid as being more acid could influence the panel member because he likes a higher acid, or converse effects could be postulated for the adipic acid.

On a weight-per-volume basis, replacement of citric acid by fumaric acid as a wine acidulant would be expected to fall between the ratio of 4 parts fumaric to 5 parts citric and 3 parts fumaric to 5 parts citric, depending on the amount of acid used and, probably to a certain extent, on the wine. If small amounts (near difference threshold) are used, the more favorable ratio would be sufficient. If larger amounts of acid are used, a ratio between 3:5 and 4:5 would seem more appropriate because of the slightly more acid taste attributed to citric acid.

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# The Direct Determination of Shear Stress-Shear Rate Behavior of Foods in the Presence of a Yield Stress

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**SUMMARY**

**The yield stress of food materials plays an important role in their flow characteristics. It is possible to determine the yield stress from the shear stress–shear rate data determined from a narrow-gap viscometer and applying Casson's equation. The yield stress determined in this manner checked with the yield stress determined from the torque exerted on a serrated cylinder at rest.**

**However, the yield stress for applesauce and tomato purée is much lower with a single-cylinder viscometer than with a narrow-gap viscometer. This is apparently due to disruption of suspension aggregates under shear, with the release of entrapped fluid at the cylinder wall. It is expected that materials in which large amounts of entrapped fluid are released under shear will not exhibit this phenomenon.**

**The shear stress vs. shear rate data from the narrow-gap viscometer could be fitted by a power-law equation.**

## INTRODUCTION

Yield stress or the shear strength of a fluid refers to the stress that must be exerted to just move one fluid layer past another. Electrical, chemical, and mechanical forces between the molecules and particles in a fluid all contribute to the yield stress. The rheological importance of the yield stress or the shear strength of food materials is often overlooked.

**Yield stress significance.** Yield stress has a great influence in laminar flow in tubes in that it determines the point in the tube where plug flow occurs (see Figure 1a).

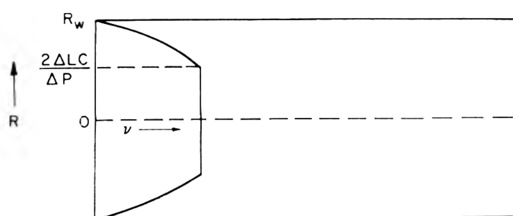


Fig. 1a. Velocity distribution in a tube when yield stress is not exceeded at all points.

Contribution 473 from the Department of Nutrition, Food Science and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts.

The point in the tube where plug flow occurs is given by

$$R = \frac{2\Delta LC}{\Delta P} \quad [1]$$

where $R$ = distance from center
$\Delta P$ = pressure drop in tube
$\Delta L$ = length
$R_w$ = tube radius
$C$ = yield stress

This is of great importance in determining the lethal effects in the holding tube of aseptic canning processes and in determining pumping requirements of fluids in pipe systems.

The yield stress is also important in its effect on the coating characteristics of fluids. For example, the amount of fluid remaining on a vertical surface after draining is given by (see Figure 1b)

$$W = CZ \quad [2]$$

where: $W$ = weight of fluid
$C$ = yield stress
$Z$ = surface area

**Indirect determination of power-law constants.** The shear stress–shear rate charac-

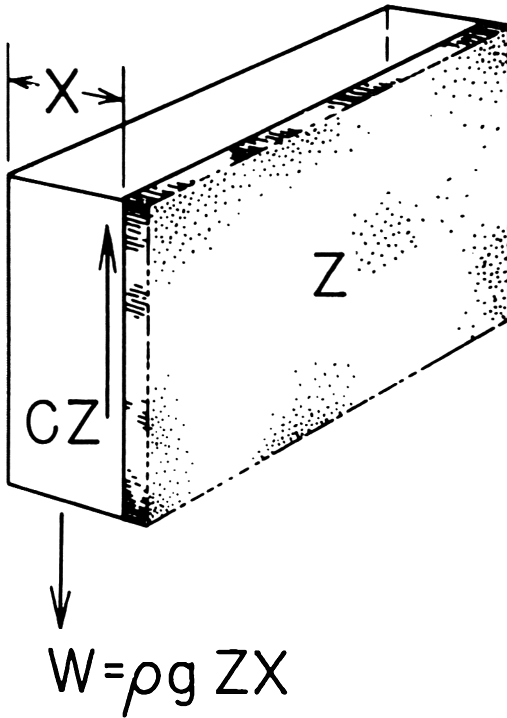


Fig. 1b. Coating on vertical surface.

teristics of many non-time-dependent food materials may be expressed by

$$\tau = b\gamma^s + C \qquad [3]$$

where: $\tau$ = shear stress
$\gamma$ = shear rate
$b, s$ = power law constants
$C$ = yield stress

It was recently shown (Charm, 1963) that the power-law constants $b$, $s$, and $C$ can theoretically be determined from a single-cylinder viscometer of the Brookfield type.

The relations between $b$, $s$, and $C$, and the speed and torque exerted on the rotating cylinder of the viscometer, are given by (see Fig. 1c)

$$2\pi N\left(\frac{b}{C}\right)^{1/s} = \int_{R_1}^{R_2} \left(1 - \frac{A}{L2\pi CR^2}\right)^{1/s} \frac{dR}{R} \qquad [4]$$

and

$$R_2 = \sqrt{\frac{A}{L2\pi C}} \qquad [5a]$$

where: $L$ = length of cylinder in contact with fluid
$N$ = speed of cylinder, RPS
$A$ = torque exerted at surface of cylinder
$R_1$ = radius of cylinder
$R_2$ = distance from center of cylinder to where stress equals yield stress and streamline velocity is zero

In order to relate torque ($A$) to cylinder speed ($N$), it is necessary to solve Eq. 4 graphically. The yield stress is determined from

$$C = \left(\frac{A}{L}\right)_0 \frac{1}{2\pi R_1^2} \qquad [5b]$$

where $\sqrt{\left(\frac{A}{L}\right)_0}$ is the intercept of the plot $\sqrt{\frac{A}{L}}$ vs. $\sqrt{N}$.

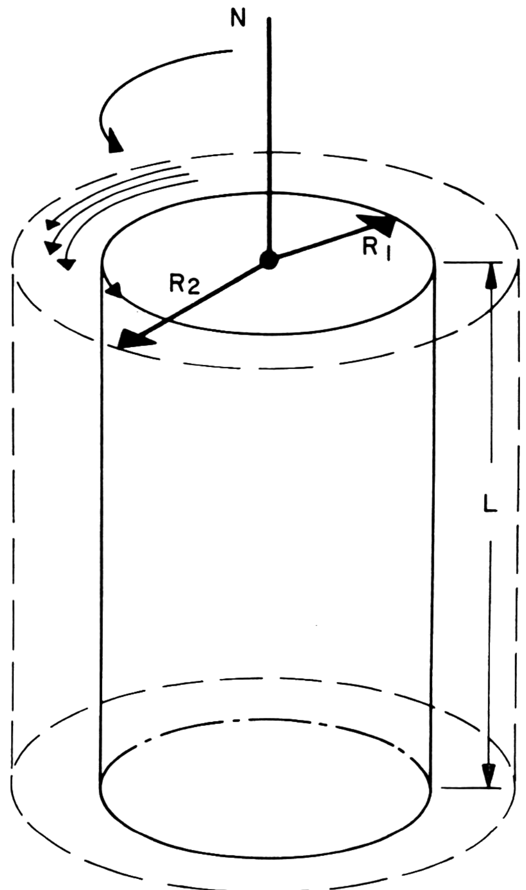


Fig. 1c. Cylinder rotating in a fluid with a yield stress.

When no yield stress exists, the following simpler relationship exists:

$$2\pi N = \frac{s}{2}\left[\frac{A}{L2\pi b}\right]^{1/s}\left[\frac{1}{R_1^{2/s}} - \frac{1}{R_2^{2/s}}\right] \quad [6]$$

The development of Eq. 5 and Eq. 6 required the assumption of Eq. 3. Therefore, the determinations of the constants $b$, $s$, and $C$ are not directly determined but are based upon the prior assumption of Eq. 3 as being the relationship between the shear stress and the rate of shear.

**Direct determination of shear stress–shear rate behavior.** Through use of a narrow-gap concentric-cylinder viscometer, it is possible to determine the shear stress–shear rate relationship of food material without the prior assumption of any $\tau$ vs. $\gamma$ relation. In this case:

$$\gamma = \frac{V}{G} = \frac{2\pi N R_1}{G} \quad [7]$$

where $V$ = linear velocity of moving cylinder
$G$ = gap width between concentric cylinders
$R_1$ = radius of inner cylinder

According to Wilkinson (1960), if $\frac{G}{R_1}$ is not greater than 0.05 the variation across the gap width in both stress and shear rate will be about 10% (for a Newtonian fluid).

The shear stress may be determined directly from the torque exerted on either the moving cylinder or the stationary cylinder since:

$$\tau = \frac{A}{L2\pi R_1^2} \quad [8]$$

**Casson's equation.** From theoretical considerations Casson (1959) found that printing-ink suspensions have the following shear stress–shear rate relation:

$$\sqrt{\tau} = K\sqrt{\gamma} + \sqrt{C} \quad [9]$$

where $K$ is a constant.

Steinert (1958) showed that melted chocolate, which is essentially a suspension of cocoa particles and sugar in cocoa butter, follows Casson's equation. Although Casson's equation is not a convenient form for expressing $\tau$ vs. $\gamma$, because of the integration difficulty when applied to flow problems, it does afford a means of evaluating the yield stress, $C$. For fluids obeying Casson's relationship, a plot of $\sqrt{\tau}$ vs. $\sqrt{\gamma}$ should result in a straight line whose intercept is $\sqrt{C}$. It is necessary to extrapolate the line to determine the intercept. Having determined the shear stress–shear rate data and the yield stress in this way, any convenient equation, e.g., Eq. 3, may be used to fit the data.

## EXPERIMENTAL

**$\tau$ vs. $\gamma$ from Haake viscometer.** The shear stress–shear rate behavior of applesauce and tomato purée was determined in a Haake viscometer.

The Haake viscometer is a concentric-cylinder viscometer with a number of interchangeable cylindrical spindles. In this case a cylinder was employed that resulted in a gap width of .0954 cm with a spindle radius of 4.08 cm. This system made

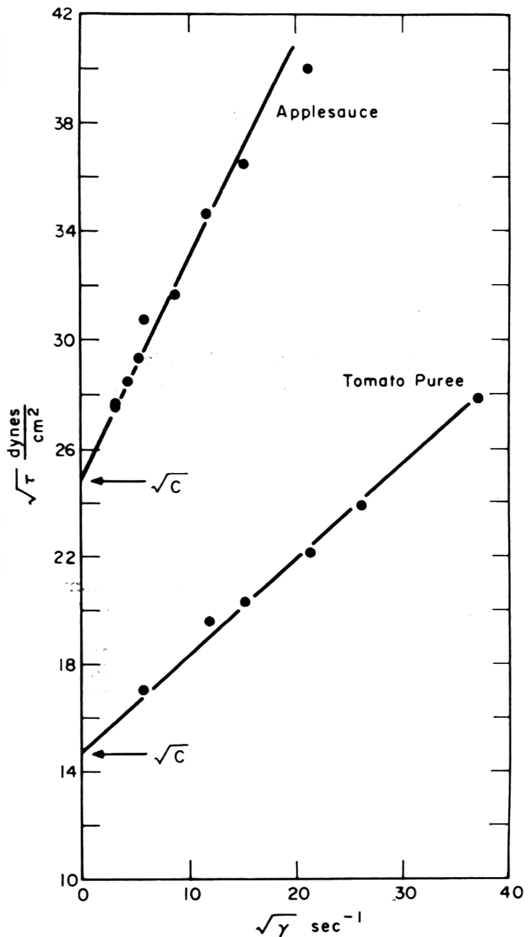


Figure 1d. Casson relationship for applesauce and tomato purée in Haake viscometer.

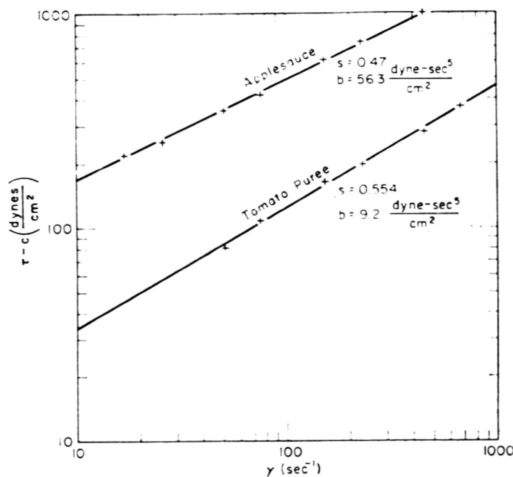


Fig. 2. Log $(\tau - c)$ vs. log $\gamma$ for applesauce and tomato purée in Haake viscometer.

possible the direct determination of the shear stress–shear rate relationship of the applesauce and tomato purée. The shear rates ranged from 8.42 $sec^{-1}$ to 1370 $sec^{-1}$ (see Table 1).

When the $\sqrt{\tau}$ vs. $\sqrt{\gamma}$ was plotted, the results fell on a straight line that was extrapolated to $\sqrt{\gamma} = 0$ (see Fig. 1d).

The yield stress for applesauce evaluated in this manner was found to be 595 $\frac{\text{dynes}}{\text{cm}^2}$, and for tomato purée 213 $\frac{\text{dynes}}{\text{cm}^2}$ (see Table 1).

**Yield stress from serrated cylinder.** It was possible to determine a value for the yield stress directly from a serrated cylinder associated with the Haake viscometer.

In this case a serrated cylinder immersed in the fluid was twisted and allowed to come to rest. The torque exerted on the cylinder at rest was related to the yield stress by Eq. 5b. The yield stress determined by this method was compared with that determined by extrapolation in Table 1.

**Fitting power law equation to shear stress–shear rate data.** A plot of log $(\tau - C)$ vs. log $\gamma$ resulted in a straight line (see Fig. 2). The slope of the resulting line was $s$. Having determined $s$, it was possible to determine $b$ by selecting a value of $\tau$ and $\gamma$ and solving Eq. 3 (see Table 1).

**Determination of yield stress from single-cylinder viscometer.** A Brookfield model HV viscometer with a cylinder 0.95 cm in radius was employed to determine the torque-speed relationship for applesauce and tomato purée. By plotting $\sqrt{A/L}$ vs. $\sqrt{N}$ and extrapolating to $\sqrt{N} = 0$, it was possible to determine the yield stress $C$ from Eq. 5b (See Table 2 and Fig. 3).

## DISCUSSION

The shear stress–shear rate relation for applesauce and tomato pureé indicates that these materials follow Casson's equation, as does chocolate (see Fig. 1d). The yield stress determined from Casson's equation and from the serrated cylinder in the Haake Viscometer agree favorably. However, the yield stress determined with the Brookfield single-cylinder viscometer is less than that found with the narrow-gap viscometer.

This is apparently due to the fact that the tomato purée and applesauce suspensions are aggregates of particles and fibers that entrap fluid (see Fig. 4). Under shear, the aggregates are broken, releasing fluid in the area of the rotating cylinder surface. Thus, less torque is exerted on the cylinder wall, with a resulting lower apparent yield stress.

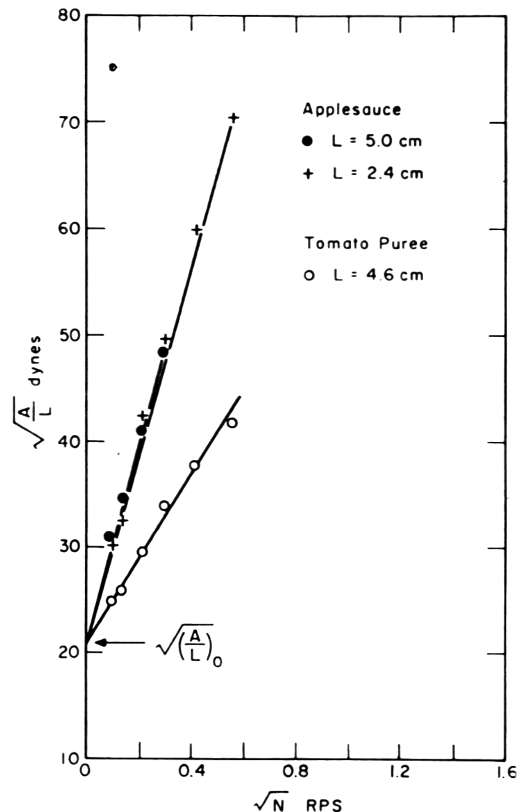


Fig. 3. Force acting on spindle as a function of speed in Brookfield viscometer for applesauce and tomato purée.

Table 1. Shear stress–shear rate behavior in Haake viscometer.

| Material | $\gamma$ ($sec^{-1}$) | $\tau$ $\frac{dynes}{cm^2}$ | Casson C $\left(\frac{dynes}{cm^2}\right)$ | Serrated cylinder $\left(\frac{dynes}{cm^2}\right)$ | h $\frac{dyne\text{-}sec^s}{cm^2}$ | s |
|---|---|---|---|---|---|---|
| Tomato purée | 50.8 | 294 | 213 | 230 | 9.2 | .554 |
| | 76 | 320 | | | | |
| | 152 | 375 | | | | |
| | 229 | 407 | | | | |
| | 457 | 490 | | | | |
| | 685 | 570 | | | | |
| | 1370 | 784 | | | | |
| Applesauce | 8.42 | 755 | 595 | 586 | 56.3 | 0.470 |
| | 16.9 | 815 | | | | |
| | 25.7 | 845 | | | | |
| | 50.8 | 945 | | | | |
| | 76 | 1010 | | | | |
| | 152 | 1210 | | | | |
| | 229 | 1340 | | | | |
| | 457 | 1600 | | | | |

Table 2. Force vs. speed for applesauce and tomato purée in Brookfield viscometer with cylindrical spindle.

| Material | N (RPS) | L (cm) | $\frac{A}{L}$ (dynes) | C $\frac{dynes}{cm^2}$ | $\frac{\text{Spindle cross-section area}}{\text{Curved-surface area}}$ |
|---|---|---|---|---|---|
| Tomato purée | .008 | 4.6 | 580 | | |
| | .016 | | 675 | 78 | 0.103 |
| | .0416 | | 892 | | |
| | .08 | | 1175 | | |
| | .16 | | 1430 | | |
| | .32 | | 1750 | | |
| | .8 | | 2210 | | |
| | 1.67 | | 2830 | | |
| Applesauce | .008 | 2.4 | 900 | 71 | 0.19 |
| | .016 | | 1230 | | |
| | .0416 | | 1820 | | |
| | .08 | | 2480 | | |
| | .16 | | 3600 | | |
| | .32 | | 4950 | | |
| Applesauce | .008 | 5.0 | 965 | 90 | .095 |
| | .016 | | 1190 | | |
| | .0416 | | 1710 | | |
| | .08 | | 2300 | | |
| | .16 | | 2610 | | |

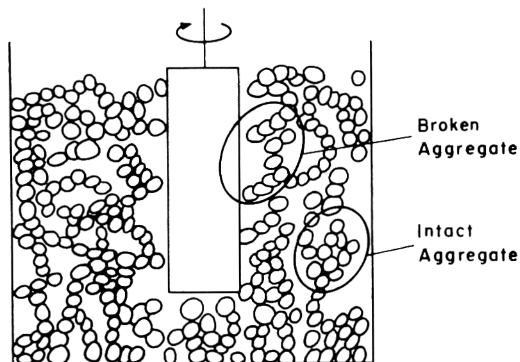


Fig. 4. Idealized concentrated suspension with immersed rotating spindle breaking aggregate.

Undoubtedly the same effect occurs in the narrow-gap viscometer. However, as pointed out by Whitmore (1959), in a narrow-gap concentric-cylinder viscometer, where a concentration gradient exists, the viscometer measures the characteristics of the average concentration rather than the fluid at the rotating surface. His development of this is as follows: "Suppose that a quantity of suspension of bulk volume concentration $c$ is introduced between two infinite, parallel, horizontal faces [see Fig. 5], and suppose that by some mechanism the suspension particles become redistributed into horizontal laminae of different concentrations. Let the thickness of the laminae be $d_1$, $d_2$, $d_3 \ldots d_n$, the mean concentration of particles in each laminae be $c_1$, $c_2$, $c_3$, ... $c_n$, and the viscosity of the suspensions in each laminae be $\eta_1$, $\eta_2$, $\eta_3 \ldots \eta_n$. Let the top plate be moved horizontally relative to the bottom plate with uniform velocity $V$ and the interfacial velocities be $V_1$, $V_2$, $V_3 \ldots V_{n-1}$, 0 respectively.

If the shear force transmitted across unit area of the plates is $F$ then, from the definition of the coefficient of viscosity,

$$F = \frac{\eta_1 (V - V_1)}{d_1} = \frac{\eta_2 (V_1 - V_2)}{d_2} = \ldots \frac{\eta_n (V_{n-1} - 0)}{d_n} \qquad [10]$$

or

$$V - V_1 = \frac{Fd_1}{\eta_1}, \quad V_1 - V_2 = \frac{Fd_2}{\eta_2}, \ldots V_{n-1} - 0 = \frac{Fd_n}{\eta_n} \qquad [11]$$

"Adding the terms

$$V = F \left[ \frac{d_1}{\eta_1} + \frac{d_2}{\eta_2} + \ldots \frac{d_n}{\eta_n} \right] \qquad [12]$$

"Now it has been shown that for many disperse suspensions the viscosity is related to the volume concentration by the equation

$$\eta = \frac{\eta_0}{1 - Kc} \qquad [13]$$

where $K$ is a constant and $\eta_0$ is the viscosity of the suspending fluid.

"Substituting from equation 13 for $\eta_1$, $\eta_2 \ldots \eta_n$ in equation 12,

$$V = \frac{F}{\eta_0} \left[ d_1(1-Kc_1) + d_2(1-Kc_2) + \ldots d_n(1-Kc_n) \right]$$

$$= \frac{F}{\eta_0} \left[ d - K\bar{c}d \right]$$

where $\bar{c}$ is the mean concentration of particles in the suspension between the plates and $d$ is the distance between the plates.

"The mean viscosity of all the suspension between the plates $\bar{\eta}$ is given, from the definition of the coefficient of viscosity, by

$$\bar{\eta} = \frac{Fd}{V} \qquad [14]$$

. . . That is, the mean viscosity of the suspension, as calculated from the velocity and shear force on the moving plate, is dependent only upon the mean concentration of particles between the plates, and is unchanged by variations in concentration distribution at right angles to the direction of movement."

In suspensions where the aggregates have

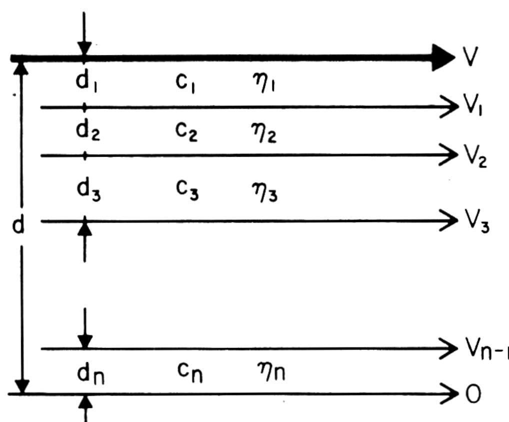


Fig. 5. Suspension divided into laminae between horizontal plates.

not entrapped large amounts of suspending fluid, it is expected that the yield stress determined by single-cylinder and narrow-gap viscometers would agree. This has been found to be true in chocolate (Walter Baker, 1962).

From Fig. 2 it appears that $\tau$ vs. $\gamma$ data can be fitted by Eq. 3.

## ACKNOWLEDGMENTS

The author thanks Walter Baker Chocolate Co., General Foods Corp., for use of the viscometry equipment employed in this study.

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# Growth and Proteolytic Activity of *Pseudomonas fluorescens* in Eggs and Egg Products[a]

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**SUMMARY**

**The growth characteristics and proteolytic activities of 3 strains of *Pseudomonas fluorescens* were determined in blended fresh egg white (initial pH 7.8-8.0), egg white adjusted to pH 7.4, egg white with added yolk, 30-day-old egg white (pH 9.0), and various egg media at the 2% protein level. In all cases the number of viable cells increased markedly during the first 1–2 weeks of incubation. Under certain conditions of incubation, strains 5 and 17 showed proteolytic activity in blended fresh egg white, and strain 10 in egg white adjusted to pH 7.4 and in egg white with added egg yolk. Growth and proteolytic activity were also observed in the 30-day-old egg white (pH 9.0). All strains showed growth and proteolytic activity in the spray-dried egg white growth medium (pH 7.4, autoclaved). Proteolysis was also determined on solid media with various egg constituents as substrate.**

Various studies have shown that psychrophilic bacteria are responsible for many undesirable changes in the flavor, odor, body and texture, and color of foods stored for extended periods under refrigeration. Detailed information on various aspects of psychrophiles—such as methods of enumeration, species involved, effect on quality of foods, sources of contamination, control, and biochemical activities—is presented in a comprehensive review (336 references) by Witter (1961). Although psychrophiles isolated from foods belong to many bacterial genera, those belonging to *Pseudomonas* are predominant. With respect to bacterial spoilage of shell eggs, pseudomonads (*P. fluorescens* in particular) are associated with various types of rots.

Our knowledge about the mode of entry of microorganisms into shell eggs has increased greatly during the last five years. Results reported by Stokes and Osborne (1956), Walden *et al.* (1956), Kraft *et al.* (1958), and Garibaldi and Stokes (1958) indicate that the shell and shell membranes act primarily as a mechanical barrier to microbial penetration. Microorganisms that manage to pass these barriers meet conditions in the albumen (such as high pH, the presence of ovomucin, ovomucoid, avidin, lysozyme, and conalbumin) that may be inhibitory to growth.

With respect to *P. fluorescens*, Ayres and Taylor (1956) indicated that an increase in the number of viable cells can occur in egg white and in egg yolk at temperatures from 2 to 20°C. Other data (Garibaldi, 1960) show that the high pH found in egg white and the iron-binding capacity of conalbumin may prevent the growth of gram-negative spoilage bacteria. Little or no information is available on the proteolytic activity of *P. fluorescens* on egg white and egg products. The present report deals with the growth characteristics and proteolytic activity of 3 strains of *P. fluorescens* in egg white, with and without added yolk, and in various other egg substrates.

## EXPERIMENTAL METHODS

**Cultures.** *P. fluorescens* strains 5, 10, and 17 were from a series of cultures isolated from eggs in an advanced stage of "green rot." They were

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identified with reference to Breed *et al.* (1957). Cultures 5, 10, and 17 were selected because of their strong proteolytic activity against gelatin, milk and alkaline egg medium (Society of American Bacteriologists, 1957). The cultures were maintained on slants of tryptone glucose extract (TGE) agar. Prior to each use, the cultures were transferred twice onto new TGE slants and then into tubes with nutrient broth.

**Collection of eggs.** Eggs were collected from the A and M College of Texas poultry plant from White Leghorn hens housed in individual cages. All eggs used were from hens fed the same ration. The eggs were collected every 30 min during the morning laying period and placed in 70% alcohol until used in the afternoon.

**Breaking of the eggs.** After removal from the alcohol, the eggs were drained, flamed, broken, separated (white from yolk), and placed in sterile glass containers. These procedures were conducted in a "breaking box." The box was made of plywood, equipped with a clear plastic lid, and provided with canvas-covered holes through which the hands were inserted. Installed in this box were a fluorescent light, a small Bunsen burner, an electrical outlet, and a small atomizer attached to a tank of nitrogen to dispense a 1% solution of Roccal into the atmosphere inside the box.

The egg white was blended for 1 min in a sterile Waring blender jar attached to a variable power transformer (Powerstat) set at 50 volts. The blended egg white was then transferred to either 1- or 2-L Erlenmeyer flasks equipped with a 7-mm glass air inlet and a 5-mm glass liquid outlet tube. The liquid outlet tube was connected to a Brewer automatic pipetting machine by a sterile rubber tube. The syringe on the pipetting machine was adjusted to deliver 5-ml samples of egg preparations into sterile flasks or screw-cap test tubes.

**Preparation of inoculum.** A loopful of a culture of *P. fluorescens* from a TGE slant was transferred twice to new TGE slants and then to a nutrient broth tube, which was then incubated for 24 hr at 25°C. One-tenth ml of the nutrient broth culture was then transferred into 50-ml Erlenmeyer flasks containing 25 ml of nutrient broth. After incubation for 24 hr at 25°C, the cells were removed by centrifugation at 0°C in 40-ml Pyrex glass centrifuge tubes in a Model-PR-2 International centrifuge at 2,000 rpm for 15 min. The supernatant fluid was decanted and the cells were suspended in phosphate-buffered distilled water (Wagenaar and Jezeski, 1952). This washing procedure was repeated. The cells were then resuspended in the buffer, and the absorbancy was measured in a spectrophotometer at 600 m$\mu$. The cell suspension was then adjusted to absorbancy values necessary to give the proper concentration of cells for the experiments. The dilution factor was established by correlating with absorbancy values the number of viable cells produced by plating on standard methods agar (APHA, 1960). One drop of the diluted inoculum was added to each flask or tube to be inoculated.

**Proteolytic activity on solid media.** Various mixtures of egg preparations and TGE agar (10 ml per plate) were poured into 15×150-mm plates. The following substrates were used: a) fresh egg white; b) alkaline egg white (Society of American Bacteriologists, 1957) without yolk; c) alkaline egg without yolk, adjusted to pH 7.4 and buffered with Veronal buffer (Colowick and Kaplan, 1955); d) alkaline egg white, without yolk, adjusted to pH 7.4 and buffered with Sorenson's phosphate buffer (Colowick and Kaplan, 1955); e) spray-dried egg white (Nutritional Biochemical Corp.); and f) alkaline egg white with yolk added. The buffers used in all these studies were added as dry ingredients to prevent dilution of the egg white. After the TGE agar plates had hardened, Whatman no. 3 filter-paper discs ¼ inch in diameter, soaked in nutrient broth cultures of *P. fluorescens*, were placed on the surface of the plate. The plates were then incubated at 10 or 25°C. At certain intervals the plates were checked and the zones of clearing were measured.

**Proteolytic activity in liquid media.** Proteolytic activity was determined by measuring: a) the ultraviolet absorption of the trichloroacetic acid (TCA) filtrates at 280 m$\mu$; and b) absorbancy of the TCA filtrates at 640 m$\mu$ with Folin-Ciocalteu reagent (Folin and Ciocalteu, 1927). In certain experiments the changes in total nitrogen and TCA-soluble nitrogen were also determined by a micro-Kjeldahl method (AOAC, 1960).

Figs. 1 and 2 describe the methods used in dilution and assay.

**Bacterial numbers.** Bacterial counts were made according to procedures described for eggs and egg products (APHA, 1960). The plates were incubated 3 days at 25°C. Sterility of the uninoculated samples was checked by the same technique.

**pH.** pH was measured with a model H Beckman pH meter.

**Composition of the substrates (growth media).** The following high-protein substrates (approx 10.5% protein) were used: a) blended fresh egg white with an initial pH of 7.8–8.0; b) blended fresh egg white with pH adjusted to 7.4 and buffered with 0.2*M* veronal buffer; c) blended 2:1 v/v fresh egg white and egg yolk, with initial pH 7.3; d) egg white with and without added iron ($FeSO_4 \cdot 7H_2O$).

Treatment of egg white for pH adjustment and

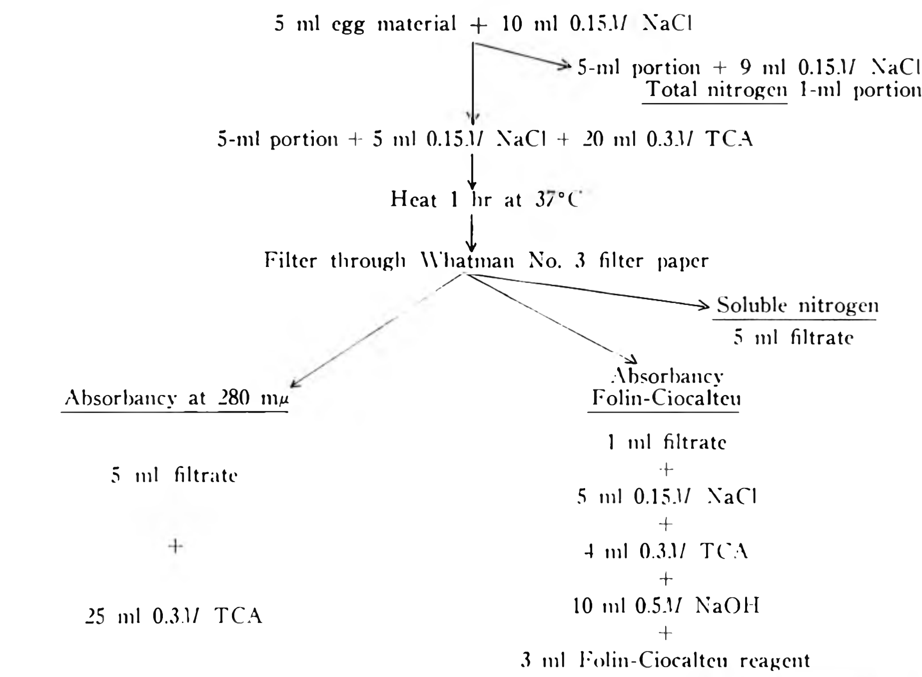


Fig. 1. Dilution procedure for high-protein substrates.

the addition of buffer was begun by placing blended fresh egg white in sterile 1- or 2-L Erlenmeyer flasks equipped with magnetic stirring bars. The quantity of sterile 1N NaOH or HCl required to adjust a 10-ml portion to the desired pH was determined. The required volume of acid or base was then added to the large flask while the magnetic stirrer was in operation. Buffer ingredients were then added, the water present being used as the diluent.

The egg white to which iron was to be added received treatment in the following sequence: a) 1,000 ml of egg white were placed in a sterile 2-L Erlenmeyer flask equipped with a magnetic stirring bar; b) $FeSO_4 \cdot 7H_2O$ (20 mg Fe per liter) was then added; and c) the egg white was dispensed into sterile screw-cap test tubes.

The following "low-protein" (2% protein) substrates were used: a) blended fresh egg white diluted with double-distilled water; b) blended fresh egg white diluted with 0.2M veronal buffer; c) blended fresh egg white diluted with 0.2M

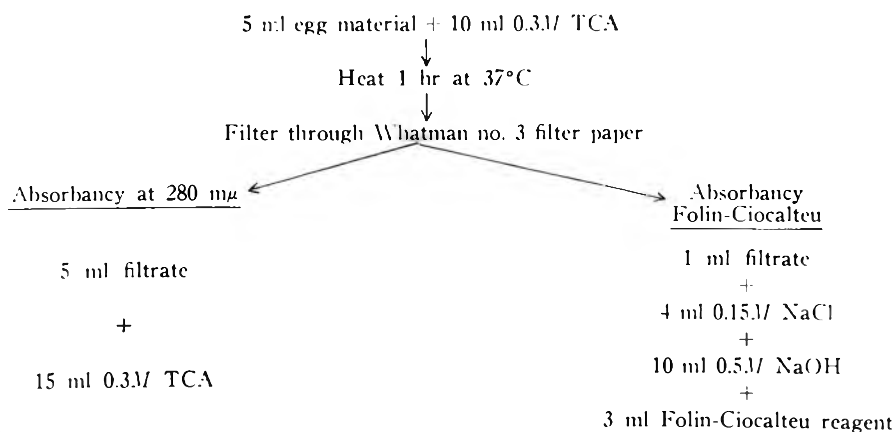


Fig. 2. Dilution procedure for 2% substrates.

phosphate buffer; d) alkaline egg white adjusted to pH 7.4, diluted with veronal buffer, placed in test tubes, and autoclaved (10 min, 121°C); e) spray-dried egg white diluted with double-distilled water, autoclaved, and then adjusted to pH 7.4; and f) spray-dried egg white, diluted with double-distilled water, autoclaved, pH adjusted to 7.4, and buffered with veronal buffer. The protein content was calculated from the nitrogen content as determined by a micro-Kjeldahl method (AOAC, 1960).

## RESULTS

**Proteolytic activity on solid media.** Preliminary studies showed that *P. fluorescens* strains 5, 10, and 17 possessed strong proteolytic activities against gelatin, litmus milk, and alkaline egg substrate. Table 1 presents data on the proteolytic activity of *P. fluorescens* strains 5, 10, and 17 on various solid media. The various media supported excellent growth and fluorescence of the 3 cultures at both 10 and 25°C. No proteolysis was noted on the fresh egg-white growth media. In general, strains 5 and 17 showed proteolytic activity on the various media at both 10 and 25°C except at some high levels of added egg material. On the media with a mixture of alkaline egg white and yolk, proteolytic activity was observed for each of the 3 cultures at both 10 and 25°C except for cultures 5 and 17 at high levels of egg substrate. Strain 10, which showed strong proteolytic activity in milk at both 10 and 25°C, showed no proteolytic action at 25°C on the solid media except in the presence of a mixture of alkaline egg white and yolk. At 10°C, however, strain 10 exhibited proteolytic activity in all substrates (except fresh egg white) at some level of substrate concentration. It was also observed that the extent of the zone of clearing decreased at the higher concentrations of added egg constituents.

**Growth characteristics and proteolytic activity of P. fluorescens in egg white and egg white with added yolk.** Fig. 3 presents data on the bacterial counts of *P. fluorescens* strain 17 in fresh egg white, egg white with the initial pH adjusted to 7.4, and egg white with added egg yolk (2:1 v/v white and yolk). The patterns of growth of strains 5 and 10 were similar to that of strain 17. In general, increases in the number of viable bacteria were marked in the 3 growth media at both 10 and 25°C. In egg white, high levels of viable population were reached after 7–35 days of incubation at 10°C, and bacterial counts then remained relatively constant up to and including 91 days of incubation. However, in egg white adjusted to pH 7.4 (strain 10) and in egg white with added egg yolk (3 strains), both marked increases and decreases in viable count were observed during this period. At a culture incubation temperature of 25°C, high viable counts were observed after 7–35 days (depending upon strain and growth medium), followed by a reduction in the number of viable cells.

Data on the measurement of proteolysis (Folin-

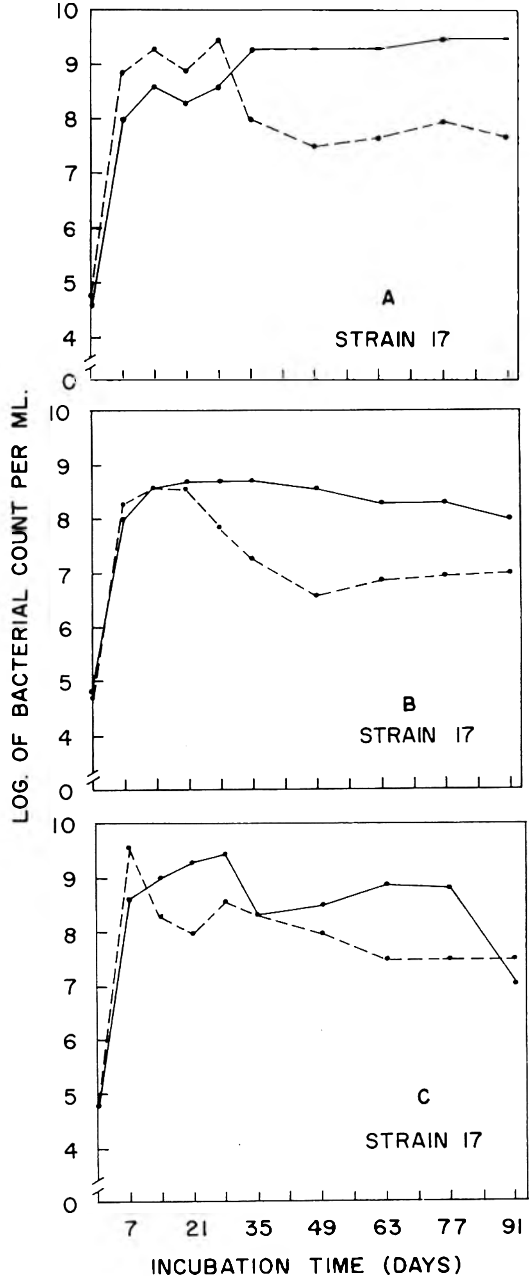


Fig. 3. Bacterial counts of *P. fluorescens* strain 17 when grown in egg white (A), egg white adjusted to pH 7.4 (B), and in egg white with yolk (C). Incubation temperatures: 10°C, solid lines; 25°C, broken lines.

Table 1. Zone of clearing when *P. fluorescens* strains 5, 10, and 17 were grown on various solid media at 10 and 25°C.

| | | 10°C | | | | | | 25°C | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 10 days | | | 17 days | | | 3 days | | | 6 days | | | 10 days | | |
| | | 5 | 10 | 17 | 5 | 10 | 17 | 5 | 10 | 17 | 5 | 10 | 17 | 5 | 10 | 17 |
| Spray-dried egg white[a] | 2[b] | 35[c] | 35 | 35 | 47 | 47 | 47 | 39 | 0 | 39 | 43 | 0 | 43 | 53 | 0 | 53 |
| | 4 | 33 | 0 | 33 | 43 | 43 | 43 | 35 | 0 | 35 | 41 | 0 | 41 | 51 | 0 | 51 |
| | 6 | 29 | 31 | 29 | 41 | 41 | 41 | 31 | 0 | 31 | 41 | 0 | 41 | 47 | 0 | 47 |
| | 8 | 29 | 0 | 29 | 41 | 0 | 41 | 29 | 0 | 29 | 37 | 0 | 37 | 47 | 0 | 47 |
| | 10 | 29 | 0 | 29 | 35 | 0 | 35 | 29 | 0 | 29 | 35 | 0 | 35 | 35 | 0 | 45 |
| | 15 | 0 | 0 | 0 | 31 | 0 | 31 | 37 | 0 | 37 | 43 | 0 | 43 | 45 | 0 | 45 |
| | 20 | 0 | 0 | 27 | 31 | 31 | 31 | 29 | 0 | 29 | 35 | 0 | 35 | 47 | 0 | 47 |
| | 25 | 29 | 29 | 29 | 31 | 31 | 31 | 27 | 0 | 27 | 33 | 0 | 33 | 45 | 0 | 45 |
| Alkaline egg white, pH 7.4, phosphate buffer added | 2 | 31 | 0 | 31 | 45 | 0 | 45 | 31 | 0 | 31 | 43 | 0 | 43 | 55 | 0 | 55 |
| | 4 | 31 | 0 | 31 | 43 | 0 | 43 | 33 | 0 | 33 | 43 | 0 | 43 | 55 | 0 | 55 |
| | 6 | 31 | 0 | 31 | 43 | 0 | 43 | 31 | 0 | 31 | 39 | 0 | 39 | 55 | 0 | 55 |
| | 8 | 31 | 0 | 31 | 41 | 0 | 41 | 37 | 0 | 37 | 39 | 0 | 39 | 55 | 0 | 55 |
| | 10 | 29 | 0 | 29 | 41 | 0 | 41 | 31 | 0 | 31 | 37 | 0 | 37 | 45 | 0 | 45 |
| | 15 | 29 | 0 | 29 | 31 | 0 | 31 | 29 | 0 | 29 | 45 | 0 | 45 | 49 | 0 | 49 |
| | 20 | 27 | 0 | 27 | 35 | 0 | 35 | 29 | 0 | 29 | 39 | 0 | 39 | 45 | 0 | 45 |
| | 25 | 27 | 0 | 27 | 31 | 0 | 31 | 29 | 0 | 29 | 31 | 0 | 31 | 43 | 0 | 43 |
| Alkaline egg white, pH 7.4, veronal buffer added | 2 | 31 | 31 | 31 | 45 | 45 | 45 | 37 | 0 | 37 | 47 | 0 | 47 | 49 | 0 | 49 |
| | 4 | 31 | 31 | 31 | 37 | 37 | 37 | 31 | 0 | 31 | 43 | 0 | 43 | 45 | 0 | 45 |
| | 6 | 29 | 29 | 29 | 37 | 37 | 37 | 27 | 0 | 31 | 35 | 0 | 35 | 45 | 0 | 45 |
| | 8 | 29 | 31 | 29 | 31 | 31 | 31 | 27 | 0 | 31 | 35 | 0 | 35 | 45 | 0 | 45 |
| | 10 | 27 | 35 | 27 | 31 | 37 | 31 | 29 | 0 | 29 | 35 | 0 | 35 | 43 | 0 | 43 |
| | 15 | 0 | 33 | 0 | 31 | 31 | 31 | 27 | 0 | 27 | 27 | 0 | 27 | 31 | 0 | 31 |
| | 20 | 0 | 0 | 0 | 0 | 33 | 0 | 0 | 0 | 0 | 27 | 0 | 0 | 31 | 0 | 29 |
| | 25 | 0 | 0 | 0 | 0 | 31 | 0 | 0 | 0 | 0 | 27 | 0 | 0 | 31 | 0 | 27 |
| Alkaline egg white, egg yolk added | 2 | 35 | 35 | 35 | 41 | 41 | 41 | 41 | 31 | 41 | 49 | 31 | 49 | 49 | 37 | 49 |
| | 4 | 33 | 33 | 33 | 37 | 37 | 37 | 41 | 31 | 41 | 47 | 33 | 47 | 49 | 37 | 49 |
| | 6 | 31 | 31 | 31 | 33 | 33 | 33 | 37 | 31 | 37 | 47 | 33 | 47 | 49 | 37 | 49 |
| | 8 | 31 | 31 | 31 | 31 | 31 | 31 | 31 | 33 | 31 | 45 | 31 | 45 | 45 | 37 | 45 |
| | 10 | 33 | 33 | 29 | 33 | 37 | 33 | 31 | 33 | 31 | 33 | 33 | 33 | 41 | 33 | 41 |
| | 15 | 31 | 31 | 0 | 43 | 43 | 43 | 0 | 33 | 0 | 33 | 39 | 33 | 45 | 33 | 45 |
| | 20 | 0 | 31 | 0 | 31 | 43 | 31 | 0 | 33 | 0 | 0 | 39 | 0 | 0 | 45 | 0 |
| | 25 | 0 | 31 | 0 | 0 | 35 | 0 | 0 | 33 | 0 | 0 | 39 | 0 | 0 | 45 | 0 |
| Alkaline egg white | 2 | 41 | 41 | 41 | 53 | 53 | 53 | 43 | 0 | 43 | 49 | 0 | 49 | 61 | 0 | 61 |
| | 4 | 35 | 35 | 35 | 49 | 49 | 49 | 39 | 0 | 39 | 45 | 0 | 45 | 55 | 0 | 55 |
| | 6 | 31 | 31 | 31 | 45 | 45 | 45 | 35 | 0 | 35 | 47 | 0 | 47 | 55 | 0 | 55 |
| | 8 | 31 | 31 | 31 | 37 | 37 | 37 | 35 | 0 | 35 | 45 | 0 | 45 | 55 | 0 | 55 |
| | 10 | 31 | 0 | 31 | 45 | 0 | 45 | 41 | 0 | 41 | 47 | 0 | 41 | 49 | 0 | 49 |
| | 15 | 29 | 0 | 29 | 37 | 0 | 37 | 31 | 0 | 31 | 43 | 0 | 43 | 49 | 0 | 49 |
| | 20 | 29 | 0 | 29 | 31 | 0 | 31 | 31 | 0 | 31 | 41 | 0 | 41 | 55 | 0 | 45 |
| | 25 | 29 | 0 | 29 | 31 | 0 | 31 | 31 | 0 | 29 | 41 | 0 | 41 | 45 | 0 | 45 |
| Blended fresh egg white | 2–25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Compounds added to TGE agar.
[b] Percentage added.
[c] Diameter (mm) of cleared zone.

Ciocalteu procedure), expressed as absorbancy at 640 mμ, indicate that with few exceptions the absorbancy values of the TCA filtrates of the inoculated samples remained relatively the same as those of the uninoculated samples. Some increases in absorbancy values over those of the controls were observed: a) in egg white with strain 5 at 10°C (after 28 days) and at 25°C (after 7 days), with strain 17 at 25°C (after 28 days); b) in egg white adjusted to pH 7.4 with strain 10 at 10°C (after 28 days); and c) in egg white plus egg yolk with strain 10 at 10°C between 28 and 49 days of incubation. Preliminary experiments showed that in egg white inoculated with *P. fluorescens* the patterns of increases were very similar in TCA-soluble N and in tyrosine and tryptophan (expressed as absorbancy at 640 mμ). A similar relation was observed in skim milk inoculated with certain pseudomonads (Vanderzant and Moore, 1955). The amount of TCA-soluble N in egg white, expressed as % of total N, was 7.5%. After incubation for 91 days, the amount of TCA-soluble N was 9.4% in egg white inoculated with strain 5 and incubated at 10°C, and 10.5% for strain 17 at 25°C. The absorbancy values of the TCA filtrates at 280 mμ also indicated that in most cases the absorbancy values of the filtrates of the inoculated samples were similar to those of the uninoculated control samples. After 63 days of incubation the absorbancy values of the filtrates of the egg white inoculated with strain 17 at 25°C remained slightly higher than those of the control samples. The pH measurements indicated that during the first 2 weeks of incubation the pH value of the inoculated egg white decreased from 7.8–8.0 to 7.0–7.5. The pH value rose sharply after 35–77 days of incubation at 10°C, but not at 25°C. Fluorescence was usually observed after 24–48 hr of incubation at 25°C, and after 5–7 days at 10°C.

**Growth and proteolytic activity in egg white with and without added iron.** *P. fluorescens* was inoculated into egg white with (20 mg Fe per liter) and without iron. The data for strain 10 incubated at 10°C indicate that the addition of iron caused a slight increase in the number of viable cells. No evidence of proteolytic activity was observed in the egg white with or without added iron.

**Growth and proteolytic activity of P. fluorescens in 30-day-old egg white.** Eggs were held in the shell for 30 days at 2–3°C. They were then broken and the yolk was separated from the white. The blended egg white was inoculated with *P. fluorescens* strains 5, 10, and 17. Eggs used in this portion of the study were from the same source and were collected at the same time as those used in the 91-day growth study. The inoculated and control tubes were incubated at 25°C. In general, the pattern of growth in 30-day-old egg white was similar to that observed in fresh egg white (91-day study), that is, a rapid increase in viable population during the first 7–21 days, followed by a decrease in viable count. Some proteolysis was observed in the egg white inoculated with strain 5 (after 21 days) and with strain 17 after 7 days of incubation. The extent of the changes in absorbancy at 640 and 280 mμ, and in the amounts of total and TCA-soluble N, were very similar to those observed with these strains under similar conditions in fresh egg white. The pH values of the inoculated samples decreased rapidly from a value of 9.0 at 0 day (30 days after collection) to 6.95–7.1 after 63 days of incubation (93 days after collection).

**Growth and proteolytic activity of P. fluorescens in 2% substrates.** Strains 5, 10, and 17 were grown in various substrates containing 2% protein. The various substrates were incubated at 10° and at 25°C for 35 days. Fig. 4 presents data on the bacterial counts of *P. fluorescens* strains 5, 10, and 17 in egg white diluted to 2% with distilled water. The pattern of growth in the other substrates was very similar to that presented in Fig. 4. In general, the number of viable cells increased rapidly and reached a maximum after 14–35 days at 10°C and after 7–28 days at 25°C, depending upon strain and growth medium used. After reaching a maximum, the viable count usually decreased in the alkaline egg white and spray-dried egg white media incubated at 25°C. This condition usually was not observed in the egg white media with or without added buffer at both 10 and 25°C or in the other media at 10°C. A comparison of the absorbancy values of the TCA filtrates of the inoculated and uninoculated media (Fig. 5) indicates that little or no proteolysis occurred in egg white or egg white with added buffer. Proteolysis, however, was apparent after 7 days of incubation in the growth medium with spray-dried egg white (E) with each of the 3 strains of

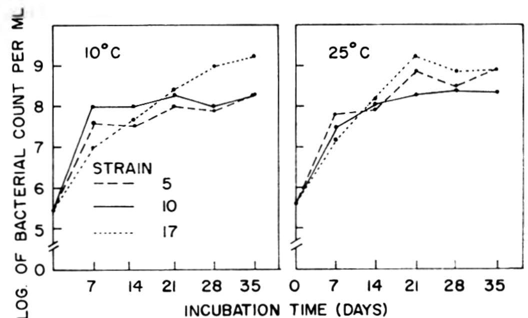


Fig. 4. Bacterial counts of *P. fluorescens* strains 5, 10, and 17 when grown in egg white diluted to 2% with distilled water.

*P. fluorescens* and at both incubation temperatures. Proteolysis also occurred after 7 days at 10 and 25°C with strain 10 in the alkaline egg white medium (D) and in the spray-dried egg white medium with veronal buffer (F). Results were similar when the absorbancy was measured at 280 mμ.

## DISCUSSION AND CONCLUSIONS

The results indicate that the number of viable cells increased markedly even without added iron or without pH adjustment. After reaching a maximum, the level of population remained relatively constant at 10°C. At 25°C, however, a reduction in viable count usually followed. Data on the growth characteristics of *P. fluorescens* in egg white from 30-day-old eggs indicate that: a) rapid growth was initiated in egg white at pH 9.0, and b) the pH of the inoculated egg white decreased from 9.0 to 7.2 during the first 21 days of incubation. Although no data were given on the pH value of the albumen, Ayres and Taylor (1956) also observed increases in the viable population of *P. fluorescens* in egg white incubated at 2, 10, and 20°C. Garibaldi (1960), however, reported that *P. fluorescens* grew in egg white at pH 7.9 but not at pH 9.1 unless supplemented with sufficient iron to saturate the conalbumin. The inhibitory action of high pH in unsupplemented egg white, according to that worker, may be explained by the relative increase in stability of the iron-conalbumin complex at higher pH values, resulting in a reduction in the availability of iron to the bacteria. In comparing the present results with those reported by others, certain facts should be kept in mind:

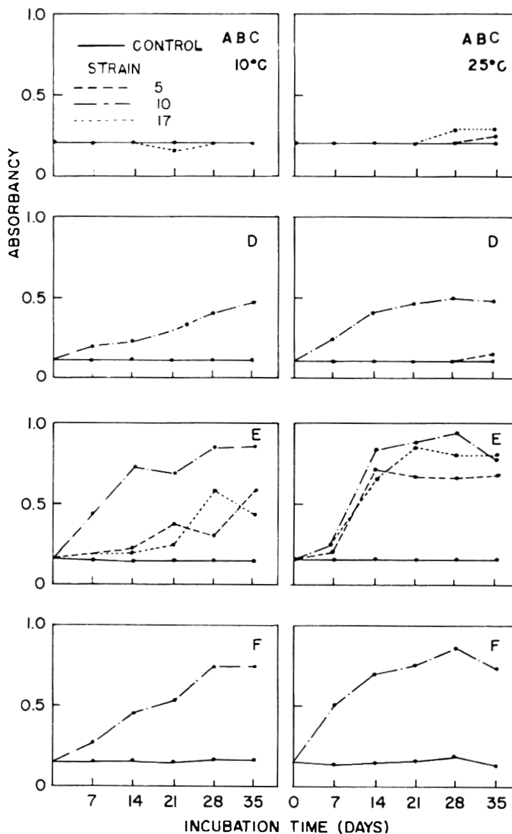


Fig. 5. Absorbancy values (Folin-Ciocalteu) of the TCA filtrates of the 2% substrates inoculated with *P. fluorescens* strains 5, 10, and 17: A) egg white diluted with distilled water, B) diluted with veronal buffer, C) diluted with phosphate buffer, D) alkaline egg white diluted to 2%, pH adjusted to 7.4, veronal buffer added, and autoclaved, E) spray-dried egg white, diluted to 2%, pH adjusted to 7.4, and autoclaved, and F) spray-dried egg white, diluted to 2%, pH adjusted to 7.4, veronal buffer added, and autoclaved.

a) In the first series of experiments (91-day study) the pH of the blended fresh egg white at time of inoculation was 7.8–8.0. The pH of the inoculated egg white decreased during the next 21 days (both at 10 and 25°C) to approximately 7.2. In view of these conditions growth could be expected to take place.

b) Initiation of growth occurred at pH 9.0 in egg white from eggs stored 30 days under refrigeration. The possibility exists that the treatment of the egg white prior to inoculation at pH 9.0 determines whether growth can occur.

c) Incubation of the egg white in the present study was at both 10 and 25°C, whereas in other studies (Garibaldi, 1960) it was at 28°C.

d) Strain differences with respect to iron requirements, secretion of iron-binding agents, and proteolytic enzymes may exert effects on growth and/or proteolytic activity. The concentration of unbound iron under the present conditions of substrate preparation and incubation may have been sufficient for these strains. Feeney and Nagy (1952) also suggest that, as the bacteria grow and utilize the unbound iron, the iron-conalbumin

complex may dissociate to supply additional unbound iron. They also considered the possibility that inhibition may take place because of metal-ion imbalance, and that the addition of metal ion may have merely adjusted the ionic relations to one favorable for growth. It is also reported (Garibaldi and Neilands, 1956) that some spoilage bacteria, when grown in media with insufficient iron, secrete iron-binding agents. The stability constants of the iron chelates of some were of similar order of magnitude as that of the iron-conalbumin compounds (Garibaldi, 1958). The ability of certain strains of *P. fluorescens* to cause proteolysis in the egg white may also be an important factor in assisting the bacteria to multiply in egg white.

Under certain conditions of incubation, strains 5 and 17 caused some proteolysis in egg white. However, no proteolytic activity was noted in egg white adjusted to pH 7.4 or in egg white with added egg yolk. On the other hand, strain 10 did not show proteolysis in egg white but did produce proteolysis in egg white adjusted to pH 7.4 and in egg white with yolk added. Little or no difference in growth pattern was observed between strains 5, 10, and 17. Other data, however, indicate distinct differences between strains with respect to proteolysis of certain substrates. With spray-dried egg white (2%, adjusted to pH 7.4, autoclaved), proteolysis was observed with each of the 3 strains. However, with added veronal buffer only strain 10 caused proteolysis. Data on the proteolytic activity of the cultures on solid media indicate strain differences with respect to incubation temperature. In many cases strain 10 caused proteolysis at 10 but not at 25°C. Strains 5 and 17, however, showed proteolysis on the various substrates at both 10 and 25°C. These results also suggest that caution should be exercised in the examination of a culture of *P. fluorescens* for proteolytic activity on solid media. Temperature of incubation and concentration of egg solids in the plating agar have a marked effect on the extent of proteolysis.

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