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An International Journal on the Microstructure and Microanalysis of Foods, Feeds and their Ingredients
ULTRASTRUCTURE STUDIES OF PASTA. A REVIEW.

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Abstract

Freeze-fracturing can be used effectively to study pasta microstructure both in the dry and cooked state. After a water-glycerol soaking, conventional raw wheat pasta shows an uncoagulated protein matrix in which the starch granules are uniformly dispersed. Starch granules appear unwoven with a spherulitic structure. Extensive protein denaturation and starch swelling may occur during processing when a temperature greater than 60°C is attained in drying. Extensive structural transformations take place in cooking. A fibrillar protein network which envelops gelatinized starch is the typical structure observed in cooked durum wheat spaghetti. Whereas, in soft wheat products, there is a less extensive protein framework with more diffuse starch particles.

Pasta cooking quality is determined by a physical competition between protein coagulation into a continuous network (I) and starch swelling with spherulite scattering (II) during cooking. If the former (I) prevails, starch particles are trapped in the network alveoli promoting firmness in cooked pasta; Whereas, if the latter (II) prevails, the protein coagulates in discrete masses lacking a continuous framework and pasta will show softness and usually stickiness. High temperature-low moisture (HT-LM) drying partially overcomes this competition by producing a coagulated protein framework in dry pasta without starch swelling.

HT-LM treatment induces protein-starch interactions and conformational changes in the starch granule fine structure during cooking. Linear and branched chain-like fibrils appear in the core of the granules and particle groupings in the outer area. The better understanding of the role of controlled starch modification which optimizes pasta processing permits better use of non-conventional raw materials in pasta preparation.

Introduction

The world-wide popularity of pasta and its increasing consumption are stimulating the development of technologies to obtain new products which sometimes show surprising cooking qualities, considering the characteristics of the raw materials. Indeed, phenomena involved in the processing and cooking of this food are not completely understood and, since our country is an important producer of pasta-making equipment, we feel the need of studying them. The objective of our investigations was to try and further understand these phenomena in order to optimize pasta production equipment and drying cycles.

Basic research on pasta, as well as on other cereal or starch based foods, requires consideration of the product’s water distribution, changes in the internal fine structure of starch granules and interactions in the protein matrix. All of these factors may be studied with high resolution electron microscopy (EM) techniques. In 1975 [69], we started to use the systematic control of the ultrastructure by freeze-fracturing (FF) technique in the pasta research. This has been recently recognized as a promising method to study cereal product structures [34]. A high water content (e.g. cooked pasta, 50-70%) may have disruptive effects in the preparation of samples [14], an EM technique inducing minimum damage is required, so we seldom use the better known freeze-etching (FE) technique [14, 16, 43]. We feel that etching might promote segregation phenomena inside the fine structures of highly hydrated components [4].

In this review, our observations of the ultrastructure of pasta made with different commercial raw materials (i.e. durum wheat semolina, soft wheat flour, rice starch and rice flour) are compared with published data, emphasizing implications of the results to consumer acceptability of the products (e.g. cooked pasta firmness and stickiness) as well as the development of new ingredient and processing technologies.

All the raw samples were soaked in a water-glycerol solution prior to FF. This step yields a detailed view (cross-fracture) of component features otherwise impossible to see in these limited moisture systems ( < 15% water). Moreover, for dry pasta soaking in water-glycerol is necessary in order to produce a cross rather than a surface-fracture, and not to break the knife. Cooked samples are usually not soaked with a cryoprotectant (see Appendix). To better understand pasta ultrastructure as shown by FF, it is necessary to consider the ultrastructure of the isolated starch and gluten, before and after heat treatment.

Key Words: Wheat flour, rice flour, heat starch modification, protein coagulation, pasta, cereal foods, drying conditions, freeze-fracturing, cooking pasta quality.
Starch

Washed starch extracted from commercial wheat flour according to Banks and Greenwood [6] and freeze-fractured reveals a compact spherulitic texture in the granules (Fig. 1a) [69, 71] in agreement with what Fretzdorff et al. report by FF [34] and many authors observed by FE [1, 8, 13, 16, 41, 43, 50, 63]. The same texture is shown in rice starch (Fig. 1b). No apparent swelling is promoted by washing, and the water-glycerol soaking does not cause visible modifications, as stated by Leonard and Sterling [50]. The diameters of the granules range from 1 to more than 20 µm [37, 39, 46]. We often found larger sizes in common soft wheat flours (i.e., without considering the hard wheat varieties) compared to durum wheat semolina, as recently confirmed by Scanning Electron Microscopy (SEM) [60]. Assuming that granule size influences its gelatinization temperature [7, 20, 37], a statistical evaluation of these differences may be useful for pasta studies, bearing in mind that cooking qualities are usually better in durum wheat than in soft wheat pasta. The granule spherulites are 5-20 nm in diameter. Mühlethaler [63] hypothesized that the range in sizes could be due to a specific arrangement of amylose and amylopeptin molecules in the spherulites. Several structural changes may take place when starch granules are heated under different time-temperature-moisture conditions: 1) progressive swelling of granules which promotes a size increase of the spherulites; 2) shape changes; 3) loss of ordered structure [gelatinization, 22, 37]; 4) collapse and 5) solubilization and reorientation of polymers [7, 47].

FF is a suitable method to follow these hydration and transition states in the granules. When examined after freeze-fracture, heat-modified rice starch granules appear swollen with a high degree of dispersion of the native spherulites still showing evidence of their polygonal type shape (Fig. 2a). Under more intense heating, wheat starch granules absorb more water, gelatinize and lose the distinctive structure of the granules (Fig. 2b). In high temperature-low moisture treated wheat pasta, the granules may exhibit, after cooking, linear and branched chains in the core and subunit groupings in outer area (Fig. 2c). A total dispersion of the spherulites is observed in solubilized rice starch (Fig. 2d). A chain-like fibril pattern is seen in retrograded starch (Fig. 2e). Recently, the fibrous features of retrograded starch have been found by FF in bread crumb [34] and starch swelling was examined by FE during cake-batter heating [16, 22, 43]. Heated tapioca starch was reported to show a honeycomb-like network by FE [2], but we were not able to find similar structures in wheat or rice starch. Since a pattern of regular hexagonal arrangements can be obtained by FE of a dilute (5%) glycerol solution [4], one might assume that some segregation phenomena are involved.

With SEM, the progressive structural changes in heated starch granules are easily observed [11, 15, 36, 39, 40]. SEM studies of starch ultrastructure deal mainly with changes in the surface morphology of the granules. Miller et al. [61] pointed out a fibrillar structure in a gelatinized starch exudate, probably formed by amylose chains [42]. However FF is more feasible than SEM to study modifications in starch fine structure due to its higher resolving power and lower risk of producing artifacts during sample preparation [14, 21]. Nevertheless, because of the lack of a three-dimensional image and the limited field of view in FF micrographs, we think that a comparison with SEM pictures may help to avoid misinterpretation in complex structural systems such as pasta.

Gluten

Gluten extracted from commercial durum wheat semolina, according to Laszity [48, 49], exhibits a loose protein framework after FF (Fig. 3a). After cooking in boiling tap water, the matrix looks more compact and contracted (Fig. 3b). These gluten modifications promoted by heating are also found in the protein matrix of cooked pasta. They play an important role in determining the ultrastructure and the cooking qualities of pasta.

The thinnest fibrils of gluten (chains of aligned protein subunits) have a thickness of 10 nm. This figure was also reported by Bernardin and Kasarda [10] for shadow-casted protein strands in wheat endosperm. Raw gluten exhibits a fibrillar shape when observed by thin-sectioning and SEM [18]. Cumming and Tung [19], using SEM, report a fibrillar protein network in commercial gluten only when it is kneaded with water and stretched. However these patterns probably present some preparational artifacts as reported by Dronzek et al. [32]. Dronzek et al. [32] reported that durum wheat gluten shows thicker fibrils than hard red spring wheat gluten.

Wheat flour

Freeze-fractured uns soaked semolina (Fig. 4a) does not show details of the components, whereas same soaked, but surface-fractured, sample (Fig. 4b) does not exhibit the interior structure of the starch granules as in soaked cross-fractured semolina.
Fig. 2. Heat-treated starch. a) dough of rice starch and water (4:1) heated at 90°C for 15 min, extruded and then dried at low temperature; rough polygonal outline of the granules is still visible. b) wheat starch cooked in boiling tap water for 15 min. c) wheat starch granules in cooked HT-LM spaghetti (see the text) with fibrils (sf) in the central area and particle aggregates (arrows) in the outer part. d) rice starch overcooked in boiling tap water. e) retrograded starch in cooked rice pasta.

Fig. 3. Durum wheat gluten. a) uncooked; b) cooked. Lipid inclusions (l), water (w), protein fibrils (p) and gelatinized starch material (sm) are shown. Note that the free protein particles (arrows) in a) are not visible after cooking.
Fig. 4. Durum wheat semolina cross-fractured without soaking (a) and, after soaking, surface-fractured (b) or cross-fractured (c). Starch granules (s), protein matrix (p), lipid inclusions (l), membrane residues (m) and water coat (arrows) around the starch granule.

(Fig. 4c). The statement of Chabot [14] concerning the advantage, in limited water systems, of a preliminary water exposure for producing high resolution micrographs is quite pertinent here. The starch granules, due to a surrounding water coat at least 30 nm wide, do not adhere to the protein matrix which looks uniformly dispersed in the soaking system. We cannot state if this water layer is due to starch shrinkage [73], protein shrinkage [74, 75], hydration of soluble proteins [8] or an osmophilic area. However, we always find it in flour, dough and dry pasta soaked in water-glycerol as well as in cooked pasta without preliminary soaking. Extensive starch-protein interactions appear only in cooked pasta manufactured under high temperature-low moisture conditions.

Taking into account the hydration effect on the protein matrix and that the water coat could not be seen by SEM, the ultrastructure of freeze-fractured semolina is in agreement with the SEM observations which showed the starch granules to be encased in an amorphous protein matrix [26, 28, 59] which, due to partial hydrations, begins to coat over the granules [45]. Protein fine structure does not differ systematically in semolina and soft wheat flour as observed by FF, even though characteristic and different features are noticed by SEM for these two raw materials [28, 60]. Moreover, SEM micrographs of Moss et al. [62] performed on the soft wheat endosperm show that the continuity of the protein matrix may be related to the grain hardness.
Pasta Ultrastructure

Conventional pasta processing involves the mixing of semolina or flour with water (~30%), kneading, extrusion at 80-90 bar and drying cycle usually below 55°C for at least 20 h. Raw commercial durum wheat spaghetti shows (after FF) a structure similar to a semolina (Fig. 5a). There are more extensive interactions inside the protein matrix (protein-protein interactions), lacking however a texturation in a compact and continuous framework (Fig. 5b). The relatively low moisture content of pasta dough and the insufficient mixing during extrusion do not allow the complete development of a gluten network as takes place in bread dough [3, 28, 59]. Furthermore, the relatively mild drying conditions (time, temperature, spaghetti moisture) avoid extensive protein coagulation [25, 69] and visible starch granule modifications [17, 23], even though Lintas and D’Appolonia [51] measured changes in the carbohydrates during conventional processing of spaghetti. No starch-protein associations are observed, contrary to what is found in bread dough [33, 34] where the interactions are reportedly intensified by yeast action during fermentation.

Contrary to what we observed in conventionally dried pasta, Banasik et al. [5] noticed that the extrusion processing step promotes a partial loss of starch granule structure. Their results are probably related to different extrusion temperatures. High temperatures may easily lead to starch and protein modifications [54], considering the high pressure and relatively high moisture of pasta (30%) in extrusion processing.

Even if the extent of the interactions inside the protein matrix of dry pasta is related to the quality and quantity of gluten [5, 35], common soft wheat and durum wheat spaghetti show a similar pattern by FF (Fig. 5c). Therefore, it is usually impossible to predict the cooking quality on the basis of the conventional raw pasta ultrastructure. The same conclusions were reached by optical microscopy [54, 55].

When viewed with SEM, raw spaghetti exhibits a different feature between the inner and the outer part. The latter presents a smooth continuous enveloping protein film, the role of which in determining cooking pasta quality is not clear [26, 28]. In the inner part, an amorphous protein matrix coats the starch granules [26, 59]. Durum and soft wheat spaghetti exhibit similar structures [60].

Cooked commercial durum wheat spaghetti shows a compact fibrillar network of coagulated proteins that envelopes the gelatinized starch granules (Fig. 6a). There are no significant differences between the inner and the outer part of pasta cross-section provided that the spaghetti is completely cooked. In the FF micrographs of cooked spaghetti the protein is easily recognized because of finer granular structure and lipid inclusions. We can compare the pattern of Figure 6a with the SEM structure reported by Angold [3] in cross-sectioned cooked durum wheat spaghetti (Fig. 6b), keeping in mind the possible artifacts introduced by both preparation methods. In these two micrographs, it is clear that the starch particles are enmeshed in the protein network and consequently inside the spaghetti. In soft wheat spaghetti protein usually tends to coagulate into discrete masses (Figs. 6c, 6d). The starch spherulite distribution, due to the discontinuity of the protein matrix, is more diffuse and therefore, the granules are easily removed from the network [69, 71, 72]. Many authors [5, 26, 35, 54] correlate the quality
and quantity of this network to the physical properties of the cooked pasta. These properties may also be affected by the starch-protein ratio at the surface of the spaghetti [78] and by the protein film coating the spaghetti surface which cannot be observed by FF.

On the basis of our EM observations we have concluded that cooking conventionally dried pasta induces two opposite phenomena: complete interaction of coagulating proteins and starch swelling and gelatinization. Since heat denaturation of gluten [80] and wheat starch modifications [23, 27] take place at approximately the same cooking conditions (moisture and temperature), both contribute to pasta quality. In the interspaces between the granules, protein coagulation and interaction leads to the formation of a continuous and strengthened network which traps the starch while the latter, by swelling and gelatinizing, occludes these interspaces. Therefore, it seems reasonable to assume that a physical competition exists between these behaviours. The faster the starch swells and spherulites disperse, the slower the rate of protein interaction and weaker the protein network inside the spaghetti. If the interaction of the coagulating proteins is more rapid than the starch swelling and gelatinization and the protein network is strong and elastic enough to prevent breakages [5, 32, 58], the cooked pasta will be firm. In the opposite case, the pasta will be soft and usually sticky. Assuming that this is true, the factors which can improve conventional pasta cooking quality are: high quality and quantity of gluten [15, 24, 26, 32, 35, 57, 58, 69, 79]; its uniform distribution in the granule interspaces [62]; no random heat denatured proteins in dry pasta [35, 54, 55] but good coagulability of wheat proteins during cooking [29] and the addition of soluble proteins with a low coagulation temperature [35, 69]. The main detrimental factor is a low starch gelatinization temperature which can be related to the type of starch [27, 40, 46], the size of granules [the larger the size, the lower the gelatinization temperature – 7, 20, 37], and the water availability [23]. Dry pasta is a very limited water system. Therefore, when cooking begins a competition for water is established between the starch and protein. The less protein surrounding the granules, the faster the rate of starch swelling and gelatinization [23, 38, 56]. Furthermore, we can assume that this competition is more critical in the peripheral part of the spaghetti where the hydration of the components takes place quickly at the beginning of the cooking.

On the basis of the above discussion, we better understand the relationships that exist between raw material characteristics and pasta cooking quality. The competition hypothesis is not only supported by the experimental data of the previously cited papers, but is corroborated by the findings that pasta prepared from the same gluten mixed with wheat starches exhibiting slightly different gelatinization temperatures show

Fig. 7. Drying cycles performed during the processing of experimental spaghetti. a) conventional conditions; b), c), d), high temperature treatments at different spaghetti moisture.
Pasta Ultrastructure

![Graph](image)

**Fig. 8.** Effect of pasta drying conditions on protein solubility in dilute acetic acid solution (0.1% v/v). a) dried pasta (see Fig. 7); b) same as a), treated at 135°C for 15 min in a dry atmosphere. Significantly different cooking qualities [20]. Relationships between the type of starch and spaghetti cooking properties were also reported by Dexter and Matsuo [27]. What we have postulated is confirmed by the following ultrastructural studies on high temperature dried pasta.

**Pasta dried with high temperature (HT) processing**

Some producers of pasta-making equipment have recently introduced drying cycles involving controlled high temperature treatment (＞60°C – HT processing). These technologies, largely used in Europe and introduced to reduce drying times [66, 67] and microbial contamination [52, 53, 64, 67], may sometimes improve pasta cooking qualities [12]. In order to understand this phenomena, we have studied the ultrastructures of HT spaghetti produced with common soft wheat under appropriate experimental conditions (see Appendix). We report here only the experimental data regarding common soft wheat spaghetti because its ultrastructure clearly demonstrates the phenomena involved in HT processing. HT-HM (high temperature dried pasta with high moisture content), HT-MM (medium moisture) and HT-LM (low moisture) indicate the HT experimental drying conditions, while LT (low temperature) indicates conventional processing. All the drying cycle diagrams are shown in Figure 7.

HT processing reduces the protein solubility as spaghetti moisture increases (Fig. 8). These results agree with what has been reported by other authors [30, 31] and confirm the relationship between heat sensitivity of vegetable proteins and moisture content [80]. HT-LM pasta exhibits the best quality after cooking, while HT-HM is worse than the control (LT) (Table 1). HT-HM raw pasta exhibits, after soaking, a partial swelling of the starch granules and a discontinuous network of coagulated protein (Fig. 9a). The starch swelling promoted by HT-HM treatment prevents the coagulating proteins from interacting and forming a continuous network and due to the extent of stretching, produces breakage provided the soft wheat has low gluten quality and quantity. After cooking, the protein network shows further breakdown (Fig. 9b) because of the stresses induced by the pasta volume increase. The resultant pasta quality is poor compared to the control (LT) (Table 1). In Figure 9b, some segregation between the swollen starch granules and protein matrix can be observed. In our opinion, this must be related to the free water content in this area and confirms that no starch-protein interactions have formed.

**Table 1:** Cooking test on soft wheat spaghetti a)

<table>
<thead>
<tr>
<th>Cooking time</th>
<th>Cooking weight (g)</th>
<th>Cooking loss (%)</th>
<th>Spaghetti quality</th>
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<tbody>
<tr>
<td><strong>A</strong></td>
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<td></td>
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<tr>
<td>LT</td>
<td>10</td>
<td>300</td>
<td>6.36</td>
</tr>
<tr>
<td>HT - HM</td>
<td>10</td>
<td>313</td>
<td>6.59</td>
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<tr>
<td>HT - MM</td>
<td>11</td>
<td>295</td>
<td>6.28</td>
</tr>
<tr>
<td>HT - LM</td>
<td>11</td>
<td>290</td>
<td>5.73</td>
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<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LT</td>
<td>15</td>
<td>280</td>
<td>5.62</td>
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<tr>
<td>HT - HM</td>
<td>15</td>
<td>291</td>
<td>6.48</td>
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<tr>
<td>HT - MM</td>
<td>16</td>
<td>280</td>
<td>5.58</td>
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<tr>
<td>HT - LM</td>
<td>16</td>
<td>278</td>
<td>5.58</td>
</tr>
</tbody>
</table>

**A:** Spaghetti dried under experimental conditions (see Fig. 7)

**B:** Dried as A and then treated at 135°C for 15 min in dry conditions.

a) Cooking is carried out on 100 g dry spaghetti in 1000 ml of tap water. Spaghetti quality is evaluated by a subjective procedure.

![Image](image)
Fig. 10. HT-LM spaghetti produced with soft wheat flour. a), b), uncooked. Unswollen starch (ug) are surrounded by a continuous protein network (pn); uncoagulated protein (p) in hydration water (w) are also seen. c) cooked. The starch material is arranged into fibrils (sf) in the core of the granule and in small regular aggregates (sa) in the outer part. The protein network (pn) shows mainly fibrils (arrows).

Fig. 11. Diagrammatic view of changes which occur in the FF ultrastructure of dry and cooked pasta, regarding protein coagulation, starch swelling and its particle scattering. Empty circles represent starch particles and black points proteins. Upper Figures (soaked dry pasta): a) (LT), b) (HT-LM), c) (HT-HM). Lower Figures (cooked pasta): d) (soft and usually sticky), e) (firm). Transition from a) to d) implies use of low quality raw material; a) to c) high quality raw material; b) is rather independent of raw material quality, while state c) is related to poor quality. No attempt is made here to show conformational changes of the starch fine structure (see micrographs).

Fig. 12. LT conventional spaghetti after heat treatment (135°C) under dry conditions. a) uncooked; b) cooked. Symbols are the same as Figures 9 and 10. Arrows indicate starch-protein interactions.
During HT-LM processing starch swelling does not take place due to the low spaghetti moisture (≈ 14%) at the high temperature step (Fig. 7d) and continuous coagulated protein fibrils envelope the granules as shown in Figure 10a. The water layers surrounding the starch granules could be due to a swelling of the dry pasta during specimen preparation which promotes the hydration of uncoagulated proteins (Fig. 10b). During cooking the magnitude of the effect of starch swelling and gelatinization on cooking quality will be reduced since a continuous protein network is already formed in the raw pasta (Fig. 10c). This is particularly true for pastamade from poor quality raw materials. The competition phenomena that takes place during LT and HT spaghetti processing and cooking are diagrammatically summarized in Figure 11. If the raw material is of good quality (e.g., good durum wheat semolina), no large differences in cooking qualities may be expected from HT-LM or HT-HM technology, due to the strength of the protein network compared to the negative effect of starch swelling (unreported data). If the raw material is of poor quality, these differences are highly significant. An enhancing effect of the HT drying treatment has been reported by other authors [30, 31, 53, 81], but they do not give any definite explanations for this phenomenon.

Cooked pasta quality can also be related to the characteristic ultrastructure of starch in cooked HT-LM spaghetti (Fig. 10c) [72]. These patterns can be obtained experimentally by treating raw LT pasta with high temperature (135°C) in a dry atmosphere. Spaghetti processed in this manner always exhibits an improvement in cooking qualities [Table 18], due to an increased amount of protein coagulation in drying (Fig. 8b) without starch swelling (Fig. 12a) and the changes in starch ultrastructure induced by cooking. Protein-starch interactions in the outer part and linear or branched fibrils in the central area of the granules are clearly seen (Fig. 12b). These modified starch patterns can be compared with the micrographs of Bechtel et al. [9] and Pomeranz [68] obtained from thin-sectioned bread after oven spring, but in our freeze-fractured cooked pasta, protein strands are interwoven with starch only in the outer part of the gelatinized granule (Fig. 12b). We can interpret the starch features shown in Figures 2c, 10c and 12b according to the statements of Sterling [76] who reports that swelling and gelatinization begin in the interior of the starch granule while the outer regions remain birefringent; during these physical changes, radial contraction and tangential expansion of branched and unbranched components take place [77].

**Pasta from rice starch or rice flour**

High quality pasta can be obtained from starch alone by taking into account the enhancing effect of the heat induced starch modifications. An enlightening example is the “Bee-Hoon”, an oriental vermicelli produced from starch of certain rice varieties. Its honeycomb-like structure both in the raw and cooked state (Figs. 13a, 13b) is due to special processing techniques that use high temperatures to effectively gelatinize the starch which readily retrogrades upon cooling [72]. These physical changes yield product with excellent cooking qualities.

On the basis of these and previously reported observations, it is possible to make good pasta starting from raw materials with low protein quality such as flour from broken rice. This
technology is described elsewhere [65]. The ultrastructure of raw pasta prepared with this technology is shown in Figure 13c. The extent of the starch strand network after cooking (Fig. 13d) can be related to pasta quality (its lack implies complete stickiness) and to starch varieties. Protein material interwoven with the starch strands is also observed in these kind of pasta. These modifications, which promote an extensive reticular texture where most of the starch material is involved, can often explain cooking qualities of pasta from unconventional raw materials [27, 65].

Conclusions
Pasta proves to be an interesting limited water-starch-protein system where starch/protein competition for water, conformational changes and mutual interactions take place during processing and cooking. The understanding of these phenomena, which may parallel that of other cereal products, can be enhanced by the study of pasta fine structure. In this type of study, freeze-fracturing proves to be one of the best techniques for observing the mild heat induced modifications of starch ultrastructure in low water systems. These changes are not easily studied with other EM or chemical-physical methods. Freeze-fracture may be a powerful tool for scientists engaged in the study of foods where starch and protein modifications play a functional role in the product's texture and consumer acceptability. In addition, freeze-fracturing micrographs, especially if compared with other EM technique images, may be beneficial in aiding technologists to optimize pasta production equipment and processes. Our research efforts have followed this direction.

Appendix
Freeze-fracturing specimen preparation
Before freezing, starch, gluten, flour and semolina are soaked in a 30% glycerol-water solution at room temperature for 10 min; dry pasta is soaked for 12 h. Cooked products are usually freeze-fractured without soaking due to their relative low free water content. When the water absorption in cooking was high, a soaking in a 10% glycerol-water solution was sometimes performed before freezing. In this case no substantial changes in the fine structures were observed. Furthermore, no modifications in the FF pattern are seen if pasta is cooked directly in a 10% glycerol-water solution (unreported data).

The FF conditions we used are those suggested by Buchheim for dairy products (W. Buchheim, 1973, personal communication). Small plugs of sample (~ 0.5 mm³) are placed onto a gold specimen holder with a drop of pure glycerol and frozen in super-cooled liquid nitrogen. This freezing medium works a little better and is more practical than Freon to prevent the Ledenfrost effect [70]. The sample is transferred into a Balzers (BAF 301) FF unit where a vacuum of at least 2 x 10⁻⁶ torr is reached. After defrosting at ~95°C for 15 min (or more), the fracture is carried out at -104 to -10°C to produce a cross fracture. Shadowing with P₄C (film thickness ~ 20 Å) and with C (film thickness ~ 250 Å) is performed immediately following the fracturing. During shadowing, replica thickness is controlled with a quartz crystal, thin film monitor. The replica is kept in 70% sulfuric acid followed by 30% sulfuric acid for at least 48 h, then washed in distilled water, acetone, and twice in distilled and double distilled water. The replica is placed onto a copper grid and observed in a Philips EM 201 electron microscope operating either at 40 or 60 kV.

Experimental spaghetti production and analytical tests
We used a commercial common soft wheat flour from the Italian market (protein 10.1%, ash 0.6% on d.b.). No hard varieties are present in this kind of raw material which always show poor characteristics for pasta making.

Spaghetti was prepared by mixing the flour with 30% water and kneading under vacuum for 15 min in a Micro C. Braibanti Press, working capacity 100 kg/h with the discontinuous procedure. The extrusion was performed in a cylinder with a teflon lined head (hole diameters 1.85 mm) at 40°C, pressure 80 atm. The experimental drying cell has an automatic system for ventilation and resting times. Air flow (3 m/sec) was parallel to the spaghetti. Detailed drying cycle diagrams are reported in Figure 7: T represents the air temperature in the drying cell; ΔT is the difference between the dry and the wet bulb temperatures (wet bulb depression). The relative humidity in the drying cell can be readily calculated on the basis of T and ΔT values by using psychrometric charts (e.g. Mollier diagram, or Carrier diagram).

Protein solubility in dilute acetic acid (0.1% v/v) is determined according to Jones et al. [44]; cooked pasta weight according to Dexter et al. [30]; material loss in cooking water and pasta organoleptic properties according to Pagani et al. [65]. Each spaghetti was cooked at its optimal cooking time, by controlling the disappearance of the central ungelatinized core.

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The authors are grateful to Dr. G. Dalbon (head of Research Centre of Braibanti S.p.A., Milan-Italy) for his technological cooperation and for helpful discussion on related aspects. Thanks are due to Prof. G. Volonterio, of this Institute, for his assistance regarding FF and EM techniques. Skillful assistance for preparation of figures by Dr. L. Pellegrino and Miss A. Mascaretti is gratefully acknowledged. We are indebted to Dr. K.E. Angold (The Lord Rank Research Centre, High Wycombe-U.K.) for his kind permission to publish Figure 6b.

References


Discussion with Reviewers

R. Moss: Could the authors provide some information of the humidity profiles used in the drying processes referred to in Figure 7? Presumably control of humidity is as important in HT drying as it is in LT drying?

Authors: The humidity profiles in the drying cell can be immediately calculated on the basis of the values of ΔT and T reported in the drying diagrams. These profiles are important both in LT and HT drying; however, for HT processings the moisture content of spaghetti at which the HT treatment is performed is critical.

E.A. Davis: Why do you not consider “defrosting” at -95°C etching? When we etch, we do this at -105°C for 2 min. Therefore etching or sublimation should be taking place during the 15 min of “defrosting”?

Authors: Since the defrosting step is performed before fracturing, it cannot influence the ultrastructures of the replica, as in freeze-etching. For this reason it is better to indicate this step as “defrosting” than as “etching”, even if a water sublimation takes place.

E.A. Davis: The gluten material shown in Figure 3 may have some freeze damage. What preparation techniques did refs. 48 and 49 use to prepare their samples prior to fracture?

Authors: Laszity’s works (refs. 48 and 49) do not refer to the freezing technique used prior to fracturing but to the method of gluten extraction from semolina. Since the ultrastructures shown in Figure 3 have been obtained according to our FF technique, we think that freeze damage in the sample is unlikely.

For additional discussion see page 98.
ENDOSPERM DEGRADATION IN BARLEY KERNELS THAT SYNTHESIZE \(\alpha\)-AMYLASE IN THE ABSENCE OF EMBRYOS AND EXOGENOUS GIBBERELLIC ACID

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Abstract

During germination at 16°C, whole seeds and distal half-seeds of Klages barley and two types of Clipper barley (Types A and B) were analyzed for \(\alpha\)-amylase. Structural changes in the endosperms of these seeds and half-seeds were examined by scanning electron microscopy. In Clipper B half-seeds, \(\alpha\)-amylase activity increased significantly, there was a detectable amount of starch granule hydrolysis and endosperm structure was markedly degraded. No starch hydrolysis and only trace amounts of \(\alpha\)-amylase activity and endosperm degradation were detected in Clipper A and Klages half-seeds. There was significant \(\alpha\)-amylase synthesis, starch hydrolysis and endosperm degradation in germinated whole seeds of all three barley cultivars. Changes were most pronounced in Clipper B. Starch degradation appeared to start in areas of the endosperm close to the embryo.

Introduction

During germination, the starchy endosperm of barley kernels is degraded by a complex series of enzymatic reactions (MacLeod et al. 1964, Palmer and Bathgate 1976). Endosperm cell walls, consisting largely of \(\beta\)-glucan, are degraded by \(\beta\)-glucanases and, probably other enzymes; the protein matrix is solubilized by proteases and starch granules are hydrolyzed by \(\alpha\)-amylase. These enzymes develop in the aleurone layer during germination and are secreted into the endosperm (MacLeod and Miller 1962, MacLeod et al. 1964). \(\alpha\)-Amylase has been the most widely studied of these hydrolytic enzymes. There is abundant evidence to show that aleurone layers treated with gibberellin acid synthesize and excrete \(\alpha\)-amylase (Briggs 1963, 1964; MacLeod et al. 1964, Warner 1964) and that gibberellin acid is synthesized in germinating barley embryos (Cohen and Paleg 1967, MacLeod and Palmer 1967, Radley 1967). These results support the attractive idea that, during germination, gibberellin acid moves from the embryo to the aleurone layer and there triggers the synthesis of hydrolytic enzymes such as \(\alpha\)-amylase. These enzymes are then excreted into the endosperm (Palmer and Bathgate 1976).

There is also compelling evidence that some hydrolytic enzymes originate in the embryo and move into the endosperm via the scutellar epithelium (Briggs 1964, Gibbons 1979, MacGregor 1980, Okamoto et al. 1980).

By manipulating environmental conditions during grain growth, Nicholls (1982, 1983) has produced two types of barley kernel, designated A and B, that differed markedly in their response to gibberellin acid. Distal half-seeds of type A, like those of normal barley, produced \(\alpha\)-amylase only when incubated in the presence of gibberellin acid. Type B half-seeds, however, produced copious amounts of \(\alpha\)-amylase after incubation in water. These kernels did not appear to contain above normal amounts of gibberellin-like material. Therefore, the growing conditions used appear to have pre-sensitized the aleurone layers of type B kernels so that they no longer required stimulation by gibberellin acid before producing \(\alpha\)-amylase.

This paper describes part of a project that was initiated to examine the physiology of these...
two types of barley kernel in more detail. Answers to two main questions were sought. Do type B half-seeds, incubated in the absence of gibberellic acid, readily produce endosperm cell wall and protein degrading enzymes in addition to α-amylase? Do hydrolytic enzymes from the aleurone layer of type B kernels play a more important role in endosperm degradation than do similar enzymes in normal barley? To answer these questions a scanning electron microscope (SEM) study was made of endosperms of germinated whole and half-seeds of types A and B Clipper barley and of normal Klages barley.

Materials and Methods

Barley Samples

The two-rowed barley cultivars, Klages and Clipper were used in this study. Klages was grown in test plots in Brandon, Manitoba in 1976; Clipper A was grown in South Australia in 1979/80 and Clipper B was grown under controlled environmental conditions as described elsewhere (Nicholls 1983).

Barley kernels were dehulled by steeping in 50% (v/v) sulphuric acid for 3 to 4 hrs. (Coombe et al. 1967) followed by thorough rinsing with sterile, distilled water. Some kernels from each sample were cut transversely and the distal halves were placed in moist sand with the cut faces uppermost. Two μL of calcium chloride solution (0.01 M) were added to each half-seed to satisfy the calcium requirement of α-amylase. Whole kernels were placed, crease down, in moist sand. All kernels and half-kernels were maintained in a humid atmosphere at 16°C and samples were removed at intervals of 24 hrs. and frozen at -20°C until analyzed. Samples were extracted and assayed for α-amylase activity as described previously (MacGregor 1976).

Kernels and half-kernels were freeze-dried at -20°C prior to scanning electron microscopy examination. Whole kernels were cracked open longitudinally through the crease and these longitudinal slices, as well as distal half-kernels, were fixed to microscope stubs with Dotite silver paint (Fujikura Kasei Co., Ltd., Tokyo) and coated with gold. Samples were analyzed on a JEOL JSM scanning electron microscope at an accelerating voltage of 10 kV. Photomicrographs were taken on Plus-X Pan Kodak film.

Results and Discussion

Klages is a good quality malting barley and germinates quickly with rapid production of hydrolytic enzymes such as α-amylase (MacGregor 1978). This is illustrated in Fig. 1 where the α-amylase content of whole kernels reached almost 4,000 units/kernel after 5 days of germination. Only small but detectable amounts of activity were present in distal half-seeds after the same period of germination. By contrast, α-amylase development in Clipper A was significantly slower and was less than 2,000 units/kernel after 5 days. This slower rate of production of α-amylase is more typical of 2-rowed malting barley cultivars (MacGregor 1978).

α-Amylase levels in half seeds were also very low, as would be expected.

![Fig. 1 Synthesis of α-amylase in whole seeds and distal half-seeds of Klages, Clipper A and Clipper B incubated on moist sand at 16°C. ○ whole seeds; ● half-seeds.](image-url)
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Fig. 2 Micrograph of distal half-seeds of a, sound Klages; b, 120 hr incubated Klages; c, sound Clipper A; d, 120 hr incubated Clipper A (section immediately under the aleurone layer). A - aleurone layer; W - endosperm cell walls; D - disjointed layer; E - area immediately under the aleurone; bar is 50 μm.

microscopy. Although the whole cut face was looked at, special attention was paid to endosperm areas immediately under the aleurone on the dorsal side of the kernel. It is from this region of the aleurone that hydrolytic enzymes might be expected to appear (Palmer 1975). In Klages barley the aleurone layer is 2 to 3 cells thick (Fig. 2a) and the endosperm consists of cells packed with large and small starch granules embedded in a protein matrix. All of these features have been discussed in detail elsewhere (Pomeranz and Sachs 1972, Palmer 1980, MacGregor 1980).

Some changes were observed in the physical structure of Klages half-seeds during incubation. After 24 hrs. the aleurone cells were wrinkled and misshapen but there was no apparent degradation of endosperm cell walls or protein matrix. Portions of the sub-aleurone layer became distorted during incubation and this was most pronounced in the 5-day samples (Fig. 2b). In these areas there was a layer (D) of disjointed fragments of cell wall material between the aleurone layer and the starchy endosperm. In many half-seeds examined parts of the aleurone layer appeared to have been torn away from the endosperm. This may have taken place during drying of the kernel after incubation. Adhesion between aleurone and endosperm tissues certainly appeared to be weakened during incubation. This may explain the disjointed layer visible in Fig. 2b but there may have been some dissolution or hydrolysis of protein and cell wall material. Because starch granules were never found in these areas these distorted fragments may represent the stretched remnants of cementing material between endosperm and aleurone tissues. Below this area stretched a band of cells in which large and small starch granules are clearly visible. Much of the protein matrix had been removed from this area and the cell walls (W) were much thinner. This more open texture of the endosperm may have been caused by simple dissolution of structural components rather than by enzymic hydrolysis but more detailed studies are required to resolve this problem. There was no evidence of starch degradation.

Distal half-seeds of Clipper A appeared to behave similarly during incubation (Fig. 2c and d). The two to three cell thick aleurone layer quickly became distorted but changes occurred slowly in the endosperm. Again, aleurone and
endosperm tissues appeared to be torn apart and areas of starch granules, free of contaminating protein, were visible at the outer edge of the endosperm (G) after 5 days of incubation (Fig. 2d). These clean areas strongly suggest that some enzymic hydrolysis of protein and cell wall material had occurred.

Below this area some protein dissolution had occurred and cell walls (W) appeared to be thinner in the 5-day sample as was the case for Klages. No evidence of starch degradation was found in any of the samples examined.

The aleurone layer (A) of Clipper B was sometimes 3 to 4 cells thick (Fig. 3a) instead of the more normal thickness of 2 to 3 cells found in Klages and Clipper A (Fig. 2). More studies on a number of barley cultivars grown under the same conditions as Clipper B would be required to determine if the particular growing conditions used caused formation of the extra aleurone cells. No published information is available on the possible variation of aleurone layer thickness with either variety or growing conditions. The structure of Clipper B endosperm appeared to be similar to that of the other two barley samples studied. After 24 hrs. of incubation many aleurone cell surfaces were wrinkled and roughened but very little change in endosperm structure was apparent. Marked changes were readily visible after 48 hrs., however (Fig. 3b). Immediately under the aleurone layer (Fig. 3b), cell walls and protein were extensively degraded and areas of free starch granules were present. These degraded areas extended only 100-200 μm into the endosperm and so they represented only a small portion of the total endosperm. There was still no evidence of starch degradation. These changes were more extensive after 72 hrs. of incubation but only after 96 hrs. was pitting of starch granules sometimes detected. By this stage a layer of starch granules virtually free of protein and cell wall material extended around the outside of the endosperm (Fig. 3c). There was little apparent increase in endosperm degradation after 120 hrs. but there was increased pitting of starch granules (Fig. 3d).

This pitting represents radial channels made by α-amylase on large starch granules as the enzyme moves into, and preferentially degrades, the central portion of these granules (Gallant et al. 1972). Pitting, therefore, does not represent the full extent of starch granule degradation nor,
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These results show that aleurone cells of Clipper B are able to produce not only $\alpha$-amylase but also protein and cell wall degrading enzymes in the absence of exogenous gibberellic acid. Therefore, aleurone cells of this particular barley sample have been pre-triggered so that when hydrated they produce and secrete the hydrolytic enzymes required to mobilize endosperm reserves.

Longitudinal sections of germinated whole kernels were also examined by SEM. Areas of particular interest are indicated on the diagram shown in Fig. 4. Results from previous work (MacGregor 1980, Gibbons 1980) suggested that endosperm degradation in germinating kernels started at the embryo-endosperm junction and moved out into the endosperm from there. Therefore, although the whole longitudinal section was scanned, attention was focused along the embryo-endosperm junction and all micrographs in Figs. 5-7 were taken in areas shown as A, B or C, representing the dorsal, middle and ventral areas of this junction, respectively.

Fig. 4 Diagram of longitudinal section through the crease of a barley kernel. All micrographs shown in Figs. 5-7 were taken along the embryo-endosperm junction at areas A (dorsal edge), B (mid-point) or C (ventral crease edge).

Fig. 5 Micrograph of longitudinal section of Clipper A kernels. a, sound (area B); b, 72 hr germinated (area C); c, 96 hr germinated (area B); d, 120 hr germinated (area A). Sc - scutellum; SE - scutellum epithelium; C - layer of crushed cells; S - starch granules; W - endosperm cell walls; G - area immediately under the aleurone; * - endosperm-embryo junction; arrows indicate pitted starch granules; bar is 50 $\mu$m.
No significant differences were found in the endosperm structures of sound samples of the three types of barley kernel studied. Micrographs of area B of each type of kernel are shown in Figs. 5a, 6a and 7a. The single layer of cells of the scutellar epithelium (SE) and the layers of crushed cells (C) adjacent to the endosperm are clearly visible. Portions of cell walls (W) and intact starch granules, both large and small, can be seen embedded in a protein matrix. These structural features of mature barley endosperms are similar to those discussed previously (Pomeranz and Sachs 1972, Pomeranz 1974, Palmer and Bathgate 1976). Endosperm breakdown in Clipper A occurred very slowly and the only change detected after 48 hrs. of germination was slight degradation of cell walls and protein matrix at the ventral edge of the embryo-endosperm junction. After 72 hrs. extensive degradation of endosperm structure had occurred in area C and some pitting of starch granules was visible (Fig. 5b). Twenty-four hours later there was some endosperm degradation mid-way along the endosperm-embryo junction (Fig. 5c), but this did not extend to the dorsal edge. There was little starch pitting in area B and none was detected in area A. After 120 hrs. of germination, extensive endosperm breakdown and starch pitting were detected in areas B and C and significant structural, but no starch degradation, was found in area A (Fig. 5d). Obviously a gradient of
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degradation existed in a narrow band along the embryo-endosperm junction from the ventral to the dorsal edge. Similar changes in germinating barley kernels have been described previously (MacGregor 1980).

Structural changes in endosperms of Clipper B kernels occurred much more rapidly. After only 24 hrs. of germination, endosperm degradation was detected in region C but no starch pitting was apparent. Twenty-four hours later, however, much of the cell wall and protein material had been degraded in region C and along the embryo-endosperm junction through area B (Fig. 6b). There were degraded starch granules throughout this area. Even at the dorsal edge some endosperm breakdown had occurred but starch hydrolysis had not yet started. After 72 hrs. a band of extensively degraded endosperm stretched all the way along the endosperm-embryo junction. Little intact protein or cell wall material remained in this area and starch granules were severely degraded (Fig. 6c). Close to the dorsal aleurone, however, only slight pitting of starch granules was detected. Endosperm degradation was even more extensive after 96 hrs. of germination, and by this stage very little endosperm structure was left at the dorsal edge. Large areas of clean starch granules with little contaminating protein or cell wall material were observed (Fig. 6d). This type of structure extended some distance along the dorsal edge of the seed immediately under the aleurone layer. The narrow band of severely degraded starch granules can be seen extending up to the dorsal edge (Fig. 6e). However, only limited starch degradation was detected under the aleurone layer, indicating that at this stage α-amylase was just beginning to move from the aleurone into the endosperm. It must be noted that these results do not preclude the presence of significant amounts of α-amylase in the aleurone layer. They indicate, merely, the start of α-amylase secretion from the aleurone into the endosperm. In the 5-day germinated sample much more starch degradation had taken place under the aleurone layer.

The rate of endosperm degradation in kernels of Klages barley was intermediate between those observed for the Clipper samples. After 48 hrs.

Fig. 7 Micrograph of longitudinal section of Klages kernels. a, sound (area B); b, 72 hr germinated (area B); c, 96 hr germinated (area B); d, 120 hr germinated (area A).
Sc - scutellum; SE - scutellar epithelium; C - layer of crushed cells; S - starch granules; W - endosperm cell walls; G - area immediately under the aleurone; * - endosperm-embryo junction; arrows indicate pitted starch granules; bar is 50 μm.
only limited cell wall and protein degradation area
only limited degradation was found and that confin ed to area C. Degradation increased rapidly during the ensuing 24 hrs. producing large areas of clean, damaged starch granules in area C and structural breakdown with some starch degradation in area B (Fig. 7b). At this stage only limited cell wall and protein degradation were detected at the dorsal end of the embryo-endosperm junction. After 96 hrs. extensive removal of protein and cell walls had occurred in a narrow band along the junction with severe starch pitting reaching area B (Fig. 7c). After a further 24 hrs., complete destruction of the endosperm had occurred along the whole embryo-endosperm junction and starch hydrolysis was detected at the dorsal edge (Fig. 7d). Most of the starch degradation appeared to emanate from the embryo but some degraded granules were found under the aleurone layer.

Results from these studies agree with the α-amylase profiles shown in Fig. 1. Only, incubated half-seeds of Clipper B showed significant levels of α-amylase activity and detectable amounts of starch degradation. In whole seeds the extent of starch degradation mirrored the α-amylase profiles. Degradation of endosperm structure always preceded starch hydrolysis, indicating that cell wall and protein degrading enzymes were active in endosperms before α-amylase. These enzymes are probably synthesized and secreted into the endosperm more quickly than is α-amylase (Gibbons et al 1980). However, more definitive studies are required to confirm this.

The high α-amylase levels observed in germinated half-seeds of Clipper B were accompanied by significant degradation of endosperm structure immediately beneath the aleurone layer. In these kernels, therefore, synthesis of the whole complement of endosperm degrading enzymes had been pre-triggered by the environmental conditions used during kernel growth.

Whole kernels of Clipper B showed increased rates of endosperm modification during germination. However, the pattern of modification did not appear to be different. Endosperm and starch degradation started at the embryo-endosperm junction and moved along the junction to the dorsal end as well as into the embryo. There did not appear to be a disproportionate amount of degradation under the aleurone, suggesting that in these kernels synthesis of hydrolytic enzymes had been stimulated in the embryo as well as in the aleurone. It should be remembered that in all samples examined endosperm degradation was limited to a relatively small area close to the embryo and aleurone. Even in 5-day germinated Clipper B much of the endosperm remained intact. Therefore, these studies relate only to initial stages of germination and endosperm modification.

Although endosperm degradation appeared to start, and was most severe, in areas close to the embryo during germination, it must not be assumed that the embryo rather than the aleurone is the major site of synthesis of hydrolytic enzymes. Synthesis of enzymes may be quite rapid in aleurone cells but active secretion into the endosperm may not take place immediately.

References

18. Palmer, G.H. The morphology and physiology


Discussion with Reviewers

R. Moss: In Fig. 2a there appears to be more intercellular cleavage of the endosperm cells (because more endosperm cell walls are visible) whereas in Fig. 2c the cleavage appears to be more intracellular (more aware where in Fig. 2c the cleavage appears to be degradation in Clipper B endosperm is more extensive than in the endosperms of Clipper A or Klages. If germination is allowed to proceed sufficiently long, surely more extensive degradation of the endosperm area is observed. Is the starch in the endosperm of Clipper B more rapidly degraded than in Clipper A and Klages as would be expected if a larger amount of α-amylase were present?

Authors: The suggestion that α-amylase synthesis may be inhibited by a feedback mechanism involving products of starch degradation refers to half-seeds only. In whole seeds these products do not accumulate because they are translocated to the embryo and metabolized. Starch degradation was detected only in half-seeds of Clipper B and not in Clipper A or Klages. This degradation was not extensive during the time period of the study and it is difficult to correlate the progress of degradation with changing levels of α-amylase in the half-seeds.

D.R. Lineback: Is there evidence for any particular type of attack of the germinating barley starch by α-amylase? In Fig. 6c it appears that many of the granules have been attacked around the "equator" of the granule. Large lenticular granules of wheat starch are normally attacked around the "equatorial groove" by α-amylases. Does this same type of attack occur in starch granules of germinating barley?

Authors: In general, α-amylase from malted barley preferentially attacks the equatorial region of large, lenticular starch granules from barley. The equatorial region of many of the starch granules shown in Figs. 6c and 6e have been significantly degraded. It is true that some α-amylase attack also takes place over the entire surface of particular granules (Figs. 6c and 3d). These, more uniformly pitted granules, appear to be rounder in shape and do not have a pronounced equatorial groove. Perhaps not all large starch granules from barley have the same physical structure. Much more research is required to determine the relationship between grain hardness and rate of endosperm modification.

R. Moss: Could the authors give more information on the manner in which they produced the surfaces examined by SEM? Were the "sound" seeds frozen before fracturing, or fractured at room temperature and at what moisture content? Moisture content can markedly influence the cleavage pattern (Moss et al., SEM III, 1980, 613) and a soft wheat variety can produce cleavage patterns similar to Fig. 2d, with a "clean" starch granule. Is it true that some α-amylase attack also takes place over the entire surface of particular granules (Figs. 6c and 3d)? These, more uniformly pitted granules, appear to be rounder in shape and do not have a pronounced equatorial groove.

Authors: A portion of the protein matrix and of endosperm cell walls in barley are water soluble. Partial dissolution of components of the adhesive material between aleurone and endosperm could have weakened this adhesion and caused the break shown in Fig. 2b. However, enzymic hydrolysis of structural components in this area is indeed a more likely explanation for the disjointed layer.

D.R. Lineback: Is the postulate of the inhibition of α-amylase synthesis by a feedback mechanism involving products of starch degradation in the endosperm of these seeds? Starch degradation in Clipper B endosperm is more extensive than in the endosperms of Clipper A or Klages. If germination is allowed to proceed sufficiently long, surely more extensive degradation of the endosperm area is observed. Is the starch in the endosperm of Clipper B more rapidly degraded than in Clipper A and Klages as would be expected if a larger amount of α-amylase were present?

Authors: The suggestion that α-amylase synthesis may be inhibited by a feedback mechanism involving products of starch degradation refers to half-seeds only. In whole seeds these products do not accumulate because they are translocated to the embryo and metabolized. Starch degradation was detected only in half-seeds of Clipper B and not in Clipper A or Klages. This degradation was not extensive during the time period of the study and it is difficult to correlate the progress of degradation with changing levels of α-amylase in the half-seeds.

W.J. Wolf: In reference to Fig. 2b, does it seem likely that simple dissolution of structural components would occur without prior hydrolysis? Cell walls and starch granules are not soluble in water hence it is difficult to see how they could have simply dissolved.

Authors: A portion of the protein matrix and of endosperm cell walls in barley are water soluble. Partial dissolution of components of the adhesive material between aleurone and endosperm could have weakened this adhesion and caused the break shown in Fig. 2b. However, enzymic hydrolysis of structural components in this area is indeed a more likely explanation for the disjointed layer.

H. Fuwa: During germination, does α-amylase from the aleurone layer of Type B kernels play a more dominant role in degradation of endosperm starch granules than does the same enzyme in normal barley?

Authors: The aleurone layer of Type B kernels appears to be more active than the aleurone layer.
of normal kernels. However, results shown in Fig. 6 strongly suggest that in Type B kernels, as in normal kernels, enzymes from the embryo are primarily responsible for starch degradation during initial stages of germination. Therefore, the embryo as well as the aleurone of these kernels has been activated so that aleurone \( \alpha \)-amylase is no more dominant in these kernels than in normal kernels.

Additional References

AN ALTERNATIVE TO CRITICAL POINT DRYING FOR PREPARING MEAT EMULSIONS FOR SCANNING ELECTRON MICROSCOPY

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Abstract

A rapid sample drying technique is described which is useful for the simultaneous preparation of large numbers of samples as an alternative to critical point drying. The cryofractured face of meat emulsions was visualized after applying this technique. The fine structure of lipids and proteins were found to be well preserved in comparison to other reports which used critical point dried meat emulsions. Lipid was readily discerned from the protein matrix by selective fixation of the components in duplicate samples. Stereo imaging was useful in enhancing the texture of the cryofractured surface and as an aid in differentiating the protein matrix from the fat component of meat emulsions.

Introduction

Meat emulsion systems as well as many other food systems have been examined with the Scanning Electron Microscope (SEM) in order to evaluate the complex structural components. Some major problems faced by many investigators, especially those doing comparative studies, have been the long periods of time required for sample preparation and difficulties in scaling-up procedures in order to process a large number of samples quickly. Several techniques for cryofracturing biological tissues have been reported (Humphreys et al., 1974, Nemanic, 1972, Boyde, 1974), some of which are very useful to food systems, while others have limited application. Preparation of meat emulsion samples requires freezing and cryofracturing prior to fixation if the surface components are to be exposed to the fixative solutions. In order to minimize ice crystal formation it is important to freeze any material for SEM very rapidly. This requires the use of a small sample size and/or replacement of sample water with a low surface tension solvent such as ethanol (Humphreys et al., 1974), acetone, ether, 1,2 epoxyp propane or amyl acetate (Boyde, 1976 and 1972). Problems associated with the use of these solvents include the potential to remove the lipids if the sample has not been osmium fixed. SEM samples must be frozen quickly utilizing an intermediate fluid which has a high boiling point and low freezing point. Commonly, fluids having relatively high boiling points and low freezing points include: Freons 12 and 22 (Boyde, 1972), isopentane (Theno and Schmidt, 1978), and acetone (Carroll and Lee, 1981). One of the drawbacks of critical point drying is that extensive dimensional changes occur during the process. Boyde et al. (1981) showed that drying from Freon 113 produced less shrinkage artifacts than critical point drying.

One drawback of SEM is the inability to clearly distinguish between lipid and protein. SEM results have been correlated with light microscopy and specialized staining techniques used as an aid in identifying lipid and protein components in serial sections of cooked meat emulsions (Ray et al., 1979). The globular appearance of the lipid component was often used as one characteristic to distinguish it from protein,
and this was confirmed by use of selective fat stains with light microscopy (Ray et al., 1977). Carroll and Lee (1981), using a combination of light microscopy studied the relationships between structure and thermal stability in beef emulsions.

Due to the difficulties in rapidly preparing large numbers of samples for comparative SEM studies, a faster procedure was developed that could accommodate a large number of samples using conventional laboratory equipment. This technique involves an initial quick freezing of small samples followed by cleavage fracture and then fixation. After ethanol-Freon 113 substitution, a desiccator drying technique is employed (Liepins and de Harven, 1978) which produces results similar to critical point drying. A distinct advantage of this technique is that it allows for the rapid preparation of a large number of samples simultaneously. In addition, using cryofractured meat emulsions and selectively preserving either the protein matrix alone or both the protein and lipid matrix together, one can readily distinguish the two components.

Materials and Methods

Two test groups of franks were prepared: 1) control, and 2) phosphate addition during processing. From each group three franks were selected, two sample slices were taken from each frank. 1.5mm x 1.5mm cubes were cut from each sample (4°C) with a chilled razor blade. Each cube was dipped into a 1% toluidine blue solution in order to facilitate identification of the fractured surface for x-ray microanalysis. Each sample was quenched in liquid Freon 12 (BP = -29°C, FP = -158°C). Once thoroughly chilled, the samples were immersed in liquid nitrogen. As shown in Figure 1, the frozen cubes were transferred to a multiwelled metal block submerged in liquid nitrogen and cryofractured by sharply striking a pre-cooled metal blade positioned over each cube. These samples were immediately removed from the liquid nitrogen and fixed in 3% glutaraldehyde (Colloidal graphite in isopropanol): SPI sputter coat was used to coat the samples with gold-palladium under a vacuum of 170 microns and an ionization current of 40 mA for 45 sec. The SEM microscope was an ISI IS-130 scanning electron microscope equipped with a LaB6 (lanthanum hexaboride) electron gun at 10 kV accelerating potential. Stereo micrographs were made with a tilt angle difference of 10 degrees.

The energy dispersive x-ray (EDX) samples were freeze-dried directly from liquid nitrogen, mounted onto stubs as described above and coated with a heavy layer of carbon in a Denton DV-502 vacuum evaporator. These samples were used to obtain the EDX spectra on a JEOL U-3 scanning electron microscope equipped with an ORTEC detector and multi-channel analyzer operated at an accelerating potential of 15 kV.

Results and Discussion

Examination of the cryofractured surface of frankfurters fixed in 3% glutaraldehyde reveals a noticeable absence of lipid material (Fig. 2a); although, the protein matrix is well preserved. The surface is pock-marked with empty voids, most of which once contained fat droplets that varied in size from 50 μm to several μm in diameter. A higher magnification of the denoted area shown in figure 2b reveals the fine structure of the protein matrix of the meat emulsion. Similar protein matrix structures have been observed by Thermo and Schmidt (1978) and Ray et al. (1979). Figure 3a depicts the cryofractured surface of frankfurters fixed in 3% glutaraldehyde and post-fixed in 1% OsO4 to preserve the lipid structure. Both the protein and lipid components are well preserved. Upon closer examination (Fig. 3b), it is evident that a large number of round fat particles are present which range in size from 50 μm to several μm in diameter for the finely dispersed small droplets. The fat globules are evenly distributed throughout the frankfurter matrix.

Stereo imaging is a very useful and informative technique for visualizing surface texture. Stereo imaging was used to further examine the cryofractured surface of glutaraldehyde fixed OsO4 post-fixed frankfurters (Fig. 4). As observed in figure 3, the protein matrix appears laden with well dispersed lipid globules. Some of the globules appear to have been drawn out from the exposed fracture face and appear as small, uniform, rounded droplets. This could be circumvented by fixing the samples initially in OsO4 prior to glutaraldehyde fixation as described by Carroll and Lee (1981). The protein matrix is seen to be very finely textured with some larger fat droplets, and a few voids can be seen where the fat droplets have been dislodged. Lipid composed predominantly of saturated fats is difficult to preserve because osmium tetroxide may not adequately fix them due to the absence of unsaturated bonds with which to react (Dawes, 1979). Also, the voids may be the result of air pockets (Carroll and Lee, 1981). When figure 4 is compared to figures 2 or 3, it is apparent that the stereo pair results in a more informative image of the frankfurter matrix.

Two groups of frankfurters were examined. Due to the difficulties in rapidly preparing large numbers of samples for comparative SEM studies, a faster procedure was developed that could accommodate a large number of samples using conventional laboratory equipment. This technique involves an initial quick freezing of small samples followed by cleavage fracture and then fixation. After ethanol-Freon 113 substitution, a desiccator drying technique is employed (Liepins and de Harven, 1978) which produces results similar to critical point drying. A distinct advantage of this technique is that it allows for the rapid preparation of a large number of samples simultaneously. In addition, using cryofractured meat emulsions and selectively preserving either the protein matrix alone or both the protein and lipid matrix together, one can readily distinguish the two components.

Materials and Methods

Two test groups of franks were prepared: 1) control, and 2) phosphate addition during processing. From each group three franks were selected, two sample slices were taken from each frank. 1.5 mm x 1.5 mm cubes were cut from each sample (4°C) with a chilled razor blade. Each cube was dipped into a 1% toluidine blue solution in order to facilitate identification of the fractured surface for x-ray microanalysis. Each sample was quenched in liquid Freon 12 (BP = -29°C, FP = -158°C). Once thoroughly chilled, the samples were immersed in liquid nitrogen. As shown in Figure 1, the frozen cubes were transferred to a multiwelled metal block submerged in liquid nitrogen and cryofractured by sharply striking a pre-cooled metal blade positioned over each cube. These samples were immediately removed from the liquid nitrogen and fixed in 3% glutaraldehyde (Colloidal graphite in isopropanol): SPI sputter coat was used to coat the samples with gold-palladium under a vacuum of 170 microns and an ionization current of 40 mA for 45 sec. The SEM microscope was an ISI IS-130 scanning electron microscope equipped with a LaB6 (lanthanum hexaboride) electron gun at 10 kV accelerating potential. Stereo micrographs were made with a tilt angle difference of 10 degrees.

The energy dispersive x-ray (EDX) samples were freeze-dried directly from liquid nitrogen, mounted onto stubs as described above and coated with a heavy layer of carbon in a Denton DV-502 vacuum evaporator. These samples were used to obtain the EDX spectra on a JEOL U-3 scanning electron microscope equipped with an ORTEC detector and multi-channel analyzer operated at an accelerating potential of 15 kV.

Results and Discussion

Examination of the cryofractured surface of frankfurters fixed in 3% glutaraldehyde reveals a noticeable absence of lipid material (Fig. 2a); although, the protein matrix is well preserved. The surface is pock-marked with empty voids, most of which once contained fat droplets that varied in size from 50 μm to several μm in diameter. A higher magnification of the denoted area shown in figure 2b reveals the fine structure of the protein matrix of the meat emulsion. Similar protein matrix structures have been observed by Thermo and Schmidt (1978) and Ray et al. (1979). Figure 3a depicts the cryofractured surface of frankfurters fixed in 3% glutaraldehyde and post-fixed in 1% OsO4 to preserve the lipid structure. Both the protein and lipid components are well preserved. Upon closer examination (Fig. 3b), it is evident that a large number of round fat particles are present which range in size from 50 μm to several μm in diameter for the finely dispersed small droplets. The fat globules are evenly distributed throughout the frankfurter matrix.

Stereo imaging is a very useful and informative technique for visualizing surface texture. Stereo imaging was used to further examine the cryofractured surface of glutaraldehyde fixed OsO4 post-fixed frankfurters (Fig. 4). As observed in figure 3, the protein matrix appears laden with well dispersed lipid globules. Some of the globules appear to have been drawn out from the exposed fracture face and appear as small, uniform, rounded droplets. This could be circumvented by fixing the samples initially in OsO4 prior to glutaraldehyde fixation as described by Carroll and Lee (1981). The protein matrix is seen to be very finely textured with some larger fat droplets, and a few voids can be seen where the fat droplets have been dislodged. Lipid composed predominantly of saturated fats is difficult to preserve because osmium tetroxide may not adequately fix them due to the absence of unsaturated bonds with which to react (Dawes, 1979). Also, the voids may be the result of air pockets (Carroll and Lee, 1981). When figure 4 is compared to figures 2 or 3, it is apparent that the stereo pair results in a more informative image of the frankfurter matrix.

Two groups of frankfurters were examined. Due to the difficulties in rapidly preparing large numbers of samples for comparative SEM studies, a faster procedure was developed that could accommodate a large number of samples using conventional laboratory equipment. This technique involves an initial quick freezing of small samples followed by cleavage fracture and then fixation. After ethanol-Freon 113 substitution, a desiccator drying technique is employed (Liepins and de Harven, 1978) which produces results similar to critical point drying. A distinct advantage of this technique is that it allows for the rapid preparation of a large number of samples simultaneously. In addition, using cryofractured meat emulsions and selectively preserving either the protein matrix alone or both the protein and lipid matrix together, one can readily distinguish the two components.

Materials and Methods

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The energy dispersive x-ray (EDX) samples were freeze-dried directly from liquid nitrogen, mounted onto stubs as described above and coated with a heavy layer of carbon in a Denton DV-502 vacuum evaporator. These samples were used to obtain the EDX spectra on a JEOL U-3 scanning electron microscope equipped with an ORTEC detector and multi-channel analyzer operated at an accelerating potential of 15 kV.
New Preparation Technique for SEM Samples

cryofractured and directly freeze dried without fixation, one set had an additional 0.5 percent phosphate added to the preblend. Energy dispersive x-ray microanalysis was performed on the fractured face of these samples. Qualitative x-ray profiles are shown in figure 5 illustrating the relative content of the major ions between the two groups of frankfurters and indicates that

**FRANKFURTER PREPARATION REGIMEN**

![Diagram of frankfurter preparation regimen](image)

**Fig. 1.** Flow diagram of frankfurter preparation regimen.

**Fig. 2.** (a) Cryofractured surface of glutaraldehyde fixed frankfurter. The protein matrix (M) is preserved with no noticeable lipid component. Empty vacuoles (E) remain where large fat globules have been dissolved away. Smaller vacuoles (arrow) which once contained smaller fat droplets are also present. Bar = 100 μm. (b) Higher magnification (10x) of area indicated by arrow in Fig. 2a. Note absence of lipid material in protein matrix.

**Fig. 3.** (a) Cryofractured surface of glutaraldehyde fixed, osmium tetroxide post-fixed frankfurter. Large globules of fat (F) are embedded in a finely textured protein matrix (M). Arrow denotes smaller fat droplet. Bar = 100 μm. (b) Higher magnification (10x) of area indicated by arrow in Fig. 3a. Small fat droplets (F) fill vacuoles depicted in figure 2a. At this magnification, even smaller, finely dispersed fat droplets can be seen (L).

**Fig. 4.** Stereo pair of glutaraldehyde fixed, osmium tetroxide post-fixed, cryofractured surface of frankfurter. The fine structure of the protein matrix is well preserved. Stereo imaging provides excellent 3-D visualization of rough, lipid (L) laden fractured surface. Small fat droplets (F) readily stand out in a finely textured protein matrix (M). The arrow denotes a few empty vacuoles where fat droplets may have been dislodged. Bar = 10 μm.
the additional phosphate added can be detected by this method. State-of-the-art instrumentation exists which allows for the quantitative x-ray profile analysis of many SEM samples; unfortunately, due to specimen preparation problems, direct application to all food products has not been realized.

In terms of preservation of structural relationships in meat emulsion systems, the techniques developed in this study provide comparable SEM results to those reported by others (Carroll and Lee, 1981; Ray et al., 1979; Theno and Schmidt, 1978). In addition, the use of osmium tetroxide post-fixation provides a quick method of distinguishing protein from lipid in meat emulsion systems and may be useful for the examination of structural changes in the lipid and protein components of many food products. Major advantages of the techniques described in this paper are the relative rapidity and capacity to handle a large number of samples simultaneously as compared to critical point drying. A typical critical point drying device consists of a rather small, high pressure cylinder which restricts the number of samples that can be processed with each run. The large desiccator technique described naturally allows for the simultaneous preparation of a large number of samples, but also is safer and less expensive.

References


Fig. 5. Qualitative x-ray profiles of cryofractured, unfixed, freeze-dried surface of frankfurter. Relatively abundant ions are labelled. (Al peak is due to x-ray emission from mounting stub.) Upper profile is the control, bottom profile is from frankfurters treated containing additional phosphate. The higher phosphorus content can be readily observed.


Discussion with Reviewers

R. J. Carroll: Have you compared this procedure which includes cryofracture followed by fixation with fixation followed by cryofracture? Do you find any differences?

S. H. Cohen: What would have happened if the samples were fixed prior to freezing? Why were the samples frozen immediately?

Authors: This study utilized only cryofracture followed by fixation and fixation followed by cryofracture was not attempted. The permeability of the fixative in this system is unknown; therefore, the degree of fixation of the internal matrix is questionable. As a consequence, fixation was performed after cryofracture to assure complete fixation of the exposed surface.

S.H. Cohen: Why was MES chosen for sample preparation?

Authors: MES buffer was used because the pKa is 5.96 and the pH of the solution was 6.00, thus it has much buffering capacity in this range.
IMAGE ANALYSIS OF MORPHOLOGICAL CHANGES IN WIENER BATTERS DURING CHOPPING AND COOKING

A.G. Kempton and S. Trupp

Department of Biology, University of Waterloo, Waterloo, Ontario N2L 3G1 Canada

Abstract

Histological changes in wiener batters during chopping and cooking have often been illustrated with "representative" fields. The practice of selecting representative fields ignores variation and leads to word descriptions that cannot be correlated with numerical scores for functional or sensory tests. If wiener is regarded as a multi-component system, objectivity can be achieved by selecting many fields for each sample according to a rigid sampling plan. Image analysis quantified parameters of both the fat and protein components. The reduction in size of fat globules during chopping of a commercial formulation, for example, was a function of area and aggregate perimeter of several hundred globules compiled by a computer. There was no relationship between wiener firmness and any feature of the microstructure; but even at a low magnification of 30x, several statistically different factors were exposed during this survey which require further study.

Introduction

The microstructure of a food is usually studied for one of two reasons. There are basic studies for which the dominant motive is intellectual curiosity, where representative photomicrographs enhance the discussion of results as well as illustrate principal observations. More often, the microstructure is examined to determine how two or more batches of the same foodstuff differ physically as a result of changes in product formulation, production methods or spoilage. In such comparative studies, the use of subjectively selected "representative" microscopic fields may illustrate differences but not quantify them. Knowing that two or more samples are different in some way, a microscopist may tend to select a field that will "represent" only the differences and ignore the similarities. The purpose of this paper is to suggest that multi-component foods be examined according to principles used in mathematical ecology, implying objective sampling plans and statistical analysis to quantify differences. This approach will produce numerical values for structural features which may then be compared to numbers generated by some functional or sensory test.

The subject of this review has been under study for more than twenty years. Hansen (1960) is generally credited as the first to conduct a microscopic examination of finely comminuted meats such as wieners. Making wieners and similar sausage products had long been an industrial art and a commercial success, but fat and water loss during processing was sometimes excessive. Although this problem provided the impetus for Hansen's work, he used low-magnification microscopy to seek an understanding of the basic theory governing the stability of wiener emulsions. He showed that the amount of chopping must be sufficient to form a protein matrix which fully enclosed the dispersed fat globules; that the salt soluble proteins appeared to concentrate at the surface of the fat globules to form a stabilizing membrane; and that the higher temperatures associated with excess chopping might partially denature the protein matrix and permit unprotected fat globules to separate during smoking and cooking. Hansen's only experiment...
involving the microstructure of the whole wiener was the one on variable chopping time that emphasized the role of the protein matrix. His demonstration of a protein membrane around the fat globules was obtained experimentally by examining a diluted slurry emphasizing the role of the protein matrix. His fat globules was obtained experimentally by involving the microstructure of the whole wiener structure, that the role played by systems, rather than further studies of microsystems involving meat proteins in the formation of stabilized systems (Swift 1963; Helmer and Saffle 1963; Saffle and Galbreath 1964; Carpenter and Saffle 1964, 1965; Saffle 1968; Acton and Saffle 1970, 1971). During this period, Borchert et al. (1967) used electron microscopy to show the protein membrane around fat globules and its disruption during cooking; and coincidently pointed to the existence of many fat bodies as small as 0.1 μm with the hope that this would stimulate further work on the nature of meat "emulsions". The significance of the work of Borchert et al. (1967) was that the protein membrane around fat globules could apparently be broken during cooking without causing fat loss.

Work on the problem of fat separation during cooking overshadowed attention to the continuous protein phase described in the microstructure study by Hansen (1960), and there was a semantic difficulty caused by the use of the word "emulsion". For example, Froeling et al. (1970) showed that salt soluble proteins were as essential to wieners made from poultry meat as they were to red meats, and by embedding sections in paraffin they illustrated this with photomicrographs. However, they described meat emulsions simply as "oil in water emulsion with the disperse phase being droplets of the oil and the continuous phase being water". In a similar paper, Hargus et al. (1970) discussed differences in structure between proteins in white and dark turkey meats. These authors seemed willing to interrelate fat and protein in their two-paragraph discussion of a histological survey, but they once again emphasized fat globule size. Angel et al. (1974) did a comparative study of three machines for mechanically deboning poultry meat, using variable chopping times and microstructural differences between raw and cooked products as bases for comparison; but in line with the existing practice, they restricted their analysis of structure to the size and distribution of fat globules.

In the colloquial sense, "emulsion" had been used by sausage makers to imply the dispersion of fat in a protein matrix; whereas, in the physicochemical sense, a true emulsion was defined by Becher (1965) as a heterogeneous system consisting of two immiscible liquids intimately dispersed in another (liquid) in the form of droplets, whose diameters in general exceed 0.1 μm. True emulsions were probably obtained by those such as Saffle (1968) who used oil titration methods to measure the emulsifying capacity of various proteins. Yet van den Oord and Visser (1973) questioned the assumption that wieners were true emulsions and shifted the emphasis from fat alone back to the fat-protein matrix system. Considering electron photomicrographs, they described wiener batters as particles of solid fat rather than droplets of liquid oil, dispersed in a salt-water-protein matrix. After cooking, the fat particles were described as being enclosed in cavities formed by the coagulated, solid, protein-like network. Furthermore, none of the products they studied had a protein membrane that was different from the original fat cell membrane.

It may be impossible to encompass all commercial products within a single theory. Theno and Schmidt (1978), using SEM, found that one brand of frankfurter had a coarse protein matrix which mechanically fixed large fat globules in accordance with the microstructure proposed by van den Oord and Visser (1973). However, a second brand had a more finely-textured protein matrix and was "emulsion like" while a third had the characteristics of a true emulsion; namely, the fat particles had been reduced to an extremely small size, they did have a proteinaceous coat, and they were bound in place by small protein cross bridges. In a related note, concerning the variability in commercial products, Cassens and Schmidt (1979) were able to statistically support the contention of Angel et al. (1974) that the firmest wieners had the smallest fat globules, but they suggested that firmness was related to the more extensive protein matrix required to surround the larger number of smaller fat globules.

Renewed emphasis on the function of proteins was most important to those who wished to replace some of the meat with comparatively less expensive vegetable and milk proteins while retaining maximum nutritional value (1979) value and desirability of functional and sensory properties. Randall et al. (1976) found that the use of extenders such as textured soy protein, powdered soy protein, soy isolate, gluten and egg white, as well as tripe and cooked beef, decreased firmness and increased water loss during cooking in proportion to the rate of substitution. However, the fat loss was not severely affected until the replacement rate exceeded 40% of the meat protein. Referring to the microstructure study of Cassens et al. (1975) for support, they surmised that the texture of the replacement protein governed the texture of the finished product. They concluded that fat and water binding are not unique factors contributing to textural properties, and that a property of the protein such as thermal coagulability exerted a greater effect. Smith et al. (1973) concluded that finely-divided protein solids can play a role in emulsion stabilization based more on their physical state than on their solubility. Comer (1979) showed decisively that conventional measurement such as the emulsifying capacity of proteins did not correlate with
either emulsion stability or texture in wiener formulations. On the contrary, various fillers which had favourable effects upon homogenate stability had negative effects upon product texture. He stressed the observation that water binding and gelation characteristics of the proteins probably account for the comparative softness of wiener containing vegetable protein. A previous report from this laboratory (Kempton et al. 1982) showed that the inclusion of nonmeat proteins in wiener formulations caused major distortions in the microstructure of the batters which were clearly visible at a magnification of 30x under a light microscope, but only in some fields. Therefore, a large number of photographs was obtained for each product by a rigid, objective sampling plan. The degree of distortion in each photograph was measured by pattern analysis and the results were subjected to computer analysis by a statistical package known as the "two-within-four randomization test". The results were expressed as the number of slide preparations on which the ingredients were "clumped" rather than having a random pattern. Emphasis was placed on the unreliability of "representative" fields, which would have been more unreliable at higher magnifications. This present study pursued the concept that the microscopic evaluation of wiener should be based on an objective evaluation of many fields selected by following a rigid sampling plan. Image analysis, coupled to statistical methods which are comparatively simple and readily available, was applied to a survey of the changes that occurred during the chopping and cooking of various industrial wiener formulations. Table 2. Characteristics of experimental products

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Designation</th>
<th>Revolutions in cutter</th>
<th>Ingredients (Protein Source)</th>
<th>Total Shrink (% weight)</th>
<th>Texture (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Coarse</td>
<td>150</td>
<td>ALL MEAT</td>
<td>10.98</td>
<td>3.00</td>
</tr>
<tr>
<td>2</td>
<td>Medium</td>
<td>75</td>
<td>MEAT + EXTENDER NO. 1</td>
<td>12.15</td>
<td>1.85</td>
</tr>
<tr>
<td>3</td>
<td>Medium</td>
<td>150</td>
<td>&quot;</td>
<td>12.04</td>
<td>1.89</td>
</tr>
<tr>
<td>4</td>
<td>Fine</td>
<td>225</td>
<td>&quot;</td>
<td>11.77</td>
<td>1.93</td>
</tr>
<tr>
<td>5</td>
<td>Medium</td>
<td>150</td>
<td>MEAT + EXTENDER NO. 2</td>
<td>11.29</td>
<td>1.96</td>
</tr>
<tr>
<td>6</td>
<td>Medium</td>
<td>150</td>
<td>MEAT EXTENDER NO. 3</td>
<td>11.05</td>
<td>1.93</td>
</tr>
<tr>
<td>7</td>
<td>Fine</td>
<td>225</td>
<td>MEAT + EXTENDER NO. 1</td>
<td>10.71</td>
<td>1.88</td>
</tr>
<tr>
<td>8</td>
<td>Fine</td>
<td>225</td>
<td>&quot;</td>
<td>10.52</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Materials and Methods

Materials

The "commercial" batters were prepared and supplied by a major producer of comminuted meats and represented a typical least-cost formulation. The precise ratio of ingredients was not divulged. A 100-lb lot referred to only as a "final blend" was placed in a Kramer-Grebe silent cutter. Grab samples of several pounds each were removed by the sausage maker after 10, 20, 30, 40 and 50 revolutions. A portion of each sample was

Table 1. Characteristics of commercial products

<table>
<thead>
<tr>
<th>Designation</th>
<th>Revolutions in cutter</th>
<th>Yield (%)</th>
<th>Fat loss (%)</th>
<th>Fitness (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coarse</td>
<td>10</td>
<td>90.55</td>
<td>0.99</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>91.08</td>
<td>0.52</td>
<td>1.95</td>
</tr>
<tr>
<td>Medium</td>
<td>30</td>
<td>92.50</td>
<td>0.22</td>
<td>2.19</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>92.44</td>
<td>0.17</td>
<td>1.94</td>
</tr>
<tr>
<td>Fine</td>
<td>50</td>
<td>92.53</td>
<td>0.18</td>
<td>1.96</td>
</tr>
</tbody>
</table>

1 All values are means of 10 determinations

2 Extender added after 150 revolutions

3 Extender and binder added after 150 revolutions

Honest Significant Difference based on Tukey's test (90% level of probability) = 0.22.
Research Institute, measured using the method described by Voisey and Ranken (1973) as modified by Randall et al. (1977). Firmness of the cooked product was measured using the method described by Voisey and Randall (1977) for the Universal Food Rheometer (UFR). Pertinent performance data are summarized in Table 1. Finished wiener were frozen, and returned for microstructure analysis.

The "experimental" batters containing significant amounts of nonmeat protein were supplied by a second industrial concern. Batters were prepared in a silent cutter. Wiener representing each batter were prepared and evaluated as described above. However, Table 2 was constructed from data supplied by the manufacturer. Complete formulations and variables in preparation were made available (Kempton, unpublished), but Table 2 contains only information required for this paper. The involvement of different industrial and governmental laboratories, and the transport of large samples, made it logistically impossible to immerse samples in liquid nitrogen. Preliminary studies showed that the structural damage caused by freezing to -18°C did not obscure the ability of image analysis to quantify similarities and differences among the various products.

Sample Preparation and Microscopy

Fig. 1 is an abbreviated illustration of the procedure used to obtain microscopic fields suitable for statistically valid analysis. For additional information see Kempton et al. (1982). Five sample units were taken at random from the 2-kg frozen sample of raw product and trimmed to 2 cm x 2 cm x 1 cm rectangular prisms, while maintaining the temperature at -18°C. For cooked products, the sample units were 5 wiener made from the batter, frozen for transport, and kept frozen at -18°C. Slices 20 um thick were made with a Cryo Cut Cryostat (Model 840, American Optical/A.O., Buffalo, N.Y.) set at -25°C. Three slices were taken from each unit by selecting the first slice, discarding the next nine, selecting the eleventh, discarding the next nine and finally selecting the twenty-first slice.

The slices were fixed on slides by immersion in a 1:1 (v/v) solution of saturated mercuric chloride and absolute ethanol for a minimum of 6 h. The fixed slices were stained for 20 min in a working solution of Oil Red O according to Disbrey and Rack (1970). The slides were washed in running water for 10 min, counterstained in 0.5% solution of Light Green (C.I. No. 42095), then washed again in running water for 10 min. Fat retains the red stain and the protein component is stained green.

The stained slices were mounted under a Nikon Aphot microscope (Nippon Kogaku K.K., Japan) equipped with a 35 mm Nikon M-35 FA camera. Each slice was sampled by photography of as many as four adjacent fields on each of two linear transects (called Planes on Fig. 1). This plan potentially yields 120 objectively selected photographs of each sample. This plan was reduced to 48 randomly selected photographs by the procedure detailed on Fig. 1. After experience with the degree of homogeneity found, the sampling was reduced to as few as 20 photographs during the course of this study.

Component Analysis

In order to analyze each component in the photographs, it was necessary to trace each separately onto a transparency sheet using a black, felt-tipped pen. Each tracing was analyzed using an Image Analyzer (Quantimet 720, Imanco, Monsey, N.Y.). The program, written in Basic Language, inputs area and perimeter for each feature and accumulates area and perimeter to give aggregate totals.

The data were analyzed by a two-way fixed effects analysis of variance using BMDP7D. The
Results and Discussion

Chopping and Cooking of an All Meat Commercial Formulation

Experiments involving variable chopping regimes have been done several times with wieners of varying composition. However, the first part of this study differs from previous work in that changes in both the fat and protein phases were described in numerical terms, the amount of void open space was measured, and quantitative measurements of the interaction between the amount of chopping and changes during cooking are also novel. Kempton et al. (1982) explained why many microscopic fields of each sample must be selected by an objective sampling plan such as Fig. 1 if the results of histological studies of wieners were assembled to illustrate mean values. In this present paper, image analysis was used instead of pattern analysis because the major expected change was that the fat component would be dispersed into more but smaller particles as chopping continued. Wiener batters were tested for fat loss and yield during chopping, and UFR measurements of firmness were made on the finished products (Table 1) so that the potential for comparing morphological changes and functional characteristics would at least exist.

Ingredients of a commercial formulation chopped for 10 revolutions in a silent cutter produced a batter which the sausage maker described as "coarse". Batters chopped for 30 and 50 revolutions described as "medium" and "fine" respectively. The performance data given in Table 1 show that even the "coarse" batter produced a wiener of acceptable firmness, and the fat loss from this batter during cooking, while higher than the others, was not considered excessive. Fig. 2 may be of interest as a reference, because as far as is known this is the first publication of a color plate in which changes in the fat and protein during chopping and after cooking have been shown using a differential stain. These photomicrographs are representative in the statistical sense because they were assembled to illustrate mean values that had been ascertained by image analysis of 36 microscopic fields of each treatment. They are presented here to discuss how appearances may be deceiving when any one photograph is selected to represent a product. Subjectively, the fat globules in the coarse batter (Fig. 2a) could be described as large amorphous masses that became more regular and ovic when chopping progressed, as well as becoming smaller (Fig. 2b, 2c). Similarly, the protein in the coarse batter could be described subjectively as thick strands retaining some evidence of tissue structure which was dispersed into a thinner, more uniform matrix during chopping. The point is that considered simple and expected changes were not substantiated by visual inspection of all the photomicrographs obtained by the random sampling plan outlined in Fig. 1. When all 108 photographs of these batters were shuffled like a deck of cards, they could not then be divided accurately into three piles corresponding to the amount of chopping.

On the other hand, all 216 photographs could be easily and accurately separated into two piles corresponding to raw batters and cooked wieners. This separation was based primarily on the darker hues of both the fat and protein phases after cooking, and secondarily on the impression that there was less material and more open space, generally, in the photographs of the cooked product. The subjective observation of greatest theoretical importance was that most of the "globules" in the raw batter appeared to be closely surrounded by a coating of protein; while after cooking (Fig. 2d, 2e, 2f) the fat could be described better as irregularly shaped "particles" which appeared to be retracted from their protein coating although most remained trapped in crevices within a protein matrix. The subjective analysis of Fig. 2d agreed with the theory proposed by Kempton et al. (1982) than with the emulsion theory. The description of fat in raw batters as amorphous masses surrounded by protein corresponds to the concept of a dispersion of solid fat, and the enclosure of fat in cavities formed by the coagulation of protein during cooking suggested a physical network rather than an emulsion. However, as it was with photographs of the raw batters, the 108 photographs of the cooked wieners could not be subdivided visually into three piles corresponding to the amount of chopping. Therefore, the program of image analysis was broadened to determine whether there were significant differences between a raw and its corresponding cooked product in either the fat, protein or open space components and to determine whether there were subtle changes during chopping which could not be discerned by visual analysis of the randomly selected microscopic fields. It was not possible to perform image analysis directly on the colored photographs by using filters to block one color while measuring the complimentary color, which would appear black. Two technical problems which prevented this preferred approach. First, the colors were not uniform and no single filter would block out all of the fat, which varied in hue between pale pink and deep red. Similarly, the color of the protein varied from blue-green to black. The second problem was that there were areas in every photograph where fat and protein were overlayed, and red and green together blocked all transmitted light and produced a black photographic image in those areas. Both problems are related to the fact that a slice has depth. This difficulty could probably be reduced by obtaining thinner slices, but it will be inherent in images obtained by transmitted light through any histological preparation. Bergeron and Durand (1977) overcame the problem of conveying histological information by transferring colored photographs with word descriptions by tracing the structures they wished to discuss. Tracing each component in the wiener separately
produced a series of three images for each photograph as illustrated by Fig. 3. Diagrams of the empty spaces (Fig. 3c, 3f, 3i), which are either air pockets or water, were used to ensure that the sum of the three components equalled the total area of the photograph. The tracing procedure involved subjective decisions as to the areas of the colored photographs where fat and protein were overlayed. Furthermore, it is unlikely that all of the very small holes in the protein matrix, and all the very small fat particles particularly evident in Fig. 2c, were accurately portrayed in Fig. 3g. However, Fig. 3a, 3d, 3g illustrate the dispersion of fat, and Fig. 3b, 3e, 3h portray the formation of a "honeycomb" protein matrix more clearly than Fig. 2. Admission of this difficulty is one reason why the procedure was evaluated first on an all meat formulation, about which there is a large body of histological knowledge and predictable results were expected. Computer printouts of data on traced images and on phase the chopping and cooking of an all meat wiener formulation are given as Fig. 4, Fig. 5, Fig. 6 and Fig. 7.

No a priori assumptions were made regarding the degree of central tendency that would emerge from analysis of tracings of 36 photomicrographs or what type of distribution around the mean could be expected. A relatively large range was expected from cursory analysis of the many fields selected by the objective sampling plan. For example, one photograph of the coarse raw batter was about 80% blank space. In spite of the range, the results approximated a normal distribution around mean values. Based on mean values, the area occupied by protein did not change at all during chopping (Fig. 4) and an apparent upward trend in the area occupied by fat during chopping was not statistically significant (Fig. 5). Since the total area occupied by fat or protein was not expected to change during chopping, these consistent mean values, each derived from 36 photographs, indicated that this technique probably quantified histological changes with greater accuracy than could be achieved by any assessment of single representative fields.

The average size of the fat globules was expected to change during chopping, by all previous reports and by visual inspection of the photographs. This could not be adequately illustrated by dividing the area occupied by fat by the number of fat globules to give a numerical "average". Kempton (unpublished) produced histograms showing that chopping caused an increase in the number of smaller fat globules; but the numerical average size was weighted in the direction of one or two large globules that accounted for as much as 50% of the fat, even in a finely chopped batter. However, image analysis showed that the aggregate perimeter of the fat phase in the raw batters increased from 1000 mm in the coarse batter to 1500 mm in the fine batter: an increase of 50% (Fig. 6.) Since the area remained statistically constant, this increase in perimeter became an indirect numerical measurement of the decreasing size of the fat globules. This increase in the fat perimeter may be a direct measurement of the increase in protein perimeter required to enclose the fat. Angel et al. (1974) and Cassens and Schmidt (1979) concluded that firmness was directly related to the size of the fat particles. That was true in their studies of various finished products, but if universally applied it would mean that continued chopping should increase firmness. In this study, UFR measurements of firmness (Table 1) did not correlate with the decreasing size of the fat globules. The decrease in size of the fat globules was the only structural change that was directly correlated with the amount of chopping; but contrary to other reports, this was of no practical significance as a criterion for performance.

All other statistically different observations showed that the medium batter, and the Wieners made from it, were distinct in several ways. For example, and to complete the discussion of changes caused by chopping, Fig. 2 shows that during the second phase the chopping and cooking of all meat wiener formulation are given as Fig. 4, Fig. 5, Fig. 6 and Fig. 7.

Further discussion of the unique nature of the "medium" chopping regime, including interactions among structural parameters, would not be productive at this time. Empirically, the medium batter was expected to produce the firmest wiener, with the least amount of fat loss and total shrink during cooking. This was not borne out by any measurements conducted by Agriculture Canada and given in Table 1. Therefore, there is no performance criterion to which the observed structural differences associated with the medium batter can be compared.

Chopping and Cooking of Experimental Formulations Containing Vegetal Protein.

Wieners made with significant amounts of nonmeat protein were known to be fundamentally different, histologically, than comparable all meat products. Pattern analysis showing clumping was one way of measuring changes visible at low magnification (Kempton et al. 1982). Image analysis exposed further differences. Fig. 8 includes an all meat control, but otherwise it is analogous to the way changes in the area of the protein were displayed in Fig. 4. The area of
Fig. 2 Photos a-c: Representative fields of commercial wiener batter chopped to coarse, medium and fine stages respectively. Fat is stained red, protein is green. Photos d-f: Representative fields of commercial cooked wiener produced from batters chopped to coarse, medium and fine stages respectively. Fat appears to be trapped in protein matrix.
the photomicrographs occupied by protein did not remain constant during chopping the way it did with all meat batters. Compared to the specific control for this experiment, the coarsely chopped amended batter appeared to have more protein, the medium batter had the same amount as the control (3300 mm²) and the finely chopped batter had less. A loss of protein area occurred during cooking, except for the finely chopped amended batter which having lost the most during chopping posted a gain during cooking. The large shrinkage in the protein component of the all meat product (Sample 1) from 3300 to 2000 mm², was totally unexpected. This may be a measure of the variation encountered in comparative studies of wieners made by different companies. Fig 9 is introduced to show that this was not an isolated occurrence. Various formulations were identical when chopped to the medium stage, and the apparent shrinking of the protein during excessive chopping could not be avoided by adding the vegetable component later in the chopping process. Changes in the area of protein in photomicrographs indicate a change in the density of the protein, not in the amount of protein present. Varying degrees of hydration would be one possible explanation for the changes noted. Whatever the explanation may be, these changes and differences were not related to wiener firmness.

Had this study been performed solely to relate microstructure to wiener firmness, there would have to be one feature emerge that would separate the control wieners that had a texture-firmness value of 3.0 kg (Table 2) from all wieners containing some vegetable protein which had UFR values in the relatively narrow range of 1.58-1.96 kg. Although this study was not successful in this respect, Fig.10 may further illustrate the wrong conclusion that could be drawn from visual assessment of photomicrographs while it serves to show what image analysis actually measured. These tracings represent mean values and there is a striking visual difference between the typical "filagree" or "honeycomb" appearance of the protein phase in the all meat control (Fig. 10a) and the severely fragmented protein in the finely ground batter that contained a proportion of vegetable protein (Fig. 10d). If there were only these two samples, it would be subjectively concluded that fragmentation is typical of nonmeat products and is the cause of the difference in texture. Image analysis again showed that, contrary to appearances,
the number of "features" (in this instance bits of protein) was not statistically different and the "average" size of the protein features was nearly the same. That is because the apparent "honeycomb" picture shown as Fig. 10a contained a number of very small features. As it was with the fat phase in the all meat products, the apparent differences in the protein strands could be measured only by the interaction of protein area (Fig. 8) and protein perimeters shown in Fig. 11. The coarse batter that had the largest protein area also had the smallest protein perimeter of the four batters. In other words, the large protein mass which occupied 4200 mm² as quantified in Fig. 8 and illustrated in Fig. 10b, had a perimeter of only 1650 mm. The finely ground batter that had only 57% as much protein area (2400 mm²) had as much perimeter as the coarse batter (1800 mm). This high ratio of perimeter to area was the true measure of the smaller size of the protein features in the fine batter shown in Fig. 10c. The "control" batter (Sample 1, Fig. 10a) and the medium batter (Sample 3, Fig. 10e) were similar in both area and perimeter (Fig. 11). Thus what was seen pictorially was caused by chopping, but it was apparently not related to firmness. The two that looked most alike (the control and the medium battery) produced wieners that were widely different in texture. Two that looked quite different (the coarse and medium batters) produced wieners that were texturally similar.

Any emphasis on the fat component at the expense of the protein component seemed unwarranted because it was the protein that was substituted. In this study, the behaviour of the fat phase was like a mirror image of the changes in the protein phase (Fig. 12 and Fig. 13). During chopping, the area occupied by fat was initially less in the coarse batter than in the all meat control and then it expanded in the fine batter (while the protein first expanded and then shrank). After cooking, the only anomaly was the very large contraction of the fat in the finely ground product, from 2400 mm² to 1200 mm² (mean values).

This finely chopped product was unique. It appeared to be composed of protein fragments (shown in Fig. 10d) enclosed in a continuous fat phase rather than the conventional view of a wiener (Fig. 2) as fat particles dispersed in the continuous protein phase. In spite of a total inversion of the accepted model this product did not lose more fat during cooking, as measured by total shrink, than the other batters. Although no conclusion should be drawn from this single
Image Analysis of Wieners During Chopping and Cooking

![Figure 6](image1.png)  
**Fig. 6** Mean values and variation in total perimeter of the fat in photomicrographs, as affected by chopping and cooking a commercial wiener formulation.

![Figure 7](image2.png)  
**Fig. 7** Mean values and variation in total perimeter of the protein in photomicrographs, as affected by chopping and cooking a commercial wiener formulation.

example, the good cook stability of this visually peculiar product tends to support the school of thought espoused by Randall et al. (1976) and Corner (1979), who placed more emphasis on the properties of the protein than on the fat emulsions.

**Summary**

Although the microstructure of Wieners has been studied for more than twenty years, more discussion seems to have centered on the use of model systems than on studies of raw batters or cooked Wieners. It was Hansen's 1960 work with model systems, rather than his study of product microstructure, that helped Saffle (1968) and others achieve an understanding of the cook stability problem, which in turn led to the development of computer controlled least-cost formulations; and it was Hansen's model that has been challenged by others such as Corner (1979).

Studies in this laboratory were commissioned to provide a visual dimension to empirical experiments conducted by industry on Wieners containing nonmeat protein. A relationship between microstructure and wiener texture was an implicit goal rather than an explicit requirement. The first decision which had to be made concerned the selection of an appropriate magnification. In the first phase (Kempton et al., 1982) a major distortion in wiener microstructure caused by the presence of nonmeat protein was observed at 30x. Unless this crude "clumping" of ingredients could be overcome, there was no practical reason to examine similar amended products under higher magnification. For comparative studies, it is suggested that the lowest magnification capable of detecting differences in structure should be used; and that the significance of these differences be reported in the statistical sense, which requires an objective sampling plan. However, pattern analysis described the problem but offered no solution. Higher magnifications obtainable by electron microscopy could be used to conduct a basic study of this aberration, which could ignore those portions of the wiener not affected.

The second decision was to give equal emphasis to the fat and protein phases. The most significant change in wiener technology in recent years has been the replacement of some of the meat proteins by vegetable, milk, egg and single-celled protein. It would seem logical to place more emphasis on the protein matrix than had been done in the past. No doubt existed regarding the
A.G. Kempton and S. Trupp

Fig. 8 Mean values and variation in area of photomicrographs occupied by protein, as affected by chopping and cooking experimental wiener formulations.

need for salt-soluble protein to impart cook stability, but the size of the fat droplets, or globules, became common currency in studies on microstructure of wiener, from Froning et al. (1970) to Carroll and Lee (1981). Yet the problem with wiener containing nonmeat proteins is not stability, but firmness (Table 2). Cober (1979) dealt with both properties simultaneously as they are affected by proteins. Cassens and Schmidt (1979), while being careful not to claim a cause and effect relationship, did show that texture was related to the size of the fat entities. Therefore, both phases were given equal attention by using image analysis.

The nature of open spaces or voids has been addressed by Carroll and Lee (1981). They are either air spaces or trapped water droplets, which one, cannot be determined after the specimen has been prepared for microscopic study. Many are large enough to be considered as features of the macrostructure and can be seen by the unaided eye when a wiener is sliced. In microstructure, at 30x they can fill some fields of view completely and they will be present in fields selected objectively. They, too, can be quantified by image analysis.

Image analysis showed several differences which were not obvious from visual assessment. In both experiments, there were greater differences between the behaviour of proteins than between the fat distribution patterns. However, image analysis failed to separate the amended products from their all meat control on the key factor of firmness.

Fig. 9 Similarities in the area of photomicrographs occupied by protein in various experimental wiener formulations chopped the same amount. Samples 1, 5 and 6 were "medium" batters. Sample 7 and 8 were "fine" batters with nonmeat protein added late in the chopping regime.

Fig. 10 Photo a: Protein component of all meat control (Sample 1). Photo b: Protein component of coarsely-chopped wiener batter containing non-meat protein (Sample 2), showing thick strands occupying large area of photograph. Photo c: Protein component of medium chopped Sample 3: not visibly different from all meat Photo a. Photo d: Protein component of finely chopped wiener batter containing nonmeat protein (Sample 4). Matrix appears fragmented.
Image Analysis of Wieners During Chopping and Cooking

The loss of firmness in products containing vegetable proteins may depend on the physical and molecular structure of the protein, and thus the mechanism may be beyond the capacity of any microscopic study. It is suggested that this be tested by further study of the morphology of the protein phase under more controlled conditions. Slices of raw batters and cooked wiener fixed on microscope slides can be defatted by a 20% solution of chloroform in ethanol without distorting the protein matrix (Kempton, unpublished). This eliminates the need to trace the protein in a multicomponent system; and it is now possible to project the image directly onto a screen without taking photographs. Chopping should be done in a Mince Master, which is used in industry more widely than a silent cutter; but the chopping time variable could be excluded. The samples should be immersed in liquid nitrogen to eliminate the distortion caused by freezing. The point is, that image analysis is objective rather than subjective and if differences among samples that vary in firmness can be detected at 30x the use of higher magnifications should be temporarily shelved. However, if the results of the proposed study confirm the similarity observed in this preliminary survey, the magnification could be increased to the limit of the light microscope using the same program of image analysis providing the number of fields analyzed was increased sufficiently to establish reliable mean values and describe the distribution about the means.

Acknowledgements

This work was performed under contract with Agriculture Canada (Contract Serial OSU78-00130). The basic language program for the image analysis was developed by J. Terry, Department of Materials Chemistry, Ontario Research Foundation. Dr. G.W. Bennett, Statistical Consultant in the Dept. of Statistics, University of Waterloo, performed all statistical evaluations. This work is dedicated to Dr. John Quinn (deceased) of Agriculture Canada who provided inspiration and encouragement.
**Fig. 13** Mean values and variation in total perimeter of the fat in photomicrographs, as affected by chopping and cooking experimental wiener formulations.

### References


Discussion with Reviewers

Reviewer I: Although this paper is unique in introducing image analysis to food microstructure, there are serious flaws. One is the selection of samples with little or no control over significant variables such as using samples prepared with different sources of meat protein. Another is the failure to distinguish distortion that results from storage at -18°C by freezing in liquid nitrogen and sectioning immediately.

Reviewer II: A very large number of products of different compositions and chopping protocols are being considered. Is this a realistic number of variables to be analysed within the limitation of sample number, etc. of this paper?

Authors: Theno and Schmidt (1978) and Cassens and Schmidt (1979) showed that theories relating wiener microstructure to performance characteristics must encompass the variation that exists among commercial brands, and Comer (1979) showed that the model developed by Hansen (1960) did not explain the performance of wieners containing nonmeat protein. Our introductory survey was intended to introduce image analysis to the study of food microstructure, and it was purposely designed to include as many variables as are likely to be encountered in further studies. There are probably some other effects in commercial wieners which are proprietary, such as variation in least-cost formulations. When all the principal variables have been identified, it may become possible to use multiple regression to develop a predictive mathematical model that will relate wiener microstructure to texture, and image analysis (or preferably image processing) is a method of obtaining the numerical values necessary to construct such a model. The primary purpose of this paper was to demonstrate the type of information that can be obtained from image analysis, and the text has been amended to explain why freezing samples in liquid nitrogen was not possible. An example of the distortion caused by freezing to -18°C was that the protein matrix surrounding some fat globules was sometimes cracked and occasionally discontinuous. However, the key parameters of total area and total perimeter of the fat and protein features were not seriously affected. Cracks in the protein matrix might have been serious if the emulsification theory had been an important factor but Borchert et al. (1967) had already shown that such cracks in the protein "membrane" occurred during cooking without causing fat loss.

E.W. Ross: Occasionally, bimodal histograms are seen, as in the cooked, all meat sample in Fig. 8. Can a visual interpretation be given for such cases?

Authors: The direct answer to your question is, no it cannot. By the time we had progressed to the study of products containing some vegetable proteins, the number of photographs subjected to image analysis had been reduced to 24 because of the relative homogeneity of the samples. Additional photographs may have re-established a more normal distribution about the mean values, but the practical difference in all meat samples in Fig. 8 is the decrease in the mean protein area, from 3300 mm² in the raw product to 2000 mm² in the cooked product. The significant difference between means seemed more important than distribution about mean values. Fig. 9 also contains bimodal and skewed histograms but the mean values (denoted by M) were identical within treatments. We wrote and we knew very well that the difference between medium and fine chopping in Fig. 9 had been obscured by variation in the shape of the histogram; but once again the consistency of mean values within a set seemed to be more important than the distribution of values about the means.

E.W. Ross: Since areas and perimeters were obtained for each feature in each drawing, it might have been possible to calculate for each feature the quantity: $I = 1 - 4\pi \cdot (\text{area})^2 / (\text{perimeter})^2$ which is a measure of the "out of roundness" of the feature.

Authors: We are aware of the potential for shape moment measurements such as reseactivity, circularity and invariant moments, but not of the comparatively simple equation you have given us. We did not measure shape because we feared that this was a parameter that was affected by the constraints that let us to examine samples frozen to -18°C (which did not affect size measurements by area and perimeter) instead of using samples frozen in liquid nitrogen.

E.W. Ross: In Table 2, the HSD is given for texture, but not in Table 1. Why?

Authors: The data in the Tables were supplied by our industrial and governmental colleagues. The methods used to express statistical differences are not standard across the meat industry.

R.J. Carroll: You may wish to explain the difference between image analysis and pattern analysis for readers not knowledgeable in this area.

Authors: This is a difficult question to answer briefly. We recommend that those not familiar with these terms read Castelman KR (1979) Digital Image Processing, Prentice-Hall Inc., Englewood Cliffs, N.J. The term "image analysis" was used in the title of this paper to imply the use of a high-quality image computer as distinguished from image processing which requires a high-resolution...
quality image display device as well (Castleman, 1979, page 13). Pattern analysis, used in the text of this paper, usually refers to a specific statistical test such as the two-within-four randomization test used by Kempton et al. (1982) to analyze spatial patterns in wiener batters. "Pattern recognition" is the accepted term for both aspects of this field of study. Cassens et al. (1975) recognized that the ingredients of wiener containing soy flour had a different distribution pattern than wiener containing only meat proteins, when viewed under a light microscope. Essentially, Kempton et al. (1982) digitized these patterns, meaning that the difference in patterns observed through the microscope could be expressed numerically. Castleman (1979) devoted two chapters (pages 299-346) to "statistical pattern recognition as applied to digital images," which he considered as little more than an introduction to the subject. Directions are given on how to select the "features" (fat and protein in our system) and how to measure size (from which we selected area and perimeter). To answer the question as briefly as possible, "pattern analysis" involves comparisons among photographs and "image analysis" determines what is in the pictures. Both procedures express microscopic observations in numerical terms.
COMPOSITION AND MICROSTRUCTURE OF SOFT BRINE CHEESE MADE FROM INSTANT WHOLE MILK POWDER

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Abstract

Comparative studies were made on the composition, microstructure and organoleptic quality of soft brine cheese made from instant whole milk powder and from raw milk. The chemical analysis of young and ripened (one and two month) cheese revealed similarity except for a higher salt content in the cheese made from reconstituted milk at the end of ripening. Electron microscopic studies showed distinct differences in the structure of the protein matrices in the ripened cheese samples, i.e. a very homogeneous structure in the cheese made from raw milk compared with a slightly aggregated state of the protein in the cheese made from reconstituted milk. The organoleptic examinations resulted in an overall acceptable quality of the cheese made from reconstituted milk except for a higher saltiness.

Introduction

The use of non-fat dried milk for cheese manufacture requires adequate reconstitution and recombination techniques, and is now common in a few developed countries.

In many developing countries, e.g. Egypt, the shortage in the milk supply requires increasing use of milk powder for dairy products. Because modern equipment and processing facilities for the reconstitution and recombination are often not available in private small dairy plants, the use of whole milk powders could be advantageous.

Reconstituted whole dried milk, however, tends to exhibit limited solubility which may result in fat destabilization and also sediment formation (14). In order to circumvent these problems, the use of instant whole milk powders could represent an alternative (22, 26).

The use of milk powder for the manufacture of Camembert cheese and the resulting changes in the microstructure during ripening have been described by Peters and Knoop (17, 18).

We now report results on the composition and structure of soft brine cheese made from instant whole milk powders.

Materials and Methods

Five imported instant whole milk powders of different origin were purchased from the local market in Egypt. The average composition of these powder samples was: 26-26 % butterfat, 25.5-26.5 % protein, 37-38 % lactose, 5.5-6.5 % ash and 2-3 % moisture. Each powder was reconstituted to 20 % total solids in water (30°C) by only gentle mechanical stirring. For control, fresh cows' milk from the University of Zagazig Experimental Farm was used.

Batches of soft brine cheese were made from each of the reconstituted milks and from the fresh milk according to a method described earlier (10, 14): The milks were at first heated to 65°C for 30 minutes, then cooled to 32°C. Subsequently 2 % starter and 0.04 % calcium chloride were added. The curd was cut after 60 minutes and filled into wooden moulds, then pressed and brine salted (16 % salt, 24 hours). The young cheeses were packed in tins containing salted sweet whey (14 % salt) and stored for two months.
at 12-14°C for ripening.

Fresh and reconstituted milks and their wheys were analysed for pH, \( \delta \text{SH} \), fat, moisture, calcium and phosphorus and salt (NaCl) (2, 5, 7). The protein breakdown of cheese was determined as soluble nitrogen (24), non-protein nitrogen (23), peptide nitrogen (6), and amino acid nitrogen (23). Cheese scoring was carried out after two months of ripening (3). Three replicate analyses of the samples by each of the analytical techniques were carried out. Mean value \( \bar{x} \) and standard deviation \( s_x \) were calculated (25).

The young and ripened cheese samples were prepared for electron microscopy by application of the freeze-fracturing technique (4, 20). Small pieces (1-2 mm³) of cheese were mounted on specimen holders, using glycerol as an intermediate layer for increasing mechanical stability. The specimens were quickly frozen by immersion into melting Freon 22 (-160°C) and stored under liquid nitrogen. Freeze-fracturing was carried out in a BALZERS BA 360 M unit at an object temperature of \(-120°C\). For replication the freshly freeze-cleaved surface was immediately shadowed with 2 nm platinum/carbon under an angle of 45° and then transferred to 5 % sodium hypochlorite (undiluted bleach) for approximately 2 hours and passed again to distilled water. Fat was removed by a short treatment in pure acetone. Electron microscopy was carried out with a Siemens ELMISKOP I at 80 kV.

Results and Discussion

Milk reconstitution

When instant whole milk powder was added to the water and mildly agitated, it formed a homogeneous reconstituted milk, showing no clumps or butteroil lenses on the surface. Analyses of fresh milks and the corresponding wheys are shown in Table 1. The values for fresh milk may somewhat differ from milks produced in countries with a moderate climate but are not abnormal for conditions in Egypt. The higher content of fat and protein of reconstituted milks as compared with fresh milk resulted from using 20 % total solids which was necessary in order to obtain a satisfactory curd firmness. Fat and protein retention in cheese made from fresh milk was higher than in those made from reconstituted milks, although the latter had 20 % total solids. The average \( \delta \text{SH} \) value of the reconstituted milks is almost double that of fresh milk which has to be ascribed to the higher protein content. The comparably high \( \alpha_f \) for \( \delta \text{SH} \) in reconstituted milk results from differences between the five milk powders used.

A precipitate was observed in the whey of cheeses made from reconstituted milks. This could be due to the protein denaturation during the drying process, resulting in insoluble whey protein aggregates. The amount of insoluble whey is dependent upon the preheating conditions of the milk and milk concentrate (13). On the other hand, a slight increase in insoluble protein can occur as a result of the drying process in all milk powders (15).

The curd from the reconstituted milks were still softer than the curd from the fresh milk despite the 20 % total solids chosen. In this respect, instant dried milk and ordinary dried milk are similar in curd formation and insoluble whey contents. This phenomenon has been repeatedly described (12, 16).

Chemical composition of cheese

The average values of pH, buffer capacity, lactose, fat, protein, moisture, calcium, phosphorus and salt (NaCl) content of cheese made from fresh milk (variant I) and cheeses made from the five reconstituted milks (variant II) are shown in Table 2. Young cheese II has a buffer capacity and a lactose content higher than that of cheese I, due to the use of 20 % total solids. After one month of ripening lactose vanishes in both types of cheese and, at the same time, the buffering capacity of the cheeses and the pH values increase.

After two months, cheese I showed a higher pH value and a lower buffer capacity than cheese II. Changes in lactose, pH and buffer capacity are in accordance with those given by Czulak et al. (5). Fat, protein and moisture contents of cheese II were less than those of cheese I.

The average value of calcium and phosphorus of all cheeses was nearly the same and was

<table>
<thead>
<tr>
<th>Samples</th>
<th>pH</th>
<th>( \delta \text{SH} )</th>
<th>Fat %</th>
<th>Protein Kx6.38</th>
<th>pH</th>
<th>( \delta \text{SH} )</th>
<th>Fat %</th>
<th>Protein</th>
<th>Protein retention %</th>
<th>Fat retention %</th>
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SOFT BRINE CHEESE

Table 2. Chemical composition of cheese. (Average of three replicate analyses)

<table>
<thead>
<tr>
<th>Age of cheese</th>
<th>Treatment</th>
<th>Index</th>
<th>Buffer capacity in 0.5NaOH</th>
<th>pH</th>
<th>Lactose %</th>
<th>Fat in D.M. (Nx6.38) %</th>
<th>Protein moisture %</th>
<th>Calcium %</th>
<th>Phosphorus %</th>
<th>Salt (NaCl) %</th>
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</thead>
<tbody>
<tr>
<td>Young</td>
<td>I</td>
<td>x</td>
<td>5.17 2.71</td>
<td>8.2</td>
<td>45.79</td>
<td>21.82</td>
<td>58.20</td>
<td>0.96</td>
<td>0.68</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>σₓ</td>
<td>0.11 0.84</td>
<td></td>
<td>0.04</td>
<td>0.24</td>
<td>0.16</td>
<td>1.22</td>
<td>0.11</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>x</td>
<td>5.11 3.19</td>
<td>1.36</td>
<td>41.32</td>
<td>20.79</td>
<td>53.19</td>
<td>0.89</td>
<td>0.69</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>σₓ</td>
<td>0.14 0.92</td>
<td></td>
<td>0.03</td>
<td>0.65</td>
<td>0.13</td>
<td>2.26</td>
<td>0.05</td>
<td>0.67</td>
</tr>
<tr>
<td>One Month</td>
<td>I</td>
<td>x</td>
<td>5.46 3.82</td>
<td></td>
<td></td>
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<td>42.83</td>
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<td>σₓ</td>
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<tr>
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</tr>
<tr>
<td>Two months</td>
<td>I</td>
<td>x</td>
<td>5.61 2.99</td>
<td></td>
<td></td>
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<td>40.06</td>
<td>21.76</td>
<td>0.71</td>
<td>4.96</td>
</tr>
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<td></td>
<td>σₓ</td>
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<td></td>
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<td>0.35</td>
<td>0.12</td>
<td>1.58</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>x</td>
<td>5.50 3.78</td>
<td></td>
<td></td>
<td></td>
<td>38.58</td>
<td>20.86</td>
<td>0.69</td>
<td>6.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>σₓ</td>
<td>0.09 0.93</td>
<td></td>
<td></td>
<td></td>
<td>0.57</td>
<td>0.14</td>
<td>1.25</td>
<td>0.12</td>
</tr>
</tbody>
</table>

I - Fresh milk cheese
II - Reconstituted milk cheeses (5 batches)

Table 3. Protein degradation of cheese (Average of three replicate analyses)

<table>
<thead>
<tr>
<th>Age of cheese</th>
<th>Treatment</th>
<th>Index</th>
<th>Total nitrogen (T·N) %</th>
<th>Soluble nitrogen (S·N) % of T·N</th>
<th>Non-protein N % of T·N</th>
<th>Peptide N % of S·N</th>
<th>Amino acid N % of S·N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>I</td>
<td>x</td>
<td>3.42 10.15</td>
<td>6.02</td>
<td>44.16</td>
<td>0.95</td>
<td>8.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>σₓ</td>
<td>0.16 1.80</td>
<td>0.91</td>
<td>5.66</td>
<td>0.24</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>x</td>
<td>3.26 12.89</td>
<td>5.95</td>
<td>47.07</td>
<td>0.61</td>
<td>4.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>σₓ</td>
<td>0.13 1.02</td>
<td>0.38</td>
<td>4.39</td>
<td>0.14</td>
<td>1.00</td>
</tr>
<tr>
<td>One month</td>
<td>I</td>
<td>x</td>
<td>3.44 18.96</td>
<td>10.12</td>
<td>51.89</td>
<td>1.56</td>
<td>9.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>σₓ</td>
<td>0.16 2.45</td>
<td>1.07</td>
<td>4.47</td>
<td>0.91</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>x</td>
<td>3.28 16.43</td>
<td>1.21</td>
<td>5.94</td>
<td>0.36</td>
<td>2.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>σₓ</td>
<td>0.11 1.66</td>
<td>1.21</td>
<td>6.32</td>
<td>1.22</td>
<td>7.01</td>
</tr>
<tr>
<td>Two months</td>
<td>I</td>
<td>x</td>
<td>3.41 24.69</td>
<td>1.09</td>
<td>4.24</td>
<td>0.48</td>
<td>3.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>σₓ</td>
<td>0.12 1.89</td>
<td>1.09</td>
<td>4.24</td>
<td>0.48</td>
<td>3.72</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>x</td>
<td>3.27 19.48</td>
<td>1.27</td>
<td>5.60</td>
<td>0.49</td>
<td>2.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>σₓ</td>
<td>0.14 1.12</td>
<td>1.27</td>
<td>5.60</td>
<td>0.49</td>
<td>2.65</td>
</tr>
</tbody>
</table>

I - Fresh milk cheese
II - Reconstituted milk cheeses (5 batches)
Fig. 1. Ripening indices of cheese made from fresh (a) and from reconstituted (x) milk. 1: young cheese; 2: one month matured cheese; 3: two months matured cheese.

Table 4. Scoring of cheese made from fresh milk (I) and reconstituted milks (II) after two months of ripening.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Finish and appearance (20)</th>
<th>Body and texture (30)</th>
<th>Flavour (50)</th>
<th>Comments</th>
<th>Total (max. 108)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1 20</td>
<td>5 10 14</td>
<td>20 20</td>
<td>White, soft, pure, slight salty.</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>2 20</td>
<td>5 9 13</td>
<td>18 19</td>
<td>Creamy, slight soft, acid, salty.</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>3 20</td>
<td>5 10 14</td>
<td>19 20</td>
<td>White, soft, pure, salty.</td>
<td>88</td>
</tr>
<tr>
<td>x</td>
<td>20</td>
<td>5 9.7 13.7</td>
<td>19 19.7</td>
<td>Creamy, smooth, slight salty.</td>
<td>87</td>
</tr>
<tr>
<td>II</td>
<td>1 20</td>
<td>5 9 12</td>
<td>18 20</td>
<td>Creamy, porous, brittle, distinct salty</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>2 20</td>
<td>5 9 12</td>
<td>16 17</td>
<td>Slight creamy, tough, distinct salty</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>3 20</td>
<td>5 8 11</td>
<td>17 18.3</td>
<td>Slight creamy, tough, distinct salty</td>
<td>80.7</td>
</tr>
</tbody>
</table>

constant throughout ripening. This result is in agreement with those of Poznanski and Kraszewska (19) and Readsveld and Klomp (21). Cheese II contained more salt than cheese I, due to the quicker penetration of the brine into cheese made from dried milks (17, 18).

Protein degradation of cheese

The breakdown of protein during cheese ripening was measured by the ripening indices as illustrated in Table 3 and Fig. 1.

In young cheese, a slight increase of soluble nitrogen was found in cheese II compared with cheese I. After one month and more pronounced at the end of two months cheese made from fresh milk had more soluble nitrogen than that made from reconstituted milk, i.e. 24.69% v. 19.48%.

The non-protein nitrogen values were the same in both cheeses at the beginning of ripening and increased during the ripening to be higher in cheese I than in cheese II, i.e. 14.01% and 12.46%, respectively.

Peptide nitrogen and amino acid nitrogen values were higher in cheese I than cheese II throughout the ripening period and affected the cheese flavour. The relative content of peptide nitrogen related to total nitrogen was 3.11% in cheese I and 2.50% in cheese II, while amino acid nitrogen relative to total nitrogen was...
Fig. 2. Young cheese made from fresh milk. The casein micelles (C) are coagulated and form an open network. In the whey phase (W) only rarely can single protein particles be seen. Generally, no adsorption of casein at the surface of fat globules (F) occurs. Bar, 0.5 μm.

Fig. 3. Young cheese made from reconstituted milk. In contrast to young cheese made from fresh milk (Fig. 2), the fat globules (F) are distinctly smaller and show a strong association with the casein (C). W: whey phase. Bar, 0.5 μm.
Fig. 4. Two months matured cheese made from fresh milk. The casein forms a nearly continuous mass of finely dispersed single protein particles. Occasionally small, particle-free areas of whey (W) occur. Bar, 0.5 μm.

4.01 % in cheese I and 3.73 % in cheese II after two months of ripening.

These analytical results show that the changes of protein degradation were more pronounced in cheese made from fresh milk.

Cheese scoring

Two months matured cheeses were scored for organoleptic properties. 20 points were given for finish and appearance, 30 points for body and texture (including holes, colour and consistency) and 50 points for flavour (aroma and taste) as shown in Table 4. (The point numbers are the maximum scores for the best cheese.) The external finish and appearance were similar in both treatments and no holes were observed. Colour of cheese I was white while that of cheese II was light creamy to creamy. The consistency of cheese I was soft and typical, while cheese II showed a tendency to be tough and brittle and sometimes had a porous texture. Control cheese I had a better flavour than cheese II.

The organoleptic properties of all cheeses were similar except for the porous structure and slightly higher saltiness of cheese II compared with cheese I.

Electron microscopy

Representative micrographs of the young cheese samples are shown in Figs. 2 and 3. The cheese made from raw milk (Fig. 2) is characterized by a loose network of partly coalesced casein micelles with whey forming a continuous phase. Because the raw milk was not homogenized and therefore the fat globules largely retained the original fat globule membrane casein is normally not associated with fat globules. The young cheese made from reconstituted milk (Fig. 3) shows a casein distribution which is roughly similar to that of young cheese made from raw milk. The main structural difference is the strong interaction between casein and fat globules which is a result of the homogenization applied during processing of the milk powders. As a result the small fat globules are largely embedded in the casein aggregates.

It is known that the development of the microstructure of various types of cheese during ripening is characterized by a varying degree of disintegration of casein micelles resulting in a penetration of whey into the disintegrated casein mass (11).

The microstructure of the cheeses after two months of ripening is shown in Fig. 4 (cheese made from raw milk) and Fig. 5 (cheese made from reconstituted milk). The main characteristic for both cheeses is the degradation of the casein into a more or less uniform matrix which is typical also for other cheese varieties (1, 8-10, 20). The protein matrix of the cheese made from raw milk (Fig. 4) is characterized by a very uniform distribution of small protein particles and by a rare occurrence of small areas (0.1-0.2 μm in diameter) of whey where protein particles are not detectable.

In contrast to this type of protein matrix structure, the cheese made from reconstituted milk (Fig. 5) shows a markedly less homogeneous distribution of protein particles. They appear to be still slightly aggregated thus making the whey phase clearly visible. The relatively loose and porous structure of the protein matrix of the cheese made from reconstituted milk must be ascribed to structural changes of the protein during the drying process of the milk (17, 18).

As a consequence of this loose structure the penetration of salt into the cheese made from reconstituted milk is more intensive than into the control cheese (see Tables 2 and 4). This has already been described for Camembert cheese (17, 18).
The structural differences of the protein matrices in ripened cheeses also may be related to the different consistencies (Table 4), especially toughness and brittleness and also the porous texture of the cheese made from reconstituted milk.

Conclusion

These studies have demonstrated that it is possible to produce a soft brine cheese of acceptable quality by use of instant whole milk powder. An increased saltiness may occur in comparison with cheese made from fresh milk due to a looser structure of the protein matrix.

Acknowledgements

This work was supported by a grant from the German Academic Exchange Service (DAAD) for M.M. Omar. The authors thank Mrs. A. Hinz for technical assistance.

References


Discussion with Reviewers

D.N. Holcomb: The $^0$SH of the reconstituted milk is almost double that of fresh milk. Is this difference solely due to the higher protein content of the reconstituted milk? Also, why is $\sigma_0$ so high for $^5$SH in reconstituted milk? Is there any relationship between cheese properties and $^0$SH?

Authors: The difference between $^0$SH of fresh milk and mean $^0$SH of the five batches of reconstituted milk must be primarily due to the different protein contents. The high $\sigma_0$ for $^5$SH in reconstituted milk reflects differences between the five different milk powders used. Pronounced relationships between cheese properties and $^0$SH were not obvious.

M. Kalab: Is it safe to assume that a close association between fat globules and casein in a soft brine cheese indicates that reconstituted nonfat dry milk had been used to make such a cheese? When you mention "representative" micrographs, do you mean that casein was attached to all fat globules, to most fat globules, or to some fat globules?

Authors: In the present study we used instant whole milk powder in which the fat phase had been homogenized. Since casein becomes closely associated with fat globules during homogenization of milk, such fat globules also subsequently become an integral part of the casein network during clotting processes. Original, unhomogenized fat globules do not show such an association with the casein network because the native fat globule membrane is a phospholipid-containing bio-membrane-like interfacial layer with quite different properties. Of course, this original milk fat globule membrane may be partly destroyed during mechanical and thermal processing of the raw milk or during the cheese manufacture and will then allow casein to become adsorbed to the fat phase to a very limited extent. This explains why in the cheese made from reconstituted milk nearly all fat globules were closely associated with the casein whereas in the cheese made from fresh milk most fat globules were not.

M. Kalab: Have you found that freeze-fracturing is more reliable than embedding in a resin, sectioning, and staining to explain the microstructure of cheese?

Authors: According to our experience freeze-fracturing is a very reliable technique for studying the microstructure of cheese, either in a young or a ripened state. In the plane of fracturing which is preserved by the platinum/carbon-replication not only size, shape and substructure of the casein aggregates, but also free protein particles of molecular dimensions and also the fat phase can be inspected in detail. Thin-sectioning techniques appear especially suitable e.g. for three dimensional structure analysis (stereo micrographs, serial sections), or for localizing certain constituents by specific staining or labelling techniques.

M. Kalab: Replicas initially floating on water are known to sink into acetone, warp, and roll. Which technique do you use and would you recommend to retrieve the cleaned replicas?

Authors: We normally transfer replicas from pure acetone to a 1:1 mixture of acetone and water and then to pure water. Often a direct transfer from acetone to water is successful but the replica may disintegrate into smaller pieces. If replicas don't completely unfurl on the water surface we put them back into acetone and repeat this procedure.

N.F. Olson: Other researchers (Green et al., J. Dairy Res. 48, 343) have observed increased coarseness of curd as the concentration of cheese milk was increased. Could the high concentration of dry milk used in treatment I have caused some or all of the coarse structure in this cheese?

Authors: It cannot be excluded that the higher concentration of dry milk used in these studies has partly contributed to the coarser structure. But according to other studies (ref. 17 and 18) this coarseness has to be mainly ascribed to effects resulting from the heating conditions during drying of milk.

Additional Reference

DEVELOPMENT OF MICROSTRUCTURE IN SET-STYLE NONFAT YOGHURT - A REVIEW

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Food Research Institute, Research Branch, Agriculture Canada, Ottawa, Ontario, Canada K1A 0C6

Abstract

The development of microstructure in natural set-style nonfat yoghurt was studied by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In addition to thin-sectioning and conventional SEM described in the literature, this review illustrates gelation of milk with micrographs obtained by rotary shadowing of casein micelles and their clusters. The existence of void spaces occupied by lactic acid bacteria in yoghurt was confirmed by cold-stage SEM of uncoated specimens. The microstructure of yoghurt is affected by the preheat treatment of milk, bacterial starter cultures, total solids content, and the presence of thickening agents. The microstructure was found to be related to firmness and susceptibility to syneresis. Suggestions on the preparation of yoghurt samples for electron microscopy have been included in this review.

Introduction

Traditionally, yoghurt is a cultured (fermented) milk product made by incubating milk with Lactobacillus bulgaricus and Streptococcus thermophilus cultures. Yoghurt (yogurt) is made with bacteria, except in Turkey, where the product is called yahourt or yaourt, jogurt or eyran, in which "a lactose fermentation yeast culture is also included with the lactic-acid organisms".

Initially, yoghurt was made from milk concentrated by boiling but today it is made from homogenized whole milk, partially skimmed milk, or from skim milk with or without added nonfat dry milk (NDM) solids. Technology, biochemistry, and quality appraisal of yoghurt have been reviewed in great detail.

In North America, yoghurt has become increasingly popular in the last 20 years and in Canada alone the production of yoghurt between 1977 and 1981 was increased by 56% (from $27.504 \times 10^6$ to $42.972 \times 10^6$ litres). Although initially yoghurt had a characteristic custard-like body and texture, recent development of the product has proceeded in various directions and yoghurt texture may vary today from a semifluid stirred yoghurt to a set-style yoghurt in which added gelatin may lead to a rigid, brittle gel. The introduction of flavoured yoghurt and yoghurt with fruits or jams has been an important factor leading to the increase in the popularity of yoghurt in North America.

The development of microstructure in yoghurt has been studied only to a limited extent in spite of the number of yoghurt varieties with different characteristics (stirred, set-style, plain, flavoured, "low-calorie", dried, frozen yoghurt, baby yoghurt etc.) on the market and in spite of the fact that some physical properties such as firmness, smoothness, ropiness, and susceptibility to syneresis are reflected by the microstructure.

The objectives of this review are to summarize recent results of microscopical studies of yoghurt and to stimulate the interest of microscopists in this milk product which is an excellent milk gel model.
Principles of yoghurt manufacture

In fluid milk, casein micelles exist as individual entities. Lactic acid produced by lactic acid bacteria (cocci and bacilli) lowers pH of the milk and destabilizes the casein micelles which become subsequently linked to each other in the form of clusters and chains. The clusters and chains are part of a protein network (matrix) in which the liquid phase of milk is immobilized and a gel is formed. The way in which casein micelles may interact depends on many variables such as the heat treatment of the milk destined for gelation, bacterial strains and the ratio of cocci to bacilli, rate of acid development and amount of lactic acid developed, temperature of incubation, total solids, presence of additives (thickening agents) etc.

Heating of milk destined for the production of yoghurt prior to the inoculation with lactic acid bacteria plays an important role in the development of the yoghurt. Grigorov et al. demonstrated that heating of milk to 85°C for 30 min led to minimum syneresis in yoghurt when compared to heating at lower or higher temperatures. Preheating the milk to higher temperatures (90° to 95°C), however, led to a decrease in the consistency of the yoghurt. Galesloot and Hassing suggested that milk should be heated to 90°C in a continuous-flow heater or to 85°C when heating is carried out in tanks. Thus, in general practice, milk destined for the manufacture of yoghurt is preheated to 90° to 95°C for 15-30 min.

Heating of milk

In a study on the effect of heating of milk upon yoghurt microstructure, Kalab et al. examined freshly skimmed milk or reconstituted low-fat NDM of an undisclosed fat content to 44, 55, 65, 75, 85, and 90°C. Immediately after the desired temperature was reached, the milk was cooled to 44°C, inoculated with L. bulgaricus and S. thermophilus cultures, and incubated at 44°C until it was gelled and a pH value of 4.3 in the yoghurt was reached. The yoghurt was then cooled to 6°C.

No differences in the appearance of the casein micelles between unheated milk (i.e. milk preheated to 44°C) and milk heated at 90°C by the authors 30 min before gelation. At approximately 16 min before gelation, casein particles in unheated milk developed short projections (appendages) (Fig. 1) and within 3 min groups containing two or three particles had joined together and these groups combined into larger clusters which started to form a three-dimensional network (Fig. 2). The appendages had vanished at that stage. Casein micelle chains were robust but "seams" between the fused casein particles were clearly visible (Fig. 3a). Finer chains were formed in yoghurt made from heated milk (Fig. 3b).

Holding milk at a high temperature was reported to have an effect on the casein micelles: in raw or in high-temperature short-time pasteurized milk the micelles had relatively smooth, uninterrupted contours compared to micelles from milk which had been heated to 90°C for 10 min or was autoclaved at 121.7°C for 15 min. In these latter milk samples, filamentous appendages bridged adjacent micelles and small amounts of the filamentous material were found free in the heated milk samples (Fig. 4). The appendages were retained until the late stages of culturing (at least 3.5 h). Although they became gradually more diffuse, their presence appeared to inhibit micellar contact and fusion (Fig. 5). In their search for the origin of the appendages, Davies et al. found that casein micelles heated in a protein-free milk dialyze were free of such appendages whereas the appendages developed on casein micelles heated in the presence of whey proteins. It was established that the presence of B-lactoglobulin was the prerequisite for the development of the appendages.

These appendages could have been formed either by heat-denatured B-lactoglobulin alone or by a complex consisting of B-lactoglobulin and κ-casein; such a complex develops in heated milk. The addition of N-ethylmaleimide (a thiol-blocking agent) to milk appreciably reduced the development of appendages when the milk was heated. However, because both the heat denaturation and precipitation of B-lactoglobulin and the formation of the B-lactoglobulin-κ-casein complex most probably involve disulfide bonding, the only conclusion made by Davies et al. was that B-lactoglobulin participated in the formation of the appendages. The hypothesis that denatured B-lactoglobulin is bound to casein micelles in milk heated to 90°C is supported by gel electrophoresis of whey separated by centrifugation from yoghurt made from heated milk. B-lactoglobulin was present in whey separated from yoghurt made from unheated milk but was absent in whey separated from yoghurt made from heated milk.

More recently, Kalab et al. examined casein micelles in unheated and heated milk using rotary shadowing with platinum and carbon. In milk heated to 90°C for 10 min the casein micelles had "ragged" surfaces, whereas micelles in unheated milk had smooth surfaces (Fig. 6). The same technique was used to examine changes in casein micelles developing during the gelation of milk by yoghurt starter cultures and the formation of casein micelle chains was also demonstrated (Fig. 7 and 8). Thus, it may be concluded that casein micelles in heated milk acquire a "ragged" appearance as the result of the fusion of denatured B-lactoglobulin or a heat-induced B-lactoglobulin-κ-casein complex to their surfaces. The temporary appendages observed by Kalab et al. in unheated milk shortly before gelation are difficult to explain unless an assumption is made that they are different from the appendages observed on casein micelles in heated milk; in their work, Kalab et al. mixed the milk with a warm agar sol and the casein micelle gel had solidified. In contrast, Davies et al. first fixed the micelles and subsequently immobilized them in agar gel. When unfixed milk is mixed with warm agar, an interaction between casein micelles "ready to gel" and the agar cannot be excluded as the cause of the temporary appendages. As the pH of the milk gradually declined below 4.3 because of the production of lactic acid by the bacterial culture, the gelation of the milk was completed and yoghurt was formed. It has already been mentioned earlier that yoghurt made from unheated milk consisted of a network considerably more robust (Fig. 3a) than yoghurt made from heated milk.
Fig. 1. A detail of casein micelle aggregation in unheated skim milk 16 min before gelation. Appendages on casein micelles (arrows) are clearly visible and are different from agar fibres (A). Reproduced by permission from Milchwissenschaft.

Fig. 2. Onset of casein micelle aggregation and formation of clusters (large structures in the left half of the micrograph) in unheated skim milk. Arrows point to small fat globules present in very small quantities in that skim milk.

Fig. 3. Protein matrix in yoghurt made from unheated (a) and heated (b) skim milk. In (a) casein micelle clusters are tightly fused and form large aggregates, yet "seams" between the particles are visible in thin sections (arrows).

In (b) the casein micelles are linked in finer chains; appendages on the casein micelle surfaces are still present at the early stages of gelation (arrows). Micrograph (a) reproduced by permission from Milchwissenschaft.

Fig. 4. Casein micelles in raw milk (a), in milk heated to 95°C for 10 min (b), and in milk autoclaved at 121.7°C for 15 min (c). Reproduced by permission from the Journal of Dairy Research.
Fig. 5. Casein micelles in raw (a and b) and in heated (95°C for 10 min) (c and d) skim milk at various intervals during fermentation with S. thermophilus and L. lactis to yoghurt. Fermentation times: a: 4.5 h; b: 5 h (both samples are from raw milk); c: 3.5 h; d: 3.75 h (both samples are from heated milk). Reproduced by permission from the Journal of Dairy Research.

Fig. 6 (left). Details of casein micelle surfaces by rotary shadowing with platinum and carbon. a: Casein micelle in heated skim milk; halo around the appendages (arrows) is caused by carbon reinforcement of the platinum replica; b: casein micelle in unheated skim milk has a smooth surface.

Fig. 7 (below). Detail of casein micelle chaining (rotary shadowing) during the gelation of reconstituted NDM.

Fig. 8 (right). Casein micelles in heated skim milk form chains and loops (arrows) which will develop into a three-dimensional matrix at later stages of gelation.
MICROSTRUCTURE OF YOGHURT

Fig. 9. SEM of yoghurt made from unheated (a and b) and heated skim milk (c and d). In yoghurt made from unheated skim milk the casein micelles are in the form of coarse clusters (a) which results in large compartments (cells) in the matrix (shown at a lower magnification in b). In yoghurt made from heated skim milk the casein micelles are in the form of chains (c) and this results in smaller compartments in the matrix (d).

Fig. 10. A schematic diagram comparing the microstructure of yoghurt made from heated milk (a) composed of small compartments formed by single branched chains of casein micelles and the microstructure of a yoghurt made from unheated milk (b) composed of large compartments formed by clustered casein micelles. Firmer immobilization of the liquid phase is experienced with (a) than with (b).

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milk (Fig. 3b). This was also confirmed by SEM\textsuperscript{22}; details of casein micelle aggregation are shown in Figs. 9a and c and the organization of the matrices is presented at a lower magnification in Figs. 9b and d. On the basis of similar micrographs a model (Fig. 10) of the different matrices had been designed earlier\textsuperscript{12}. In the diagram in Fig. 10, the same number of casein micelles is shown in sections of a fine network (a) and a coarse network (b). The liquid phase (whey) was immobilized in the fine matrix which consisted of small compartments but the whey separated more easily from larger compartments in the coarse matrix. Susceptibility to syneresis in both yoghurts was measured by the volume of whey separated from the milk gels by centrifugation at several different centrifugal forces for 10 min\textsuperscript{12,14} (Fig. 11) and by draining the yoghurts for up to 1 h\textsuperscript{24} (Fig. 12). It is evident from these measurements that yoghurts made from unheated milk were considerably more susceptible to the separation of whey than yoghurts made from heated milk. Tarodo de la Fuente and Alais\textsuperscript{45} explained that heating increased solvation of casein micelles and that after heating to 90°C for 2 min
there was about twice as much non-solvent water in the micelles as in the raw milk micelles. Denatured β-lactoglobulin either alone or in the form of a complex with κ-casein in heated milk inhibited micelle fusion, which is in agreement with an earlier postulate by Knoop and Peters. The formation of a fine protein network in yoghurt made from heated milk resulted from this inhibition of micelle fusion. In addition to the preceding experiments which had been carried out using fresh milk or reconstituted spray-dried NDM, yoghurts were also made from reconstituted roller-dried and freeze-dried NDM. Although the milk in the roller-dried NDM had received a severe heat treatment during production, yoghurt made from this source was of a very poor quality as far as flavour, texture, and susceptibility to syneresis were concerned. This is probably associated with the formation of fused micellar aggregates and the inability of the casein micelles in roller-dried NDM to freely disperse in water. Additional heating of the reconstituted NDM suspension had no effect on the dimensions of casein particles in that yoghurt. However, in yoghurt made from reconstituted freeze-dried NDM the differences between casein particle dimensions depending on the heating of milk were greatest of all types of NDM.

**Total solids content**

Dimensions of the compartments in the protein matrix of yoghurt and, thus, susceptibility to syneresis, are also affected by factors other than the preheat treatment of milk prior to gelation.

One of these factors is the total solids content in the milk. In practice, the total solids content is largely dictated by legal standards of the country concerned. It may vary from 9 to over 20%, but bacterial activity is retarded at levels exceeding 25% total solids. The range most generally recommended lies between 14 and 18% according to Robinson and Tamime or between 15.5 and 16.0% according to Koshe et al. The increase in the total solids content can be achieved in several ways. By maintaining the same casein to non-casein protein ratio, there is a higher number of casein micelles per unit volume at a higher total solids content which results in smaller compartments occupied by a smaller volume of the liquid phase. This is evident in Fig. 12, where the protein matrices are shown in yoghurt made from low-heat spray-dried reconstituted NDM containing 10 to 20% total solids. In addition to smaller compartments, a higher number of casein micelles and a larger amount of whey proteins per unit volume bind a larger proportion of water. This was demonstrated by draining the different yoghurts using the procedure described by Modler et al. (Fig. 12) and by centrifuging the yoghurts at different centrifugal forces for 10 min (Fig. 11). Only a part of the aqueous phase in the yoghurt may be separated from the protein matrix by centrifugation because the other part is firmly bound by the proteins. As the total solids content in the yoghurt is increased, the volume of the aqueous phase which may be separated is decreased. Matrices of low-solids yoghurts are subject to noticeable compaction whereas matrices of...
Fig. 13. Protein matrices in yoghurts made from heated reconstituted nonfat dry milk containing 10.0% (a), 15.0% (b), and 20.0% (c) total solids. The dimensions of compartments (pores) in the matrices are decreased as the total solids contents are increased. At higher (15 and 20%) total solids contents void spaces around lactic acid bacteria become more clearly evident.

Fig. 14. Comparison of protein matrices in an unfortified yoghurt (a) and a yoghurt fortified with a whey protein concentrate (b). In unfortified yoghurt (a) the casein micelles form chains by contacting each other (arrows) whereas in the fortified yoghurt (b) the casein micelles are linked to each other with fine aggregates of whey protein (arrows). Reproduced by permission from the Journal of Dairy Science 28.

Microstructure of yoghurt

The total solids content in milk may be increased by evaporation, ultrafiltration (which selectively increases only the fat and protein content), reverse osmosis, or by addition of NDM, milk protein concentrate, whey powder etc. Microstructure of yoghurt made from skim milk (3.5% total protein) fortified to 5.0% total protein was studied recently28,29. Sodium caseinate, NDM, milk protein concentrate, and whey protein concentrates, commercially obtained by electrodialysis, ultrafiltration, and ion exchange were used as the fortification agents. Because of the different composition of the additives, the casein to non-casein protein ratio was 4.86 : 1.00 in yoghurt fortified with sodium caseinate, 2.85 : 1.00 in yoghurt fortified with NDM and milk protein concentrate, and 1.08 : 1.00 in yoghurt fortified
with whey protein concentrates.

Sodium caseinate significantly increased the dimensions of casein particles in the fortified yoghurt and formed a microstructure similar to that in yoghurt made from unheated milk. Because the addition of sodium caseinate decreased the relative non-casein protein content in the milk mixture to 18%, it may be hypothesized that the casein micelles in the milk thus fortified were insufficiendy coated with denatured β-lactoglobulin or with the β-lactoglobulin-κ-casein complex and, thus, were not prevented from excessive fusion. However, the true mechanism has yet to be studied. Interestingly, susceptibility to syneresis was lowest (8%) and gel strength was highest (117.9 g) in the yoghurt fortified with sodium caseinate. NDM and milk protein concentrate reduced susceptibility to syneresis and gel strength as compared to unfortified yoghurt but had no significant effect on the microstructure.

Whey protein concentrates considerably altered the microstructure of the fortified yoghurts as compared to unfortified yoghurt (Fig. 14). Instead of casein micelles tightly fused into compact clusters containing the milk with natural calcium to non-casein protein ratio, the casein micelles were linked to each other at relatively long distances with finely flocculated whey proteins (Fig. 15). Gel strength of the fortified yoghurts was approximately the same (77-79 g) as that of yoghurt fortified with NDM when the whey protein concentrate used was obtained by ultrafiltration. The gel strength was lower (50-55 g) with whey protein concentrates obtained by methods in which electrodialysis and ion exchange were part of the process.

Thickening agents

It is not always feasible to increase the total solids content of yoghurt in order to improve texturality. Susceptibility to syneresis, there may be various reasons for such a step, for example the concern for the joule (calorie) value, the lactose content, retardation of the bacterial action and hence prolonged coagulation time, titratable acidity, etc. There is a wide variety of thickening agents available to facilitate the immobilization of the yield phase in yoghurt: gelatin, pregelatinized starch, cellulose derivatives, alginites, and various gums have been used commercially. Effects of a great number of thickening agents on the quality of yoghurt were studied by Radema and Van Dijk. Some thickening agents were found to have a tendency to decrease the rate of the lactic acid production. Kalab et al. examined the microstructure of yoghurt as related to the presence of gelatin, carrageenan, and pregelatinized starch. At a 0.5% concentration, gelatin did not significantly affect gel strength but considerably reduced syneresis to less than 2.5%. However, yoghurts containing this concentration of 225 Bloom gelatin were indistinguishable by SEM and TEM from yoghurts not containing any additive. No change in the microstructure of the protein matrix was observed even with the gelatin concentration increased to 10% when the yoghurt resembled a gelatin gel rather than a typical yoghurt. This was probably because neither of the electron microscopic techniques used was suitable to detect collagen fibres of gelatin in the milk gel. Although the authors referred to an earlier report showing a gelatin gel composed of thin sheets, such sheets represented typical artefacts formed by slow freezing of the gel whereby large ice crystals developed, and the gelatin was compressed into the form of thin sheets between the ice crystals. After freeze-drying and sublimation of the ice, these sheets were visualized by SEM.

Carrageenan and pregelatinized starch were reported to considerably change the microstructure of yoghurt. Both additives led to the clustering of casein micelles as was demonstrated in thin sections of embedded specimens; the density of the casein micelle clusters was higher in the presence of carrageenan. Interactions with carrageenan, which led to the aggregation of casein micelles, were studied by Snoeren and Hood and Allen. Although short fibres with free terminals and sheets were reported by SEM to be present in yoghurt containing pregelatinized starch, it is highly probable that the initial microstructure of the specimens had been altered during preparatory steps such as freeze-drying and that the structures found were actually artefacts. Several authors pointed to critical-point drying as a technique not suitable to dry food samples containing pregelatinized starch. The only proper electron microscopic technique to visualize gelatinized starch is freeze-fracturing followed by replication with platinum and carbon. Hence it follows that results obtained by examining the effects of thickening agents on the microstructure of yoghurt should be revised.

Lactic acid bacteria

In yoghurt, the milk is gelled by a combined action of cocci and bacilli which digest lactose and convert it into lactic acid, thus lowering pH of the milk which leads to its gelation. In addition, lactic acid bacteria cause a significant degree of proteolysis in yoghurt, which leads to changes in the physical structure of the product, although these bacteria are usually considered to be only weakly proteolytic.
Yogurt starter bacteria, *L. bulgaricus* and *S. thermophilus*, are thermoduric, homofermentive lactic acid bacteria whose characteristics were summarized by Tamime and Deeth\(^{43}\). The ratio of *L. bulgaricus* to *S. thermophilus* determines flavour and body characteristics of the ripened yogurt. In yogurt manufacture, ratios of 1:1 to 1:3 have been used. However, Tamime and Deeth\(^{44}\) noted that the term "ratio" as it appears in the literature, is somewhat vague because it may refer to the colony-to-colony, clump-to-clump, chain-to-chain, or cell-to-cell ratios of *Lactobacillus*-to-*Streptococcus*. According to the starter manufacturers, a ratio of 1:1 means 5 to 10 cells of *S. thermophilus* to one cell of *L. bulgaricus*\(^{43}\).

SEM examination at low magnifications of smooth...
Fig. 19. Cold-stage SEM of uncoated yoghurt revealing the presence of void spaces occupied by lactic acid bacteria. Absence of a conductive metal layer led to some charging artefacts (lines and excessively light areas) but in general, the void spaces are clearly visible.

Fig. 20. Dead lactobacilli (arrows) are tightly embedded in the protein matrix of a yoghurt made by culturing the milk with viable streptococci. No void spaces are formed around dead bacteria.

Fig. 21. Mucogenic (slime-producing) lactobacilli in a set-style yoghurt, demonstrating the presence of an exocellular polysaccharide (filaments). (The yoghurt sample was provided by Dr. A.Y. Tamime).

Fractures obtained by freeze-fracturing reveals numerous cavities (void spaces) in the protein matrix (Fig. 16). The presence of lactic acid bacteria in such void spaces becomes apparent as the magnification is increased (Figs. 17 and 18). The void spaces around the bacteria were attributed to bacterial action, although C.J. Thomas (personal communication) suspected that they may be artefacts arising from differences in the shrinkage of the protein matrix and the bacterial clusters during the preparation of the yoghurt samples for electron microscopy. A hypothesis that the void spaces are caused by proteolytic enzymes produced by the bacteria was tested in several kinds of experiments: (a) One of the strains in the mixed starter culture used to make the yoghurt (cocci or bacilli) was killed by physical (radiation) or chemical (formaldehyde) means. The dead bacteria were incorporated in the milk at a concentration 1,000-fold higher than that of the viable strain; the reason for this difference was that the viable bacteria propagated in the milk whereas the dead bacteria did not. (b) Cold-stage SEM was used to examine yoghurt unaffected by fixation, dehydration, and drying and the micrographs were compared with those obtained by conventional SEM of glutaraldehyde-fixed, freeze-fractured, and dried yoghurt samples. (c) Viable bacterial cultures differing in the
amount of protease produced (i.e., so-called protease-positive and protease-negative strains) were used to make yoghurt and their effects on the microstructure of the protein matrix were studied by conventional SEM.

Cold-stage SEM of freeze-fractured yoghurt not coated with gold clearly demonstrated the existence of void spaces with bacteria in the protein matrix; this is evident (Fig. 19) in spite of the poor quality of the micrograph caused by the examination of the frozen yoghurt samples without coating them with gold which means that conductivity depended on the ions present in the yoghurt. However, this approach had the advantage in that the matrix was examined during freeze-etching as the ice present in it gradually sublimed off and exposed the underlying structures.

SEM of yoghurts containing viable and dead bacteria showed void spaces only around viable bacteria whereas dead bacteria were embedded tightly in the protein matrix (Fig. 20). Dead bacteria, however, did not retain their initial shapes and were subjected to changes, particularly shrinkage. Protease-positive bacteria formed void spaces but protease-negative bacteria did not; however, the studies on the relationship between the dimensions of the void spaces and the production of proteolytic enzymes by the bacteria have not yet been concluded.

Another possibility for the development of the void spaces was mentioned by N.F. Olson (personal communication) who suggested that casein micelles which are closest to the bacteria are coagulated by lactic acid first because its concentration is highest in the vicinity of the bacteria which produce it. As the coagulation proceeds outwards, the freshly formed gel may separate from the bacteria and lead to the development of void spaces.

There is evidence in some yoghurts that the casein micelle matrix surrounding the void spaces was subjected to stress. Casein micelle chains are stretched and oriented in one direction, which is particularly clearly visible in pairs of stereo micrographs (Figs. 17 and 18), and which may support the hypothesis by Olson.

In general, higher total solids in yoghurt make it easier to detect the void spaces. In some yoghurts the number and dimensions of the void spaces may reach significant proportions affecting the integrity of the matrix.

Bacteria contribute to the microstructure of yoghurt in an additional manner. Many lactic acid bacteria possess the ability to produce extracellular polysaccharides, particularly when grown in the presence of sucrose. So-called slime-producing, mucogenic, or "ropy" lactic acid bacteria cultures were developed in the Netherlands for use in yoghurt. By SEM, such cultures are characterized by the presence of filaments which attach the bacteria (bacilli in Fig. 21 and cocci in Fig. 22) to each other and to the protein matrix. As extracellular polysaccharides have some of the properties of the thickening agents, the use of mucogenic cultures makes it possible to prepare viscous yoghurt without the use of exogenous additives.

Electron microscopy

Examination of fixed and dried samples by conventional SEM and TEM of thin sections of yoghurt samples embedded in a resin were most frequently used to study the microstructure of this product. In this review, micrographs obtained by rotary shadowing of casein micelles and their clusters at the early stages of gelation are also presented. For this purpose, casein micelles fixed in a glutaraldehyde solution were attached to freshly cleaved mica sheets pretreated with poly-L-lysine, dehydrated in a graded ethanol series, critical-point dried, and shadowed with platinum and carbon at a 45° angle while the samples were rotated. The replicas of the casein micelles or their clusters thus obtained were cleaned in a 3% sodium hypochlorite solution, washed with water, placed on 400-mesh grids, and examined in an electron microscope operated at 60 kV. Pro tease-negative samples were made using 35-mm 135-ASA film.

Embedding of casein micelles in a resin was usually preceded by their fixation and immobilization by either mixing them with a warm agar solution or by encapsulating them in agar gel tubes. The solidified samples were postfixed in a 2% OsO4 solution in a 0.05 M veronal-acetate buffer (pH 7.5) or in a 0.2 M cacodylate-HCl buffer (pH 7.2). Samples (~1 mm^3 ) of finished yoghurt were fixed and postfixed in the same way as immobilized casein micelles, embedded in a Spurr's low-viscosity medium or Araldite, sectioned, and stained with uranyl acetate and lead citrate for electron microscopy.

The incubated yoghurt mix was sampled before gelation at regular 3 to 4 min intervals. Embedding of immobilized casein micelles and of small yoghurt samples is easy as the porous matrices are rapidly impregnated with the resin monomer. However, Schmidt warned that so-called immobilized casein micelles may be artefacts arising from the preparatory steps. The appendages observed in casein micelle thin sections were not found when similar samples were freeze-fractured and replicated with platinum and carbon; instead of threads, an increased number of free particles of submicellar dimensions was observed. Schmidt (personal communication) suggested that at least a part of the "spikes" may be due to precipitated calcium phosphate.

Freeze-fracturing followed by replication with platinum and carbon is better suited to examine the progress of gelation of milk in greater detail and can also be used to elucidate interactions of casein micelles with additives such as gelatin, starch, alginates etc. and fortifying agents such as whey proteins. However, this method has yet to be employed in such studies. One reason for not using it to its potential may be that unless quality, and thus expensive equipment is used both to rapidly freeze the specimen and to fracture and replicate them, the artefacts arising from the inappropriate execution of the technique may be a deterrent. It is evident from the previous discussion that the onset of gelation and interactions with additives need to be studied in greater detail.
Conventional SEM of the gelled yoghurt is easy to carry out provided that certain rules are followed. As was mentioned earlier, fixed samples may be freeze-fractured either with the aqueous phase present or replaced with absolute alcohol. In the former case, the fragments are freeze-dried whereas in the latter case they are melted in absolute alcohol and subsequently critical-point dried. This procedure has no harmful effects on the yoghurt matrix.

Freeze-fracturing leads to images superior to those obtained by dry-fracturing. Smooth fracture planes obtained by freeze-fracturing are suitable for studies of the porosity of the matrix, linkages of casein micelles in clusters and chains, distribution of void spaces etc. Dry-fractured particles, on the other hand, are characterized by ragged surfaces of a complex topography which are difficult to both photograph and interpret (Fig. 23) as the structure elements are not viewed in the same plane.

Mounting of the fragments on SEM stubs has to be done carefully to provide a base for a conductive path for the electrons after the particles are coated with carbon and gold. In practice it means that the particles should have relatively small fracture planes (<1 mm²) and be low (<0.5 mm). It is advisable to position the particles on top of a droplet of a conductive cement of a proper consistency (free solvent should not penetrate the matrix) with the fractured plane facing up and to paint the sides of the particle with the cement as close to the fracture plane as possible (Fig. 24).

Coating of the fragments with carbon prior to gold coating improves the final SEM image. The angles (a minimum of two) at which coating with gold is carried out by the evaporation technique should be properly selected. Coating at an angle too acute (low) fails to provide enough gold to form an uninterrupted conductive path on the casein micelles and clusters. Also the amount of gold used is important as the large total surface of the protein matrix requires more gold to be evaporated than does coating of compact surfaces such as fractured cheese. The presence of a small number of nitrogen molecules was used²⁸ to induce collisions with the gold atoms and their deflections;

this resulted in their deposition on the protein matrix from various directions making the coating uniform. Sputter coating of yoghurt samples in the authors' laboratory failed to produce better images than evaporative coating.

The porous matrix of yoghurt is susceptible to electron beam damage and, consequently, focussing of the electron beam should be carried out rapidly.

Cold-stage SEM of uncoated samples produces inferior images and is justified only under specific circumstances. However, continuous scanning of the freeze-fractured sample makes it possible to study the gradual emergence of the protein matrix from the aqueous phase as the ice sublimes off; needless to say this technique contributes to the contamination of the microscope.

Conclusion

Yoghurt has become an important milk product which is made in a great variety of styles differing in texture, flavour, and flavour (caloric) content. Microstructure and related properties such as susceptibility to syneresis and firmness depend on the heat treatment of milk, total solids contents, bacterial starter cultures, conditions of culturing, presence of additives, and other factors. A wide field is open to studies of the microstructure of set-style as well as stirred and frozen yoghurts. Electron microscopical techniques used should not be limited to thin-sectioning and conventional SEM but should also include freeze-fracturing and freeze-etching followed by replication with platinum and carbon. In addition to points already raised in this review, it would be interesting to study the role of fat globules in yoghurts made from whole unhomogenized and from homogenized milk, particularly in view of the hypothesis postulated by van Vliet and Dentener-Kikkert⁶ that a fat globule membrane reacting with milk proteins will increase the strength of the resulting milk gel as opposed to a nonreactive membrane.

It is evident that electron microscope can play an important role in the study of the gelation of milk and in anticipating and explaining some physical properties of newly developed yoghurt. The objective of this review was to stimulate such studies.
Acknowledgments

Skillful assistance provided by Mr. J.A.G. Larose and Mr. Sierk Itz is acknowledged. The authors thank Dr. F.L. Davies and the Journal of Dairy Research (National Institute for Research in Dairyling, U.K.) for permission to reproduce Figs. 4 and 5 and to Dr. E.J. Thomas and Dr. N.F. Olson for personal communications on the origin of void spaces in yoghurt. Dr. A.Y. Tamime provided samples of yoghurt, the micrograph of which constitutes Fig. 21. Dr. R.P. Sinha carried out experiments on the inclusion of dead lactic acid bacteria in yoghurt.

Figs. 1 and 3a are reprinted by permission from Milchwissenschaft, Fig. 10 is reprinted by permission from the Journal of Texture Studies (Food and Nutrition Press, Inc., U.S.A.), Figs. 14 and 15 are reprinted by permission from the Journal of Dairy Science (American Dairy Science Association), and Figs. 11 and 12 are from a manuscript submitted by Dr. V.R. Harwalkar and M. Kalab for publication in Milchwissenschaft. Useful suggestions provided by Dr. H.W. Modler are acknowledged. Electron Microscope Centre, Research Branch, Agriculture Canada in Ottawa provided facilities. This review is Contribution 535 from the Food Research Institute, Research Branch, Agriculture Canada in Ottawa.

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4. Dairy Facts and Figures at a Glance. Published by Dairy Farmers of Canada, 111 Sparks Street, Ottawa, Ontario, Canada K1P 5B8, Table 21.


M. KALÁB, P. ALLAN-WOJTAS, and B.E. PHIPPS-TODD


43. Tamime AY. (1977). The behaviour of different starter cultures during the manufacture of yoghurt from hydrolysed milk. Dairy Ind. Internat. 42(8), 7-11.


Discussion with Reviewers

M. Rüegg: You have observed that acid coagulation of casein micelles leads to the formation of clusters and chains. In the case of rennet coagulation, the predominant form of aggregation of the casein particles seems to be the chain formation. Both theoretical considerations and experimental observations indicate that linear chains are formed with the highest probability, followed by branching. Do you think that cluster formation is typical for acid coagulation?

Authors: Chains as the predominant form of casein micelle aggregation in yoghurt are found in all samples made from preheated milk; preheating of milk is always practised in yoghurt manufacture. In milk that had not been preheated and was gelled by a yoghurt starter culture, however, the predominant form of micelle aggregation is clustering; there is sufficient electron microscopical support for this statement although no theoretical considerations have yet been made on this type of casein micelle aggregation.

A.Y. Tamime: The authors have suggested in their discussion that the protein/protein interaction takes place only between β-lactoglobulin and κ-casein; I wonder, however, whether α-lactalbumin is also involved in such interactions.

Authors: We have found no references which would indicate that α-lactalbumin is involved in the formation of "spikes" on casein micelles in heated milk.

D.N. Holcomb: What artefacts, if any, result if casein micelles are postfixed with OsO4 without first being immobilized in agar?

Authors: Postfixation of casein micelles with OsO4 is a logical step in preparing casein micelles for...
embedding in a resin, be it executed by immobilizing them in agar or centrifuging and embedding them in the form of a pellet. Shimmin and Hill\textsuperscript{29} even used postfixation with OSO\textsubscript{4}, in the case of casein micelles destined for negative staining and metal-shadowing. When casein micelles are immobilized in agar, it is easier to approximate the OSO\textsubscript{4} solution from the agar gel particles than from casein micelles freely dispersed in the postfixing solution. The question thus can be reformulated as to whether any artifacts develop in casein micelles fixed and postfixied only after they had been immobilized in agar gel. This is an interesting question and will be answered by practical experiments.

D.N. Holcomb: The authors identify agar fibres in Fig. 1. Should these also be visible in Fig. 2? Authors: Fig. 1 was obtained at a high magnification where agar fibres are clearly distinguished. They are not evident in Fig. 2 because of the considerably lower magnification.

M. Rüegg: This review is concerned with nonfat yoghurts only. The residual fat content of skimmed milk is usually smaller than 0.05%. Nevertheless, this small percentage corresponds to at least $10^5$ to $10^8$ fat globules per 1 g of milk or yoghurt. Did you observe these fat globules or were they removed during the preparation procedure? Authors: We did not observe fat globules in nonfat milk yoghurts although no attempts were made to remove them from the samples on purpose. It is possible, however, that the fat globules were removed during the preparation of the samples for SEM. This preparation consisted of fixing the samples in a glutaraldehyde solution, dehydrating them in a graded alcohol series, and freeze-fracturing. The resulting fragments were melted in absolute alcohol and were subsequently critical-point dried from carbon dioxide. The fat globules were, thus, exposed to absolute alcohol and carbon dioxide, both to be known lipophilic solvents. It is probable that the fat was extracted during those steps. Otherwise, there would be approximately 0.01 to 1 fat globule within the field of vision in the scanning electron microscope used at a 1,200X magnification considering your assumption that there were $10^5$ to $10^8$ fat globules in 1 g of yoghurt.

M. Rüegg: The interface between the coagulated milk and the walls of the container plays an important role for the syneresis of yoghurt. Certain surfaces retard and others promote the shrinkage of the casein network. Has this phenomenon been studied using electron microscopy? Which preparation technique could be recommended for such a study?

Authors: It is regrettable that no reference has been made concerning this phenomenon. In their book, Rašić and Kurmann compare the advantages and disadvantages of various packaging materials. So, for example, glass prevents gas diffusion and does not interact with the product. Polyvinyl chloride (PVC) and polyvinylidene chloride (PVDC) have a relatively low permeability to water vapour, oxygen, nitrogen, and carbon dioxide, whereas poly-stryre and polystyrene demonstrate a high permeability for the above gases. Waxseed cotton, susceptible to degradation and discoloration in fruit yoghurts, indicates a selective absorption of some substances from the yoghurt. However, no relationship between the packaging materials and syneresis in yoghurt has been mentioned. We consulted commercial yoghurt manufacturers in Canada to answer your question. One of them assumed that if differences in syneresis are noted in yoghurt transported in various packaging materials, such differences can result from differences in the way vibrations are transmitted to the yoghurt during transportation. The other manufacturer reported no differences in syneresis among yoghurts made in stainless steel pots and tanks, in glass bottles, and in different types of plastic containers provided that other variables such as the type of culture used, product formula, and processing conditions were the same.

Interactions between yoghurt and the packaging materials could be studied by immersing test strips of uniform dimensions in the milk and by SEM of the material adhering to the strips in the finished yoghurt.

F.L. Davies: In experiments to determine the cause of void spaces surrounding bacteria, the authors report that proteinase-positive bacteria (lactobacilli) formed void spaces whereas proteinase-negative variants did not. It should be remembered that S. thermophilus has little or no proteolytic activity, depending, in yoghurt, upon L. bulgaricus to effect the initial breakdown of proteins. Examination of figures 9b, 9d, 13a, 13b, 13c, 17, and 18 suggests that void spaces are equally (if not more) evident around the non-proteolytic streptococci as around the strongly proteolytic lactobacilli. This argues against proteinase activity as the cause of void spaces and perhaps adds weight to Olson's hypothesis concerning local concentrations of lactic acid.

D.G. Schmidt: In view of the results presented, which clearly show a relationship between the voids around bacteria and proteolytic activity, I consider Olson's hypothesis inferior to the proteolysis hypothesis unless a definite answer can be given to the question "Why should the gel separate from the bacteria?"

M. Rüegg: The readers should be informed whether the void spaces shown in Fig. 19 were formed after the sublimation of ice or whether they correspond to gas bubbles.

Authors: At present we are unable to draw any definite conclusion concerning the origin of void spaces around lactic acid bacteria in yoghurt, because the evidence presented is insufficient to support any hypothesis. The suggestion to more closely examine the nature of the void spaces (fluid or gas) is important, particularly in view of the work by Driessen et al.\textsuperscript{31}; these authors showed that carbon dioxide produced by S. thermophilus is need-

D.N. Holcomb: What SEM conditions (beam voltage, working distance, etc.) did the authors use for cold-stage SEM? Would use of a so-called "charge neutralizer" (e.g., C.K. Crawford: Charge
neutralization using very low energy ions. Scanning Electron Microsc. 1979; II: 31-46) help to improve the quality of the cold-stage SEM micrographs?
Authors: Accelerating voltage was 10 kV, beam current was 50 to 70 μA, and the working distance was 7.1 mm. The charge neutralizer might help improve the quality of the micrographs, particularly those taken at a low magnification, but we have no practical experience with this SEM accessory.

Additional References


FIELD SPECTROSCOPY IN THE FOOD PRODUCTION CHAIN

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Abstract

This paper reviews the application of field spectroscopy in the food production chain, particularly how field spectroscopy aids research and production of agricultural crops, and helps to understand the process of converting these crops into food.

The review discusses the nondestructive application of field spectroscopy such as ultraviolet, reflective, visible, near infrared and fluorescence techniques using conventional or laser light sources, to determine qualitative and quantitative properties of agricultural crops and produce.

Field spectroscopy can provide data on soil constituents and moisture, crop maturity, crop vigor, crop health, variety, variety within crops, and protein and moisture content in a standing crop. It provides data on gas particulates in cold storage, and growth rooms (CO₂ and O₂).

Introduction

The term of "Field Spectroscopy" implies that spectroscopic measurements are performed in the field without disturbing the object to be measured, be they growing crops, soil, harvested produce or processed food, in other words a nondestructive method.

Spectroscopy measures the electromagnetic spectra (EMS) generated by the interaction of incident radiant energy (solar or artificial) with the material to be measured or analyzed. The EMS produced by this interaction produces two types of spectra: a) emission; b) absorption. In spectro-radiometric measurements these spectra are characteristics of transitions whose energy requirements are related to specific wavelengths where transition energies can be measured by the amount of energy absorbed or reflected. In the field spectroscopy domain this is confined to the ultraviolet (UV), visible and infrared (IR) spectral ranges.

In the food production chain, the application of field spectroscopy is diverse, and requires spectroradiometers of different kinds both in principle and technical arrangement.

The theory of spectroscopy is well recorded in the literature [2,4,13,19,21,22,23,25,27,34,35,41,42,43,52,53]. Several practical applications are described by authors in scientific and technical papers of various aspects of spectroscopy applied to soils, crops and produce. This paper will review these applications along with the work accomplished at the Engineering and Statistical Research Institute in cooperation with various Research Stations of Agriculture Canada and Universities.

The objective of this paper is to focus the attention of food scientists on field spectroscopy since it is gaining importance in the evaluation of agricultural crops by remote sensors.

KEY WORDS: spectroscopy, radiometry, reflectance, maturity detection, stress detection, protein detection

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Fundamental Concepts

In the investigation of the role of radiant energy in biological systems, one must consider both the complexity of photochemical processes in biological systems and the proper selection and application of appropriate techniques of radiation production, control, and measurement.

Radiant energy is propagated through space as electromagnetic waves. Interaction with matter, this energy behaves as "quanta" or "photons". In field spectroscopy we utilize that region of the spectrum in which the photons have sufficient energy to alter the outer electronic energy levels of atoms, i.e. the UV, visible and IR regions, but not the regions of very high energy which result in complete ionization.

The visible spectrum extends from about 380 nm in the violet to 720 nm in the red, as determined by the limits of the spectral sensitivity of the average human eye. The ultraviolet region, useful in field spectroscopy as a fluorosensor range, extends from 250 nm to 380 nm.

The infrared range extends from 720 nm to tens of thousands of micrometers. The region of principal biological interest, especially for field spectroscopy, is between 720 nm and 2.4 μm. Radiant energy is characterized quantitatively by wavelength (Å) or frequency (Hz), and expressed as:

\[ c = \nu \lambda \text{ or } \lambda = c/\nu \]  

where \( c \) = velocity of electromagnetic waves in vacuo,  
2.998 x 10^8 cm/sec;

In addition to wavelength and frequency, radiant energy may be characterized by the energy of its smallest element, \( \varepsilon \) the quantum, or photon and is expressed as:

\[ \varepsilon = h \nu = h c/\lambda \]  

Equation 2 indicates that the photon energy is proportional to frequency and inversely proportional to wavelength, and expresses the energy of one molecule. A more practical unit introduced is the Einstein (E):

\[ E = N \varepsilon = N h c/\lambda \]  

where \( N = 6.02 \times 10^{23} \) molecules/gram-molecule.

Equation 3 indicates that the number of molecules photochemically activated must equal the number of photons absorbed. The quantum or photon yield (\( \delta \)) provided by the radiant energy is calculated from [47]:

\[ \delta = \frac{N_m}{N_q} \]  

where \( N_m \) = the number of molecules reacting and \( N_q \) = the number of quanta absorbed.

Growing plants or plant materials are composed of carbohydrates, fats, protein, etc. with each having various quantum yields at various wavelengths [1, 4, 7, 16]. The reflectance of the leaves of a plant is related to its cellular structure. Therefore, its absorption, reflection and transmission (optical) properties can be interpreted for various plant stresses, growth stage and maturity detection.

Relationship Between Reflectance and Leaf Structure

Plants depend upon radiant energy to carry on photosynthesis and other physiological processes [51]. The leaf of a plant is the primary photosynthesizing organ as they interact with electromagnetic radiation. The optical characteristics of leaves (transmission, absorbance, reflectance, refraction, scatter) are a function of the wavelength of the incident energy.

Figure 1 shows micrographs (Dr. Gausman, USDA, personal communication) of various transactions of corn, wheat, cotton and avocado leaves.

The literature on optical properties of leaves is extensive [18, 28, 29, 30, 35, 59, 60] and as Figure 1 indicates, it depends on the age of the leaves which is given for the agricultural crops by Gausman [29] as 0.2 mm. Generally, the reflectance increases as the thickness of the leaves increases. A thin leaf transmits a larger amount of the incident energy than a thick leaf.

The reflectance spectra of leaves over the 380 nm to 2600 nm are divided into three wavelength bands. The visible wavelength (380-720 nm) is highly absorbed by plant pigments especially chlorophylls and carotenoids present in chloroplasts which are integral parts of palisade cells. The mesophyll scatters the near infrared and affects the 750-1350 nm wavelength band, whereas the 1350-2500 nm band is affected by the concentration and distribution of the leaf water content. Later on, the spectra of lettuce and wheat cultivars are shown, which indicate a strong water absorption band at 1450 nm. The wheat spectra also indicate that maturity greatly affects the reflectance of the leaves, because more mature plants demonstrate higher infrared reflectance, this is related to structural changes in the leaves as indicated in the micrographs (Fig. 1).

Measurement

The food production chain starts by culturing the soil, in which the crops for food grow. For healthy crop production, the mineral, moisture and nutrient content of the soil are of great importance and can be analyzed with field spectroscopy techniques. For mineral content in soil the line depth (FLD) measurement of the Fraunhofer lines (FL)
Figure 1. Transections of leaves; a, wheat cultivar Penjame (S stomata); b, wheat cultivar Milam (M mesophyll); c, corn seedling (M mesophyll); d, mature corn (D dorsal side); e, cotton seedling; f, mature cotton (P palisade cells); g, young avocado; h, mature avocado. (courtesy of Dr. H.W. Gausman, U.S.D.A.)

were used successfully[46,57,58]. This technique indirectly measures the soil mineral content by measuring the growing plants.

Fraunhofer lines are sharp dark lines in the solar spectrum caused by the selective absorption of light by gases in the upper part
Figure 2. Fraunhofer lines appear on spectra curves of sun, soil, and oat crops. Hydrogen (H) lines; C, F, H; Oxygen (O2) lines a and b; sodium (Na) lines D1; Iron (Fe) lines d, e and G, L, M, H; Magnesium (Mg) line b; calcium (Ca) line K.

of the solar atmosphere. Line widths range from 0.01 nm to several tenths of a nanometer and are most numerous in the uv, visible and near-ir regions of the electromagnetic spectrum. Line depth measurement of the Fraunhofer lines involves observing a selected FL from the solar incident radiation and measuring the ratio of the central intensity of the line to a definable point on the continuum a few tenths of a nanometer distant. The solar ratio is then compared with that observed from a conjugate spectrum of the experimental material or crop. Both ratios are normally identical, however, luminescence (L) is indicated where the material ratio exceeds that of the solar ratio.

The luminescence component is given by [32, 48]:

$$L = \frac{(c - a \cdot d/b)}{(b - a)}$$  \hspace{1cm} (5)

where:
- a is the central line intensity FL observed directly from the reference
- b is the nearby component of the reference
- c is the central line intensity at the same FL, and
- d is the nearby component of the soil line intensity

In 1973, Watson et al. [57] demonstrated that luminescence is an indicator of geochemical stress produced by metal toxicity. Fraunhofer line depths were obtained from a helicopter and an aircraft to measure the luminescence of stressed and nonstressed trees, both on a diurnal and seasonal basis. Plascyk [45] discussed the feasibility of performing luminescence measurements from a spacecraft with an FLD imaging system.

Strong FL's (Figure 2) appear in soils and crop measurements at 589.5 nm and 588.9 nm (Na) which are sodium lines, iron (Fe) lines show up at 526.5, 516.8, 516.7, 495.7, 438.3 and 430.7 nm. Magnesium (Mg) lines appear at 518.3, 517.2 and 516.7 nm and calcium (Ca) lines appear at 430.7, 422.6, 396.84 and 393.6 nm. FLD can be measured with a high resolution spectroradiometer which measures the incoming and reflected radiant energies simultaneously, and can be related to iron or magnesium deficiencies on crops.

Laser fluorescence techniques are also used [6, 36, 37], to determine soil types or characteristic differences among soils. Using a 20 mWatt Cd He laser in which energy is focused by a lens system on to the slit input of a monochromator, soil was made to fluoresce at a wavelength and energy level usually not attainable with a conventional filtered light source. Using this fluorosensor, soil materials such as clay, chlorites and sands exhibited pronounced fluorescence over a range of 380 to
which may affect spectra obtained from laser-fluorescensing of airborne remote sensing missions over open vegetated and non-vegetated areas. To use field spectroscopic techniques for crop measurements, it must be recognized that during the growing season, the reflected energy from a field varies with changing crop cover, rate of plant development, concentration of plant pigments and degree of water stress. The spectral characteristics are influenced greatly by physiological and biochemical changes in the plants.

Various spectroradiometers used to measure plant optical characteristics and the results obtained from them, have been reported in the literature[20,31,33,50]. At Engineering and Applied Research Institute laboratories, several application-oriented spectroradiometers have been developed[34-36]. The basic concept of a spectroradiometer is indicated in Figure 3, where the reflected radiance $N_\lambda$ from the plant into the spectroradiometer is given by:

$$N_\lambda(Watt \cdot cm^{-2} \cdot \mu m^{-1} \cdot steradian^{-1}) = \frac{1}{\pi} (E_\lambda \cos \theta_D)$$

The irradiance $E_\lambda$ from the sun falls on the plant at an angle of $\theta_S$ and the radiance from the plants is collected by a Schmidt-Cassegrain telescope via a flat folding mirror $M$ with 4 degrees of freedom. The detector $D$ converts the radiance into electrical signals, which are measured by a photon quantum meter or lock-in amplifier, displayed on a recorder $R$, and recorded by a paper tape punch for computer processing. The monochromator can be replaced by interference filters, field stage by various lens arrangements. The detector can be a photomultiplier or silicon detector for the visible spectrum, and lead sulphide (PbS), lead selenide (PbSe), gallium arsenide (GaAs) or indium antimonide (InSb) for the infrared spectrum. Each spectroradiometer is constructed to suit its application.

Lettuce Maturity Detection: Two kinds of experiments have been tried to detect lettuce maturity: optical and laser fluorescence spectroscopy[8,12,44]. The objective of these experiments was to determine the correlation between lettuce reflection or fluorescence and its stage of maturity. Anatomical studies with a scanning electron microscope showed that the surfaces of young head leaves were covered initially with capitate-stalked glandular trichomes (Fig. 4a) which at later stages were broken and appeared as septate trichomes. At maturity the surface of the leaf appeared free of trichomes (Fig. 4b).

Cross sections of the first head leaf for all four cultivars showed a rather dense and uniform cell arrangement (Fig. 5). Well defined, spongy and palisade tissues were apparent only in the exterior leaves and cotyledons (Fig. 6). In the young (44-day-old) heads the cells were closely packed with few,
small intercellular spaces (Fig. 7, micrographs a and b). In the mature heads the number and volume of the intercellular spaces, especially those immediately below the epidermis were larger (Fig. 7, micrographs c and d). As a consequence of cell enlargement, the subcellular organelles, especially chloroplasts, were more dispersed in old than in young tissues.

The absence or presence of trichomes on the leaf surface (or anatomical structure of the leaf) may cause variation of reflectance, cellular enlargement may have a twofold consequence, a change in light: 1) scattering; 2) reflection.

Field experiments were conducted with the spectroradiometers housed in a mobile laboratory. The incoming radiant energy (vertical sighting) falling on the lettuce was measured and at each wavelength point the reflected energy (oblique measurement) was normalized by dividing it by the incoming energy to eliminate cloud effects. Visible and infrared incoming and reflected spectral curves of lettuce are shown for 41 days after planting (Fig. 8a) and the spectral curves of the same plot, at 61 days after planting (Fig. 8b). While the incoming radiant energy did not differ greatly, the reflected energy in the visible as well as in the infrared wavelength range showed a marked difference between the two dates which is due to cell structural changes. Plant leaves reflect incident radiation due to its pigmented cells containing water solutions. As indicated in Figure 8a, the UV blue and red reflectance are low and the green high, due to chlorophyll content. The water absorption bands (Figure 8a, 8b) at 900, 1100 and 1450 nm are due to liquid water in the leaf. The spectral curves were taken at approximately the same time of day (11:32 vs 11:13 hours).

Experiments using field spectroscopy techniques were conducted on various grain crops (wheat, barley, oat, corn, soybean) to determine agronomic conditions from their spectral (optical) properties [7,9,31,36,39,49,54].

The experimental set up and instrumentation was similar to those used in the lettuce maturity measurements. The spectral data collection and analysis done in 1976 is presented where spectral data were collected from experimental field plots of various crops and varieties. The crop spectra were obtained at wavelengths from 350 to 1840 nm with a spectroradiometer having a grating resolution in the order of 0.1 nm. The data were recorded and digitized onto magnetic tape for computer processing at a reduced accuracy of 1 nm. Calibration was performed regularly by measuring the incident solar intensity and checking for the

Figure 5. (to the left). Cross-section view of the first head leaves of different lettuce cultivars at the same stage of maturity. a. Evergreen; b. Fulton; c. Great Lakes; d. Ithaca. No defined palisade or spongy mesophyll cells are apparent.

Figure 6 (facing page - top). Cross-sectional view of the cotyledon and leaves of lettuce (cv. Fulton) at the sixth-leaf stage. a. Cotyledon; b. Sixth leaf; c. Fifth leaf; d. Fourth leaf; e. Third leaf; f. Second leaf; g. First leaf. Palisade (P) and spongy mesophyll (S) cells are apparent in the cotyledon and leaves up to the fourth leaf.

Figure 7 (facing page - bottom). Cross-section view of the first head leaf of two lettuce cultivars. a,c. Ithaca and Great Lakes, 44-day-old plants. b,d. Ithaca and Great Lakes, 63-day-old plants. Compactness of leaf structure decreases with maturity and chloroplasts are more dispersed in older leaves (b,d). Arrow indicates airspace between cells.
stability of the measuring system. Preceding the measurement of the crop spectra, reflected radiation was obtained from a Kodak standard gray card for the visible region and from an aluminum plate for the near infrared region. Correction for the viewing geometry and atmospheric conditions were applied using atmospheric models. By averaging the 600 spectra according to seeding dates (D_1, D_2) in two different replicates, the complexity in the interpretation was minimized. An error less than 20% was estimated near the
750 nm region but it was less than 5% within the spectral regions of 350-750 and 750-1840 nm.

Principal-component analysis was conducted to obtain information at 10 nm band-widths which would be independent of daily standardization and atmospheric variations. The computations were done for all 10 nm bands-widths from 350 to 1840 nm (190 10 nm bands) using 35 spectra available from the crops and cultivars within the period from early August to late September, 1976. The analysis was based on the determination of the covariance matrix that was calculated from the crop spectra. As a measure of the amount of information within a specific band, the variance of the reflectances of a number of spectra was computed. There were six to eight spectra available for each of the crops covering the growth stages V (early heading) to X (ripe). Crops seeded May 27, 1976 (D1) were more advanced in their growth stages than those seeded June 23, 1976 (D2); thus, there was overlap in growth stages for part of the period. The analyses for the D1 and D2 crops were carried out separately.

Certain spectral bands may be differently absorbed and reflected by certain crops features. For example, near the growth stages V to VI, the chlorophyll of green plants absorbs red radiation (640 to 690 nm) but turgid leaves greatly reflect the near-infrared (e.g. near 740 nm). Later both bands may reflect similarly. Thus, certain pairs of bands, having a negative correlation, may be more useful for discriminating crops and separating crop features than individual bands.

The changes in the reflectance spectra for wheat (cult. Neepawa) over the infrared spectral range of 750 to 1840 nm are shown for four growth stages from the boot stage to full maturity (Figure 9). The spectral bands of water absorption are clearly shown by the minima near 950, 1150 and 1450 nm. The absorption bands near 950 and 1150 nm regions disappear as the maturity of the wheat plant reaches the growth stage X.

For each cultivar, the spectral values at each nm interval were averaged for the different measuring dates. The standard deviations of the periodic measurements were calculated and coefficient of variation (CV) was obtained and plotted.

\[
CV = \frac{\sigma}{\mu}
\]

(7)

\(\sigma\) = the standard deviations
\(\mu\) = the average spectral value for each 1 nm bandwidth

Figure 10a shows that the soil has a smoother spectrum than the sod except in the water absorption regions. The CV of sod varies mainly in the 450-700 nm region which corresponds to chlorophyll and carotenoids whereas the CV of soil lies in the 1350-1600 nm region due to moisture.

The CV varied among the wheat cultivars throughout the entire spectrum (Figure 10b). Among the wheat varieties (Sinton, Neepawa, Hercules, and Marquis SR6), Sinton (D2) had the largest CV (60 percent) while Marquis SR6 (D1) had the smallest (15 percent to 20 percent). The general pattern of the CV throughout the spectral range 350 to 1850 nm was somewhat similar. Large CV's occurred near 500 to 550 nm, 650 to 700 nm, and 1250 to 1350 nm, which correspond physiologically to the wavelength regions of minimal radiation absorption by plants, chlorophyll and plant water, respectively. Among the barley cultivars (Figure 10c) Fergus (D1) showed the maximum CV of 45 percent and Conquest (D2) the lowest CV of 10 percent. The pattern for Fergus was distinctly different from the others, having a number of broad plateaux with low indentations near 750, 925, 1125 and 1350 nm. Among the oat varieties (Figure 10d) Garry showed a high CV, 40 percent, particularly in the range of 550 to 660 nm, whereas Terra (D1) was low, <40 percent.

It is worthwhile to note that the diseased Garry variety had a markedly larger CV than the healthy variety. This technique indicates that with field spectroscopy the identification of crop variety, cultivar within a crop and crop maturity is possible. It also indicates that a diseased (stressed) crop can be detected.

Protein and moisture analysis on ground grain products have been established [5, 40], and commercial laboratory protein moisture analyzers of grain products are available. However, little is reported in the scientific literature on protein analysis of standing grain crops. In 1980 and 1981, protein measurements on standing crops were attempted [3] with field spectroscopic techniques. In this experiment, absolute relationships between reflectance and grain protein content was not conclusively achieved but a number of important points were noted.

1) The highest correlation between near infrared reflectance and protein content was at the 2.07 to 2.11 μm wavelength band, with a secondary absorption band at 2.15 to 2.17 μm. 2) The relationship between near infrared reflectance and protein content was found to be negative, that is, the protein content increased as the reflectance of the plant decreased. 3) The growth stages from the beginning of heading to maturity yielded better results than the earlier preheading stages. 4) The growth stage which yielded the best results was growth stage 6 (beginning to head) where the correlation coefficient for the regression of protein content on reflectance was equal to -0.72 at 2.11 μm (Table 1). 5) The correlation between reflectance and protein content was not high enough to develop a model for predicting protein content on the basis of near infrared reflectance.
FIELD SPECTROSCOPY IN FOOD PRODUCTION

Figure 8. Visible and infrared spectral distribution of incoming and reflected radiation energies from lettuce: a) 41 days after planting; b) 61 days after planting. Incoming energy scale is multiplied by 100.

Figure 9. Reflectance spectra of a wheat cultivar (normalized infra-red) from 750 to 1840 nm at four growth stages from early heading to maturity.

Figure 10. Coefficient of variation of the spectral values for selected crops and soil around the mean value for each 1.0 nm bandwidth.
Further research is ongoing to refine the technique of measuring protein, also moisture and fat in standing crops during their growth.

In breeding experiments, it is useful to monitor the growth of plants and the rates of the growth of the plant which occur in response to applied stimuli or changes in the environment.


A fast and reliable method for the determination of differences in the morphology of plant species, and between cultivars of the same species at different ages, would be welcomed by plant scientists. Figure 11 presents reconstructed holograms of plant leaves which are characterized by the shape and slope of the fringes, the distance between fringes and the number of veins in the hologram. The measurement of the slope angle "α" is indicated by the inset drawings of Figure 11.

The number of fringes in the hologram is determined by the setting of the subject lens or its magnification factor. Therefore, one must always compare holograms with the same number of fringes. Larger numbers of fringes shown in the hologram indicate a larger cross section of plant leaf. Figure 11 as well as Table 2, indicates that in any age group and with the same number of fringes, species can be identified by the slope angle α.

**Plant CO₂ Uptake as a Measure of Crop Growth:**

Under the influence of light energy, plants perform the overall reaction:

$$\text{n} \text{hv} \quad 6 \text{CO}_2 + 6 \text{H}_2\text{O} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6 \text{O}_2$$

where \(\text{C}_6\text{H}_{12}\text{O}_6\) stands for carbohydrate in general. Equation 7 indicates when the plant is stimulated by the photon \(\text{hv}\), it converts \(\text{CO}_2\) using available \(\text{H}_2\text{O}\) to organic matter \(\text{C}_6\text{H}_{12}\text{O}_6\) and oxygen \(\text{O}_2\). The carbon content \(\text{C}_c\) of \(\text{C}_6\text{H}_{12}\text{O}_6\) is:

$$\text{C}_c = \frac{\text{C}_6}{\text{C}_6\text{H}_{12}\text{O}_6} = \frac{72.06}{72.06+12.011+31.998} = 0.3979-0.40\%$$

The ratio of the molecular weight of \(\text{CO}_2\) to carbon is:

$$\frac{\text{CO}_2}{\text{C}} = \frac{12.011 + 31.998}{12.011} = 3.6$$

The amount of \(\text{CO}_2\) assimilated \((\text{tCO}_2)\) by the plant is:

### Table 1. Summarized results of the analytical procedure of correlation between protein content and wavelength band for all of the growth stages of hard red spring wheat, Triticum aestivum L., CV. Neeapawa.

<table>
<thead>
<tr>
<th>Growth stage</th>
<th>Wavelength band of highest correlation (μm)</th>
<th>Highest correlation value (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2.34 - 2.36</td>
<td>-0.40</td>
</tr>
<tr>
<td>5</td>
<td>2.23 - 2.25</td>
<td>-0.22</td>
</tr>
<tr>
<td>6</td>
<td>2.08 - 2.11</td>
<td>-0.72</td>
</tr>
<tr>
<td>7.5</td>
<td>2.06 - 2.09</td>
<td>-0.44</td>
</tr>
<tr>
<td>8</td>
<td>2.06 - 2.09</td>
<td>-0.42</td>
</tr>
<tr>
<td>9</td>
<td>2.00 - 2.02</td>
<td>-0.50</td>
</tr>
</tbody>
</table>

### Table 2. Holographic identification of variety of species (B,O,R,T,W) between species (NC, OL, OS...) and age (0, 1, 2) by the value of slope angle "α".

<table>
<thead>
<tr>
<th>Variety</th>
<th>Number of Fringes</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley (BVO)</td>
<td>2</td>
<td>25.62</td>
</tr>
<tr>
<td>Oat (OCO)</td>
<td>2</td>
<td>19.98</td>
</tr>
<tr>
<td>Rye (RSO)</td>
<td>2</td>
<td>38.65</td>
</tr>
<tr>
<td>Wheat (MF0)</td>
<td>2</td>
<td>36.86</td>
</tr>
<tr>
<td>Barley (BHO)</td>
<td>3</td>
<td>53.13</td>
</tr>
<tr>
<td>Oat (OLO)</td>
<td>3</td>
<td>25.01</td>
</tr>
<tr>
<td>Rye (RPO)</td>
<td>3</td>
<td>26.56</td>
</tr>
<tr>
<td>Barley (BH1)</td>
<td>4</td>
<td>38.33</td>
</tr>
<tr>
<td>Oat (OS1)</td>
<td>4</td>
<td>30.35</td>
</tr>
<tr>
<td>Triticale (TS1)</td>
<td>4</td>
<td>26.56</td>
</tr>
<tr>
<td>Wheat (MF1)</td>
<td>4</td>
<td>42.27</td>
</tr>
<tr>
<td>Oat (OF1)</td>
<td>5</td>
<td>56.30</td>
</tr>
<tr>
<td>Wheat (WR1)</td>
<td>5</td>
<td>22.38</td>
</tr>
<tr>
<td>Oat (OC2)</td>
<td>5</td>
<td>53.74</td>
</tr>
<tr>
<td>Rye (RS2)</td>
<td>5</td>
<td>45.00</td>
</tr>
<tr>
<td>Triticale (TS2)</td>
<td>5</td>
<td>20.55</td>
</tr>
<tr>
<td>Barley (BHO)</td>
<td>6</td>
<td>21.80</td>
</tr>
<tr>
<td>Oat (OLO)</td>
<td>6</td>
<td>27.89</td>
</tr>
<tr>
<td>Rye (RPO)</td>
<td>6</td>
<td>30.96</td>
</tr>
</tbody>
</table>
Figure 11. Reconstructed holograms of: a) young oat leaf variety C.I. 4492; b) old oat leaf variety Foothill; c) triticale leaf variety Rosener; d) old barley leaf variety Betzes.

\[ T_{CO_2} = W \cdot C \cdot (CO_2)_C \]
where \( W \) is the growth rate of the plant gDM/day
\[ DM = \text{dry matter} \]
If \( W = 100 \) gDM m\(^{-2}\) day\(^{-1}\), then,
\[ T_{CO_2} = 100 \times 0.4 \times 3.6 \]
\[ = 146.56 \text{ g CO}_2 \text{ m}^{-2} \text{ day}^{-1} \]

By measuring the assimilation of CO\(_2\) by a crop, one can then measure its rate of growth and measure its retardation due to environmental stresses. For that purpose, an open path CO\(_2\) analyzer was developed\([11]\]. The analyzer is based on the differential infrared absorption by CO\(_2\) at 3.7 and 4.3 \( \mu \)m over a 1.5 m measuring path. It measures the CO\(_2\) concentration with 0.3 ppm sensitivity by volume with a time constant of 0.075 s. The normal operating range is between 240 to 400 ppm. The concentration CO\(_2\) in the atmosphere is 330 ppm, therefore measuring the difference in CO\(_2\) concentration between the air moving upward and downward above a crop canopy, estimates the CO\(_2\) assimilated by the crop.

Desjardins et al.\([24]\) mounted this analyzer on an aircraft and measured the exchange of CO\(_2\) above a cornfield, a forest and a lake at midday. Repeated test flights over these ecosystems were conducted. Mean CO\(_2\) absorption values obtained from these measurements were 3400, 1200, and 100 mg m\(^{-2}\)hour\(^{-1}\) for corn, forest and water, respectively (Table 3).

The CO\(_2\) analyzer was also adapted to control the CO\(_2\) content in plant growth rooms and greenhouses. Horticultural and ornamental crops grow faster with a greater yield at a high CO\(_2\) content in the atmosphere (800 -1800 ppm) thus shortening the period for producing the crops and increasing the productivity. The open path of the CO\(_2\) analyzer was reduced to 50 cm, since there is no need for more than a 10 ppm sensitivity. A digital controller was added to regulate the CO\(_2\) supply into the greenhouse. If too much CO\(_2\) is in the room (e.g. in the early morning, due to CO\(_2\) release by the crop) the controller opens inlets admitting fresh air. The required upper and lower limits can be preset on the analyzer.

Adapting the analyzer to monitor and control the CO\(_2\) content of atmospherically controlled storage for apples is underway.
## Table 3. Carbon dioxide and heat flux densities for passes over corn, forest, and water around midday on August 28, 1980.

<table>
<thead>
<tr>
<th>Altitude (m)</th>
<th>CO₂ flux density (mg.m⁻².h⁻¹ x 10²)</th>
<th>Heat flux density (W.m⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>-14</td>
<td>14</td>
</tr>
<tr>
<td>24</td>
<td>-34</td>
<td>42</td>
</tr>
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<td>25</td>
<td>-35</td>
<td>70</td>
</tr>
<tr>
<td>33</td>
<td>-36</td>
<td>70</td>
</tr>
<tr>
<td>33</td>
<td>-47</td>
<td>84</td>
</tr>
<tr>
<td>35</td>
<td>-36</td>
<td>63</td>
</tr>
<tr>
<td>Average</td>
<td>-34</td>
<td>57</td>
</tr>
<tr>
<td>Forest</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>-8</td>
<td>56</td>
</tr>
<tr>
<td>34</td>
<td>-10</td>
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</tr>
<tr>
<td>47</td>
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<td>48</td>
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<td>61</td>
<td>-18</td>
<td>56</td>
</tr>
<tr>
<td>Average</td>
<td>-12</td>
<td>44</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>29</td>
<td>-4</td>
<td>14</td>
</tr>
<tr>
<td>31</td>
<td>-3</td>
<td>28</td>
</tr>
<tr>
<td>Average</td>
<td>-1</td>
<td>21</td>
</tr>
</tbody>
</table>

### Conclusion

Field spectroscopy and its usefulness to research and production in the agricultural industry was presented. The technology of field spectroscopy and its application is relatively new and therefore not fully explored. As more researchers in agricultural research and production get acquainted with the technology, additional applications will be discovered, besides detecting stress in plants, identifying varieties, measuring protein and estimating biomass. Concurrently, the potential for developing equipment is being enhanced by rapid developments and cost reductions in computer technology.

### References


Discussion with Reviewers

Reviewer III: The techniques as discussed in the paper may be related to microstructural components of food or plant related systems, but the exact relationships and interrelationships are not made parallel to one another in terms of macroscopic or macrostructural versus microscopic or microstructural parts of the systems being evaluated. The techniques at best measure overall protein, fat, moisture, etc. and are not specific analysis (even at specified wavelengths). Please comment.

Are plants being treated as optical systems or as objects containing atoms and molecules of specific types that interact with radiation of specific wavelengths, therefore, characterized by the vibrational spectral transitions and atomic electronic spectral transitions?

Do you believe the molecular and atomic spectral differences are averaged measurements of a great number of different (but perhaps related molecules) which absorbs, emits and reflects radiation at specific wavelength positions?

Author: I agree with your comment, but in field spectroscopy we don't intend to study the specific atom and molecule of the standing crop, we rather observe the spectral changes on the surface of the leaves caused by molecular changes within the plants.
THE STRUCTURE OF FRESH AND DESICCATED COCONUT

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Abstract

Light and electron microscopy have been used to study the structure of fresh coconut endosperm tissue and the changes which have occurred when this has been processed to the desiccated form. In fresh tissue, a marked gradation of cell size, shape and contents exists between inner and outer endosperm regions. Adjacent to the brown testa, cells are rigid, compact and lipid-filled while those cells lining the central water-filled cavity have thin, easily deformed cell walls and contain little lipid. Desiccated coconut being derived from fresh tissue, is similar in morphology but moisture is reduced from 50% to 2-3%. During the cutting and disintegration stages of the process, a fatty "rind" develops around individual particles and tissue damage may occur.

A comparison has been made of a range of desiccated coconut samples from Sri Lanka and the Philippines. Variation in structural characteristics appears to correlate with known differences in processing that exist between the two countries. Significant factors relate to particle size, shape, colour and tissue damage. In the subsequent manufacture of products, such factors could clearly lead to differences in processing behaviour such that for a given recipe, products may be formed which vary in both texture and taste.

Introduction

Coconuts are the fruit of the palm, Cocos nucifera L. It is the most economically important member of the Palmae and is grown over a wide geographical area. Practically all parts of the plant are considered important and the products of the fruit alone range from coir or husk used in matting, to the dried meat or copra as a source of coconut oil. The fresh white meat or endosperm is probably the most important constituent as a foodstuff and in the desiccated form is used widely in the bakery and confectionery industries. Prior to desiccation, the meat may be cut into many forms including flakes, shreds and chips. Each of these cuts can be significant in imparting optimum texture to a final product. They may also be used as a bulking agent or simply for decorative purposes.

Most widely known in the confectionery industry is a "medium" cut or grade of desiccated coconut and this is considered to give the desired eating properties to products both in terms of particle size and shape. Understanding the structural basis and the extent of such variation is important in assessing the use of desiccated coconut as a raw material. Sri Lanka and the Philippines represent two of the major sources of commercial, desiccated coconut used today. The coconut operation in these countries was observed from harvest through to the packed, desiccated form and a range of desiccated coconut samples was collected at source for subsequent microscopy examination.

The distribution of the major constituents of coconut meat has been examined by Sabularse (1970), and Vaughan (1970) gave a brief general account of the structure of the whole fruit including observations using the light microscope. Dieckert and Dieckert (1973a) carried out a specific study relating to the apparent effect of water stress on both seed and endosperm development in coconut and extended this to a study of changes in ultrastructure of the developing solid endosperm (Dieckert & Dieckert, 1973b).

This paper describes the use of light and electron microscopy to examine fresh, fully matured coconut endosperm tissue and uses this
information as a basis for studying the microstructure of desiccated coconut and the changes which have occurred during its production. The structure of a range of desiccated coconut samples is compared.

Materials and Methods

Samples of whole, fresh coconut were obtained from local sources after shipment from the Philippines. Desiccated coconut material was obtained directly from Sri Lanka and the Philippines and a total of eighteen samples was examined. These represented products from different processing mills within the two countries and included a range of sizes (or grades) of material. The majority of samples studied had been supplied as "medium" grade.

Light Microscopy

Cryostat Sectioning Samples of fresh tissue were taken from various regions of the endosperm. Preliminary work showed that in order to achieve good sections of desiccated coconut samples, the material has first to be rehydrated. For all material, small samples (1mm²-1cm²) were supported in Tissue-Tek and frozen onto a 2cm aluminium support stub using liquid nitrogen at -196°C. The stub was placed in a Brights Open-top cryostat and sectioned at 10-15μm with a freezing microtome at -25°C. Sections were collected on chilled microscope slides coated with a thin layer of glycerin albumen.

Resin Embedding Small samples of material (2mm x 4mm) were fixed in 2% Palade's osmium tetroxide (Palade, 1952) at pH 7.2 for up to 24 hours. Material was dehydrated through a graded series of acetone solutions and finally infiltrated and embedded in low viscosity Spurr resin (Spurr, 1969). Embedded material was sectioned using a Reichert-Jung Ultracut to a thickness of 2μm. Sections were collected on a brush and secured to a microscope slide using a drop of distilled water.

Light Microscopy Stains

Toluidine Blue Toluidine blue was used as a general histological stain to study cellular morphology. Slides were immersed in a 0.1% aqueous solution of toluidine blue for 30 seconds. The sections were rinsed well with distilled water, mounted in a suitable aqueous mountant and covered with a glass cover slip.

Sudan IV (Scarlet R) Sudan IV was used to stain lipid in the specimens. A few drops of 1% Sudan IV in 70% ethanol were added to the sectioned material on microscope slides. A cover slip was placed over the stained material before immediate viewing using the light microscope.

Transmission Electron Microscopy (TEM)

Resin Sectioning Pieces of fresh and desiccated coconut material (~2mm²) were processed for TEM. Samples were fixed, dehydrated and embedded in Spurr resin as previously described. Sections, 50nm thick, were cut on the ultramicrotome and collected on uncoated copper grids.

Sections were stained for 30 minutes in 1% uranyl acetate followed by three minutes in Reynolds lead citrate solution (Reynolds, 1963). Grids were then coated and examined using a JEOL 1200EXB TEM operating at 80kV.

Scanning Electron Microscopy (SEM)

Samples of desiccated coconut particles were examined after routine preparation procedures for SEM or by using low temperatures in both the preparation and examination steps. In the former, surface lipid was removed to avoid problems of instability under high vacuum or the electron beam. This was achieved by two extractions with a 2:1 mixture of chloroform/methanol. Material was then supported on conventional SEM stubs using an Enscop SPS500A diode Sputter unit. Examination was carried out in the JSM 35CF SEM operating at 20kV.

For examination at low temperatures, samples of material were first supported between two aluminium rivets and quick-frozen in liquid nitrogen at -196°C. Frozen material was transferred to a Hexland cold temperature preparation unit attached directly to a Philips 505 SEM. Cryo-fracturing and gold sputter coating were carried out in the unit under vacuum at -150°C before direct transfer of the sample onto the cold stage of the SEM which was held at -180°C.

Fig. 1. Diagrammatic representation of fresh coconut tissue. L.S. - longitudinal section. T.S. - transverse section.
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Fig. 2. (a-c). Longitudinal section of fresh tissue viewed with transmitted light and representing inner (a), middle (b) and outer (c) regions of the endosperm. Adjacent to the outer endosperm is the brown testa (t). Bar = 100μm.

Fig. 3. (a-c). Same as 2 (a-c) but viewed by crossed polars. Crystalline lipid (l) cell walls and associated lipid material appear birefringent. Bar = 100μm.

(Same magnification for a, b, c).
Results

Fresh Coconut

Figure 1 is a diagrammatic representation of fresh coconut. The white, fleshy, inner nut meat or endosperm is used directly as a foodstuff or is processed further to form desiccated coconut. The diagram illustrates the varying types of cells from different regions of the endosperm viewed in both longitudinal and transverse section. The corresponding light micrographs (Figures 2 a-c) in longitudinal section, illustrate this difference in cell size and shape across the endosperm. The presence of crystalline material in the same sections is demonstrated using crossed polars (Figure 3 a-c). The lipid material within the cells, cell walls and an associated lipid layer appear birefringent.

These two sets of micrographs show a marked gradation of cell size, shape and contents between the inner and outer endosperm. Cells adjacent to the brown testa are rigid, compact and lipid filled, in contrast to those lining the central water-filled cavity, where cells have thin, easily deformed walls and show little evidence of crystalline lipid.

The corresponding transmission electron micrographs confirm these findings. Figure 4 (a-c) shows fresh endosperm and corresponds in origin to Figure 3 a-c. Cells of the inner region are again seen to be thin-walled and almost void of contents (Figure 4a). In contrast, Figure 4c shows the intact and rigid nature of cells from the outer region and the smooth appearance of the cell walls. The middle endosperm as represented by Figure 4b shows an intermediate stage with cells generally intact but the organisation of the cell contents beginning to disrupt.

Desiccated Coconut

The colour of the desiccated coconut samples examined ranges from white, associated with material from the Philippines to a creamy, yellow colour common to Sri Lankan samples.

The morphology of the individual particles is seen to be very similar to that of fresh tissue. Figure 5 represents a cryostat transverse section stained with toluidine blue, used in this case to reveal general morphology. Cells appear as a regular, rigid honeycomb and correspond to the outer, more mature endosperm of fresh tissue. In contrast, Figure 6 shows material prepared in the same way but cells are seen to be misshapen and thin-walled, similar to those in the inner endosperm.

All cell types found in fresh tissue are also found in desiccated coconut samples.

The location of lipid material can be demonstrated with Sudan IV. The lipid stains a dense red/brown colour and is present throughout the section (Figure 7a). The apparent difference in appearance of the stain between those cells at the edge of the particles and those in the centre indicates a difference in the precise nature of the lipid present. Viewing the same section using crossed polars, the peripheral lipid is seen to be birefringent and is assumed to be crystalline (Figure 7b). A consistent observation in all of the samples examined is the presence of an outer fatty "rind" around individual desiccated coconut particles (Figure 8a).
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Fig. 5. Transverse section of desiccated coconut stained with toluidine blue. Arrangement of cells indicates material has originated from the outer endosperm of fresh tissue. Bar = 50μm.

Fig. 6. Transverse section of desiccated coconut stained with toluidine blue. Arrangement of cells indicates material has originated from the inner endosperm of fresh tissue. Bar = 50μm.

Fig. 7a. Transverse section of desiccated coconut stained with Sudan IV. Lipid is apparent throughout the whole section. Bar = 50μm.

Fig. 7b. Same as Fig. 7a but section is viewed under crossed polars. Crystalline lipid appears birefringent and is restricted to the peripheral cell layers. Bar = 50μm.

The position of the rind corresponds to the outer layer of damaged cells produced during the cutting/disintegration process of manufacture. Figure 8b is a scanning electron micrograph of frozen-fractured desiccated coconut and confirms this observation. Cell walls around the periphery of the particle are seen to be incomplete and are surrounded by an envelope of fatty material.

The process of desiccation results in a drastic reduction in moisture content from 50% to 2-3%. Associated with this is considerable shrinkage of the tissue. A comparison of desiccated and rehydrated material, indicates this is on the order of 50%.

A comparison of the range of samples of desiccated coconut has shown that one of the striking differences between them is particle size and although the particle size range is similar, the distribution of particles within the various classes is very different.

Sri Lankan material (code SL) (Figure 9a) has a fairly even particle size distribution within the range 1-4mm wide and 1-10mm long, and fine material is virtually absent. In contrast, the extremes of particle size are clearly evident in Philippine material (code PI) (Figure 9b), in particular a large proportion of fines (<1mm x 1mm) are present. Figures 10a and b are examples of other grades of desiccated coconut, Philippine material termed “fancy shred” (Figure 10a) and a “coarse flake” (Figure 10b) from Sri Lanka represent the largest particle sizes of the samples examined, ranging up to a length of 40mm and 100mm respectively.
The shape of particles also differs significantly between samples. Scanning microscopy clearly distinguishes the two extremes of shape commonly found within the medium grade range of samples. Material from Sri Lanka (code S2) has particles which are particularly blunt-ended and with similar dimensions in both the long and short axes (Figure 11). In contrast, Philippine material (code P2) has particles which appear long and tapering and with an overall fibrous appearance (Figure 12). Other samples of medium grade material fit between these extremes although occasionally samples contain the two distinct populations of particle shape within the same bulk.

Light microscopy and SEM complement each other in showing the nature and extent of tissue damage which can arise during the cutting/disintegration process. Scanning microscopy of a "tender flake" from the Philippines (Figure 13) shows an example of considerable surface damage to a particle and numerous fissures are readily apparent. Examination of the edge of this particle, however, shows it to be no more than five cells thick. Fissures within such a sample may eventually result in its fracture. Fissures and tears in the tissue are also apparent in Figure 14 which represents a sample of medium grade material from Sri Lanka.
Fig. 11. SEM - medium grade desiccated coconut from Sri Lanka (S2). Note the particulate nature of this material. Bar = 500μm.

Fig. 12. SEM - medium grade desiccated coconut from the Philippines (P2). Particles appear fibrous in nature. Bar = 500μm.

Fig. 13. "Tender flake" sample from the Philippines. Surface tissue damage (D) is apparent in the form of fissures. Examination of the edge of the flake (E) shows this to be no greater than five cells thick. Bar = 50μm.

Fig. 14. SEM - medium grade sample from Sri Lanka. Surface fissures and tears (D) in the tissue are apparent. Bar = 500μm.

Fig. 15. SEM - "Extra fancy shred" sample from the Philippines. Little surface damage is present. Bar = 100μm.

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Fig. 16. Desiccated coconut as in Figure 15 but sectioning for light microscopy reveals an internal fracture (F). Bar = 100μm.

A Philippine sample of "extra fancy shred" shows little surface damage when viewed by SEM (Figure 15), however, sectioning for the light microscope reveals a large internal fracture with a number of broken cells (Figure 16). Samples of desiccated coconut from Sri Lanka in general show greater damage than the equivalent Philippine material. The presence of extensive surface damage is also seen to be associated with a particularly heavy fatty "rind". Figure 17 is a scanning micrograph of a Sri Lankan sample (code S4) and shows extensive surface damage. This sample has a generally "oily" appearance and feel, and light microscopy reveals a heavy rind similar to that shown in Figure 8a.

Discussion

The production of desiccated coconut from the fresh coconut tissue results in a series of changes which considerably alter the properties of the tissue. These changes occur as a result of the cutting/disintegration of the fresh, white endosperm combined with a drying stage taking the moisture content down from 50% to around 2%. The natural variation in cell types that exists within fresh tissue is still observed in individual particles of desiccated material. Other structural differences, however, are also apparent between the various sources of desiccated material.

The coconut industry in Sri Lanka is based, at present, on a series of relatively small-scale operations each with differences in both standards and procedures. In contrast, the Philippine industry consists of only a few large scale operations which together process up to ten times the total Sri Lankan volume and overall are more sophisticated and controlled. The type of variation between samples, as described during this study, shows good correlation with these known differences in processing. In the Philippines, for instance, tissue is treated by passing it through a hot water tank followed by a sulphite-blanching/sterilisation bath, whereas in Sri Lanka a hot water tank only is used. Such differences can readily explain the colour variation between samples.

The work described above, has also shown that particle size and shape vary within and between samples. Both these properties will directly influence the relative surface area of a particle. These factors could clearly lead to differences in processing behaviour when desiccated coconut is used as an ingredient in the food industry, such that for a given recipe, products may be produced with differences in both texture and taste.

The cutting/disintegration operation is seen to result in an envelope of broken or damaged cells around individual coconut particles. The heat generated during cutting melts the lipid present in the tissue and this is liberated from cells. Blunt or badly maintained cutters will therefore result in extensive bruising leading to a particularly heavy "rind". Subsequent incorporation into a product may well lead to problems when excessive free fat becomes dispersed. Another consequence of this free fat is the increased likelihood of rancidity in a product.

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References

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Discussion with Reviewers

J.F. Chabot: What is in the cells of Fig. 4c, The Transmission electron micrograph?

Authors: Fig. 4c shows what we consider to be a cell with relatively intact contents including some lipid, amorphous protein and a number of vacuoles, some containing crystalline protein. It is quite likely that a large proportion of the unstrained region may correspond to lipid which has been removed during processing for microscopy examination. Unfortunately coconut oil is composed of approximately 50% saturated fatty acids and these will not react readily with osmium and so be prone to extraction.

J.G. Vaughan: Coconut endosperm contains protein reserves. Have the authors made observations on this material?

Authors: Although no specific study was made, it was noted that both crystalline and amorphous material were present in the fresh endosperm, with crystallloid protein, up to 30μm in size, being more apparent in the middle and outer regions.

J.F. Chabot: Why is there birefringent lipid in the cells of fresh tissue, but only the peripheral lipid is birefringent in the processed tissue?

Authors: Although Fig. 8a shows only peripheral crystalline lipid, the centres of individual coconut particles were also frequently observed to contain crystalline lipid. The consistent observation here was of a continuous birefringent "rind" present around the periphery of individual desiccated coconut particles. This was true whether the material originated in the outer, central or inner endosperm.

It is also quite likely that the liberated lipid has changed its form during heating and exposure to air resulting in a more stable crystalline state.

J.W. Dieckert: Do you have any information concerning the developmental age, post-flowering of the nuts examined or harvesting, handling or shipment procedures? How do the usual processing conditions in Sri Lanka and the Philippines relate to these factors?

J.F. Chabot: Variation in structural characteristics was related only to differences in processing. Are there other factors which could be important such as variety of coconut or environmental conditions during growth and maturation?

Authors: The material we used as our control was dehusked, fully mature nuts from the Philippines which had been cellophane wrapped prior to shipment. This would be the normal raw material for the desiccation process. Sri Lankan material would differ from this in that it would be harvested one month earlier and allowed to mature on the ground before processing. In both countries, the extent of natural variation in the coconut crops and the varieties used are difficult factors to control. Such inherent variation will inevitably be carried through to the desiccated product. We would expect all these factors to exert some influence on the structure and behaviour of the tissue during processing and it would be interesting to carry out a more detailed study in these areas. On the basis of our results, so far, however, we would consider differences in processing between bulk samples to be more significant than variations in environmental conditions or variety of the fresh coconut.

J.F. Chabot: The processed product had been subjected to several treatments other than simple cutting and desiccation. How can these be related to the final structure?

Authors: Apart from the effects of types of cutters maintained to different levels and the different types of driers used, the only other major variable would be the two different Blanching pretreatments carried out in Sri Lanka and the Philippines. We have noted this causes a colour change and possibly differences in the levels of microbial contaminants which may in turn lead to storage problems. We would consider these materials, however, to be structurally similar in terms of their subsequent use as a raw material.

J.W. Chabot: There was a broad generalisation drawn between the economic organisation of coconut processing plants in the two countries. For the purposes of the structural and functional characterisation of processed coconut meat, would not more careful laboratory experiments be useful?

Authors: Carefully controlled laboratory experiments to emulate the production processes "in situ" would be desirable but difficult to
control. The handling of material from the various stages of desiccation for microscopy may also prove difficult although this may be the easier part of the process to monitor. Scaling down of the cutting procedure to laboratory size may prove more difficult. The gear/tooth ratio of the cutters, for example, could not be altered without changing more than simply the volume of material processed.

The original aim of this study was to evaluate samples produced at the commercial level and although ideally, empirical investigations should be carried out, preferably in the country of origin, it was not possible within the scope of this work.
EFFECT OF PRERIGOR PRESSURIZATION ON BOVINE LYOSOMAL ENZYME ACTIVITY

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Abstract

Longissimus muscle from 8 dairy cows was prerigor pressure (PRP) treated at different pressure levels (0, 34.5, 68.9 and 103.5 MNm^{-2}). a-Glucuronidase (indicator of lysosomal enzymes) activity in the unsedimentable (U) and sedimentable (S) fractions was fluorometrically assayed at ½, 24 and 168 hr postmortem. At ½ and 24 hr postmortem, the specific activity of a-Glucuronidase in the U-fraction from PRP treated samples was significantly (P<0.05) higher than that in the U-fraction from the control. However, at these times, the sedimentable fraction from the control sample had higher (P<0.05) enzyme activity than the sedimentable fraction from the PRP sample. Prerigor pressurization caused a pronounced Z-line degradation. However, there was no Z-line degradation in the control samples, either at ½ or 24 hr postmortem. Z-line degradation could have been caused by a lysosomal enzyme released early as the result of the pressure treatment.

Introduction

Tenderness is one of the most important characteristics that render meat acceptable to consumers. Tenderness is a dynamic property, and as such, even originally tender meat can be rendered tough by the postmortem handling e.g. chilling in a holding cooler. Several tenderization techniques have been found to restore tenderness, including prerigor pressurization (PRP) (Elgasim, 1977; Kennick et al., 1980; Macfarlane, 1973). In the past few years PRP has been intensively investigated at our laboratory and its beneficial effect on tenderness has been well documented. Also, several other physico-chemical properties were affected by PRP (Elgasim and Kennick, 1982).

Several investigators have provided evidence for the presence of lysosomes in skeletal muscle (Canonico and Bird, 1970; Ono, 1970; Stagni and Bernard, 1968). Lysosomes are subcellular organelles that house several proteolytic enzymes. Many researchers have reported on the role of lysosomal enzymes in meat tenderness (Moeller et al., 1976; Dutson et al., 1980). Also these studies suggested that unless the lysosomal membrane is disrupted, the lysosomal enzymes are inactive. Disruptive processes involving several chemical and physical factors e.g. pH, freezing and thawing, thermal activation and detergent treatment, have been implicated in lysosomal disruption (Sawant et al., 1964). Ivanov et al. (1960), Ikka and Opi (1966) and Joseph and Harrington (1968) concluded that muscle proteins are affected by hydrostatic pressure. Enzymes are proteins and are likely to be affected by pressure treatment. Hydrostatic pressure in the order of 706.6 and 588.6 MNm^{-2} inactivated trypsin and chymotrypsin respectively (Horigawa and Suzuki, 1963a, b). Also sarcoplasmic reticulum subjected to 150 MNm^{-2} lost its extra Adenosine Triphosphatase (ATPase) activity (Horgan, 1981).

The mechanisms by which pressurization tenderizes meat have not been fully elucidated. In a previous study, Elgasim (1977) hypothesized that lysosomal enzymes are likely to play a role in the tenderness improvement induced by PRP. This study was conducted to see if PRP caused an early release of lysosomal enzymes as monitored by a-Glucuronidase.

Materials and Methods

Sample Preparation and Treatment

Samples were obtained from 8 utility grade dairy cows (approx. wt 450 kg) slaughtered at the Oregon State University Meat Science Laboratory. After slaughter, skinning, evisceration, splitting and washing the carcass, the left side of the carcass was assigned for various pressure treatments, whereas the right side served as the control (CON). The longissimus muscle (from the last lumbar vertebra to the 8th thoracic vertebra) was removed (=35 min. post slaughter) from the side designated for treatment and pressure treated according to the procedure of Elgasim et al. (1982) except that the pressure was applied at the rate of 2.6 MNm⁻² sec⁻¹. Unless otherwise stated the pressure level used throughout the experiment was 103.5 MNm⁻² (1021 atm.).

Four pressure levels, namely 0, 34.5, 68.9 and 103.5 MNm⁻² were used to study the effect of the pressure level on the activity of β-Glucuronidase. Longissimus muscle between the 6th and 8th thoracic vertebrae was removed from 6 sides designated for the pressure treatment in the study. Each two sides were assigned for 34.5, 68.9 and 103.5 MNm⁻² pressure treatment, whereas all the muscles from the corresponding sides were used for the 0 (CON) MNm⁻². For this part of the study the activity was measured at 1½ hr postmortem in the unsedimentable (U) fraction only.

The pH of the CON and pressure treated samples was measured at 1½, 6, 24 and 168 hr postmortem using a Corning pH meter, Model 125, equipped with a combination glass electrode which was inserted in a freshly made incision each time pH was taken.

Enzyme Extraction

A method similar to that employed by Moeller et al. (1976) was used for the enzyme extraction. At appropriate times postmortem (1½, 24 and 168 hr) samples were removed from each CON and pressure treated muscle, trimmed of external fat and connective tissue and ground in a Hobart meat grinder. Ten grams of the ground meat sample was homogenized in 50 ml homogenizing solution containing 0.25 M sucrose, 0.02 M KCl and 2.0 mM EDTA, pH 7.0) for 45 sec. in an Osterizer blender. The homogenate was filtered through two layers of cheese cloth and the pH of the filtrate was adjusted to 7.25 to 7.30 with 0.5 M KOH. The filtrate was centrifuged at 105,000 G for 2 hr to give unsedimentable (U) and sedimentable (S) fractions. The U-fraction was filtered through glass wool and the S-fraction was resuspended in 25 ml of the homogenizing solution.

Enzyme Assay

β-Glucuronidase standard and 4-methylumbelliferyl-β-D-glucuronide were obtained from Sigma Chemical (St. Louis, MO). The enzyme activity was measured according to methods described by Moeller et al. (1976).

Electron Microscopy

Observations were made on samples from two animals. Within 1½ hr postmortem, samples were removed from the CON and pressure treated samples and fixed immediately in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 for 4 hr, then transferred to a fresh phosphate buffer and left overnight. The samples were then fixed in 1% osmium tetroxide (OsO₄) in 0.1 M phosphate buffer, dehydrated in 50, 70, 90 and 100% acetone solutions and stained in a saturated solution of uranyl acetate. Specimens were infiltrated, embedded in Spur's epoxy formulation and cured overnight at 70°C. Silver-grey sections were cut using a diamond knife on a Porter-Blum MT-2 ultramicrotome, stained with Reynold's lead citrate and examined with a Philips EM-300 transmission microscope. At 24 hr postmortem a sample was removed from the CON muscle and similarly prepared for examination with the electron microscope.

Results and Discussion

β-Glucuronidase activity was determined after treating muscles at 0 (= CON), 34.5, 68.9 and 103.5 MNm⁻² for 2 min. to determine whether the enzyme activity is pressure dependent. There was a progressive increase in activity with increase in pressure level (Fig. 1), however, the change was observed to be non-linear. The change in activity/pressure unit was higher between 34.5 and 68.9 MNm⁻². The cause for this non-linearity was not clear however, it could be that more enzyme was released but some of it was inactivated. Pressure in the order of 51.8 MNm⁻² caused an increase in the activity of sarcoplasmic reticulum extra ATPase, however, the activity decreased as the pressure was increased to 103.5 and 155 MNm⁻² (Horgan, 1979).

The specific activity of β-Glucuronidase in the U and S-fractions at different postmortem times is shown in Table 1. At 1½ and 24 hr postmortem, the activity in the U-fraction from the PRP sample was significantly (P<0.05) higher than its corresponding CON (approximately 23% and 20% increases in free activities at these postmortem times, respectively). At 7 days postmortem, the free activities of both CON and PRP samples were the same (P>0.05). Dutson et al. (1980) reported that electrically stimulated ovine samples had higher percent free activity for β-Glucuronidase and cathepsin D. The increase in the activity of β-Glucuronidase in the U-fraction, immediately after the treatment (1½ hours postmortem), was not surprising because, besides other factors, the PRP treatment caused the pH of the sample to be acidic (Fig. 2). At the acidic range observed in this study, Sawant et al. (1964), reported that ary1 sulfoxidase became more available. Divalent
Lysosomal enzyme activity of PRP muscles

Fig. 1: Changes in the specific activity of β-Glucuronidase (relative intensity) with changes in pressure levels. Temperature (37°C) and duration (2 min.) of pressurization were kept constant. The activity was measured in the U-fraction immediately before and after pressurization (1½ hr postmortem). Each point is a mean of 2 samples with 2 observations per sample, except the 1st point, at 0 MNm⁻², which is a mean of 6 observations.

cations, especially Ca⁺⁺ and Mg⁺⁺ also enhance the availability of lysosomal enzymes (Sawant et al., 1964). In a previous study using electron microscopy, Elgasim and Kennick (1982), observed that the mitochondrial and sarcoplasmic reticulum systems were both affected by PRP resulting in the release of Ca⁺⁺ in the cell cytoplasm which was evident by the contraction (48% of its on carcass length) observed after PRP treatment (Kennick et al., 1980). However, the effect of pressure on the pH of prerigor muscle cannot be used to explain the differences observed in the β-Glucuronidase activities between PRP and CON samples at 24 and 168 hours postmortem (Table 1). At these times, the pH differences between the two treatments vanished and the samples have the same ultimate pH (Fig. 2).

At this stage our attention was focused on possible molecular changes that could have dictated these differences in the activities of CON and PRP samples. Examination of electrophoretograms (Fig. 3) of the CON and pressure treated samples reveal that the PRP sample from the U-fraction (gel 1) has two bands less than the CON sample (gel 2). The two missing bands were observed to be in the 173,000 and 153,000-dalton regions and it is suggested these two bands were

Fig. 2: Changes in the pH of the control (— ) and pressure treated (○—○) longissimus muscle with postmortem times. Conditions of the pressure treatment: 103.5 MNm⁻², 37°C, 2 min. duration. Each point is a mean of 8 observations.

Table 1 - Changes in specific activities of β-Glucuronidase in the sedimentable and unsedimentable fraction from control and pressure treated bovine longissimus muscles with postmortem times.

<table>
<thead>
<tr>
<th>Post-mortem Time(hr)</th>
<th>Treatment</th>
<th>Specific Activities of Fraction</th>
<th>% Bound Activity</th>
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<tr>
<td></td>
<td>U</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>1½</td>
<td>CON</td>
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<td>0.202^h</td>
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<tr>
<td></td>
<td>PRP</td>
<td>0.231^d</td>
<td>0.113^h</td>
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<td>CON</td>
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<td>0.341^f</td>
<td>0.141^k</td>
</tr>
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<td>PRP</td>
<td>0.392^g</td>
<td>0.072^k</td>
</tr>
</tbody>
</table>

a Specific activities were expressed as nM substrate hydrolyzed/mg protein/min. Each value is a mean of eight observations. U = unsedimentable fraction; S = sedimentable fraction.
b CON = control; PRP = prerigor pressurization.
c % bound activity = S-activity / (U-activity + sedimentable activity).
d, e, f, g, h, i, k means in the same postmortem period and the same column, carrying the same superscript are significantly different (P<0.05)
mobilized by the PRP treatment. The nature of these two bands is not known, but we believe them to be natural proteinase inhibitors. A third band at 130,000-dalton region almost vanished from the PRP sample (gel 1). When these gels were scanned, the intensity of some other minor bands were observed to be affected by the pressure treatment. There were no differences in the banding pattern of the electrophoretograms obtained from the total extract of the PRP (gel 3) and in the CON (gel 4). Only minor differences in the intensity of these bands can be observed.

Fig. 3: SDS-gel electrophoresis of total extract and U-fractions prepared from CON and PRP samples 1½ hr postmortem. Gels 1 and 2 are U-fractions from PRP and CON samples respectively. Gels 3 and 4 are total extracts from PRP and CON respectively. Conditions of the PRP: 103.5 MNm², 37°C and 2 min. duration. Note that CON has two bands (arrows) more than the PRP samples.

Figure 4 shows the electron micrographs of pressure treated and CON samples. It is obvious that the Z-lines of the pressure treated samples were extensively disintegrated. Calcium-activated factor (CAF) (Penny, 1974; Dayton et al., 1976) and cathepsin D (Eino and Stanley, 1973) were implicated in postmortem Z-line degradation. At a pH <6.0 Suzuki et al. (1982), observed that CAF was less effective in degrading the Z-line of the myofibrils, and in our study, the pH of the muscle is below 6.0 immediately after pressurization. Several studies (Eino and Stanley, 1973; Robbins and Cohen, 1976) have indicated that cathepsin D is effective under these conditions. Since cathepsin D is lysosomal in origin (Canonico and Bird, 1970) the Z-line degradation observed here (Fig. 4) can be taken as evidence of an early release of lysosomal enzymes induced by PRP treatment.

Fig. 4: Transmission electron micrograph of longissimus muscle sampled and fixed 1½ hr postmortem: a) longitudinal section from CON sample; b) longitudinal section from PRP sample; c) longitudinal section from CON sample, sampled and fixed at 24 hr postmortem. Note Z-line disintegration of PRP (arrows). There was no Z-line disintegration in the CON samples even with aging up to 24 hr postmortem. Bars in a and b equal 3.1 μm, bar in c = 2.0 μm.
In view of the results presented here, there is no doubt that PRP caused an early release of lysosomal enzymes at a time when the temperature and pH conditions of the muscle were conducive to a greater and faster enzymatic action. Therefore, at least part of the improvement in tenderness induced by PRP treatment could be attributed to the early action of the lysosomal enzymes.

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References


Ivanov II, Berg IN, Labedeva NN (1960). Changes in some properties of myosin, actomyosin and actin under the influence of high pressure. Biokhimia. 25, 505-510.


Discussion with Reviewers

P.B. Addis: The authors report that pressure causes a more rapid pH drop in muscle. However, this experiment is confounded by the fact that the control side was intact whereas the treated side was excised, and excision by itself is able to stimulate contraction and pH decline. Furthermore, pH decline is a factor which can stimulate release of lysosomal enzymes.
Authors: We measured pH before and after excision and we observed very little change as a result of excision. It is to be noted that our measurements were taken within ½ to 1 min. after excision. We think excision will become a factor in pH decline if there is a time gap between excision and pH measurement. We are quite confident that the substantial change in the pH of the pressure treated sample is due to the treatment itself and not the excision of the muscle. We do agree with you that pH decline is a factor that can stimulate release of lysosomal enzymes and this is the main reason for presenting the pH results in this study.

T.R. Dutson: Was care taken not to remove samples from any area of muscle that had previously been cut for pH measurement?
Authors: Generally we avoided any muscle area that had been exposed for a longer period.

R.J. Carroll: Why were utility grade cows used? Would not the connective tissue content be higher, and, therefore, less tender meat?
Authors: It happened that they were the samples available to us. We agree with you that they are less tender, however, we think it is due mainly to the quality of connective tissue rather than the quantity. Connective tissue becomes more cross-linked with maturity, thus more stable to heating (cooking).

Reviewer VI: Can you please comment further on the gel electrophoresis?
Authors: Two techniques were used to perform gel electrophoresis. One was Laemmli (1970) with some modification (slab gel instead) and the other was the Porzio and Pearson (1977) technique. The latter has given a much better resolution and a clear electrophoretogram is presented (Fig. 3). With Laemmli's procedure the two missing bands were observed at 179,000 and 126,500-dalton. With Porzio and Pearson's procedure the two missing bands were observed at 173,000 and 157,200-dalton. A third band at the 130,000-dalton region almost vanished.

T.R. Dutson: Are these micrographs (Fig. 4) representative of the micrographs of all samples? How many animals and how many samples per animal were evaluated?
Authors: Samples were obtained from two animals with two observations per animal. Over the years we have taken many electron micrographs and feel confident that the effect of pressure on the ultrastructure of muscle is very consistent.

R.J. Carroll: In the 'Results' section in discussing Table 1, you mention "approximately 23% and 20% increases in free activities...". Can you please explain this further?
Authors: Free activity is the enzyme activity in the unsedimentable fraction (supernatant). 23% and 20% came from Table 1 by subtracting and rounding to whole numbers, the % bound activity in the PRP sample from that of the CON at 1½ and 24 hr postmortem, respectively.

Reviewer VI: Please give further details about the β-glucuronidase standard.
Authors: Purified enzyme with known protein content and enzyme activity (3,500,000 units/gm solid) obtained from Sigma Chemical, St. Louis, MO.

R.J. Carroll: ...48% of carcass length contraction after PRP treatment (Kennick et al., 1980), but on page 77 (Elgasim and Kennick, 1982, Food Microstructure) the sarcomeres for longissimus muscle were only 8.2% shorter. Can you clarify this discrepancy?
Authors: There is no discrepancy. In the first study (Kennick et al., 1980) we are looking to the overall shortening of the whole muscle relative to its resting length. In the second study (Elgasim and Kennick, 1982) the measurement was on the sarcomere, which is the smallest unit of the muscle. We have encountered some difficulties measuring the sarcomere length of the pressure treated samples. It is observed that the contraction generated by the pressure treatment is not uniform. We observed areas where the sarcomeres are stretched and in other areas supercontracted.

T.R. Dutson: Were gels produced from the tissue before 105,000 XG centrifugation or were the two fractions combined after centrifugation?
Authors: After centrifugation.

Reviewer VI: In connection with Fig. 3, can you please explain relative intensity further? What was the activity of S-fractions for these samples?
Authors: In our case β-glucuronidase enzyme solution with the highest concentration was used to set the relative intensity scale at 80. Usually quinine sulfate solution is used to calibrate the machine, but we did not use it in our study because the excitation and emission wave lengths of our samples are different from those of quinine. Unfortunately with our limited resources we did not measure the activity of the S-fraction, but our assumption is that it will show a pattern similar to that in Table 1.

P.B. Addis: Does prerigor pressurization affect rancidity of tissue? The disruption of membranes could, if severe, lead to phospholipid oxidation.
Authors: This study is in progress. We observed that there is more free fatty acid (FFA) in the PRP sample than their corresponding control. However, the peroxide values of the PRP samples are less than those of the control. It is too early to speculate what happened exactly.

Reviewer V: Another way to look at the data in Table 1, which I think is easily defensible, is to sum the activities of the "U" and "S" fractions and look at the total activity. If this is done, one finds differences due to treatment that do not appear to be significant. Thus, it may be postulated that the treatment only affects the equilibrium between the two fractions. Please comment.
Authors: There is more than one way of looking at the data. Based on the objective that we have stated for this study we looked at the data from
the distribution point of view (distribution of \( \alpha \)-Glucuronidase in the S and U-fractions). We don't see any way of reaching the conclusion that the treatment has affected the equilibrium (we believe you mean distribution) between the two fractions without presenting the data the way we did in Table 1. Dutson et al. (1980) found that electrical stimulation affects the distribution of lysosomal enzymes. Prerigor pressurization effect is similar to electrical stimulation, just more vigorous than it, therefore it is not surprising that it affects the distribution in the manner discussed in this paper.

R.J. Carroll: Since the pH is a major variable in this study, why did you not fix each sample at the pH of the muscle at the time of sampling to minimize possible artifacts?

R.J. Carroll: Have you carried a restrained muscle through the prerigor pressurization treatment? How would this effect sarcomere lengths? Meat tenderness?

Authors: We agree with you that the effect of pH is very important. This study as well as the study you mentioned in your second question are in progress at this time and we hope to report about them in the near future.

P.B. Addis: Are any commercial applications of this technology currently being utilized?

Authors: So far no, but we think we are getting there.

S.H. Cohen: The authors should make note of the range of activity of the CAF vs catheptic enzymes, since the cathepsins are active at postmortem pH, whereas CAF is not.

Authors: CAF was dealt with in a separate study and its manuscript is under preparation.

Reviewer V: There is no evidence to support the statement that, the Z-disc removal is evidence of lysosomal activity.

Authors: A study on CAF from two different muscles (Koohmaraie, Kennick and Elgasim, in progress) shows that the control had more CAF activity, at all postmortem periods investigated, than the PRP samples. Our preliminary results with cathepsin D indicate that the PRP samples have more activity than their corresponding controls, that is immediately after the pressure treatment (=1 1/2 hr postmortem). Since cathepsin D and CAF are the two enzymes implicated in the Z-disk removal and since the conditions of the pressure treatment are not in favor of the CAF activity, one would tend to lean towards cathepsin D (a lysosomal enzyme) as a possible causative of the Z-disk degradation observed in the study. Also please refer to S.H. Cohen's comment above.

P.B. Addis: Does hyperbaric treatment of the type reported here result in more dissolved gases (nitrogen and oxygen) in the aqueous phase of the meat?

Authors: Both subjective (consumer panel) and objective (Hunter Color Difference Meter) evaluations indicate that PRP improves the color of the meat. The mechanism(s) by which it does so is not clear. Some possible mechanisms are: 1) pressure treatment induces the expansion of muscle pigments (Marshland, 1944); 2) accelerated lactic acid production or 3) the improvement observed in color is due simply to more oxygen dissolved in the aqueous phase of the meat. We do not know.

Discussion References


Additional discussion with reviewers of the paper "Ultrastructure Studies of Pasta. A Review" continued from page 12.

E.A. Davis: Figure 4 shows "water" around starch and membrane residues. In our studies we observe this "water" or "ridge area" forming just as the granule begins to swell. What model system evidence do you mean by "membrane residue"?

Authors: By FF a ridge area of the granule cannot be clearly shown, because etching is not performed. The material around the granule that we indicated as "membrane residue" ("m" in Fig. 4c) exhibits the characteristic features of the material shown at the surface of the granule after a surface-fracture (Fig. 4b).

J.E. Dexter: How did the authors determine cooking time? Why was group B cooked 5 minutes longer than group A? Assuming strand diameter is similar for all samples I would not expect cooking time to vary by more than one minute between samples.

Authors: Cooking time is determined as written in the Appendix. Group B always shows a much longer cooking time than Group A even with the same strand diameter. We think that this is probably due to the starch and protein modifications caused by the heat treatment producing perhaps a lower rate of water penetration into the strand of the spaghetti (Wyland and D'Appolonia, [81]). However, in our opinion, it is difficult to foresee the cooking time of unknown products on the basis of conventional pasta cooking time.

R. Moss: How are protein and starch components identified, particularly in cooked pasta – in many of the Figures the protein appears to have a finer granular structure than the starch? Is this the case?

Authors: In FF cooked samples the protein exhibits always a finer structure than the starch because, under hydrated state, the protein subunits have a diameter at least 3 times smaller than starch spherulites. This is one of the differential features of the two components. Furthermore, the protein matrix shows often inclusions of lipid and other material, never found into the starch, which help in the interpretation of the micrographs.
A REVIEW OF THE MUSCLE CELL CYTOSKELETON AND ITS POSSIBLE RELATION TO MEAT TEXTURE AND SARCOLEMA EMPTYING

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Abstract

A review of the muscle cytoskeleton is presented. Current evidence leads to the concept of a muscle cell cytoskeleton consisting of at least two elements - gap filaments which are located parallel to the fiber axis and provide intracellular elasticity and tensile strength and intermediate filaments found in the Z-disc area that function to connect adjacent Z-discs and promote lateral registration. The former constituent consists of the high molecular weight protein connectin (titin) while the latter is composed of the smaller protein desmin (skeletin). Both proteins exist in filamentous form, are susceptible to proteolysis and are insoluble in physiological solutions. It is proposed that these two elements may interact in the region of the Z-disc to form a three-dimensional network that functions to hold myofibrils in place and provide an ordering of the contractile mechanism. Degradation of the cytoskeleton during post-mortem conditioning of muscle may be partially responsible for the tenderizing phenomenon observed in aged meat. Original work is described in which beef skeletal muscle cell segments are induced to empty, leaving behind the sarcomemal sheath. Conditions necessary for this reaction to occur included the presence of Ca$$^{2+}$$ ions and six days of post-mortem conditioning at 0-5°C. On the basis of these data, it is further proposed that muscle cell emptying may be a consequence of the action of an endogenous proteolytic enzyme that breaks down the cytoskeleton.

Introduction

Meat texture is an important, if not the most important, quality factor dictating its acceptance as a food. Scientists have used genetic, nutritional, chemical, microstructural, instrumental and sensory methodology in order to understand the factors responsible for tenderness - toughness in meat. At present, the most widely accepted theory of meat texture holds myofibrillar contraction and intramuscular connective tissue to be the two major factors determining physical properties of muscle tissue (see Harris, 1976 for review). More recently, Currie and Wolfe (1980) have introduced the concept of intrafiber water as a third factor to be considered in meat texture. These authors found a high correlation among the tensile and adhesive properties of muscle strips undergoing rigor and changes in extracellular space, which, in turn, is inversely proportional to intrafiber water content. High levels of intrafiber water are thought to facilitate slippage of the myofibrils under tensile and adhesive forces.

The purpose of this review is to consider another structural factor which may play a significant role in meat texture. As will be seen, the muscle cell cytoskeleton, although postulated for many years, has only recently come under close scrutiny and its true relation to meat texture is unknown. However, both theoretical and experimental reasons exist for carefully considering the cytoskeleton as a potential contributor to the physical properties of meat.

The Cytoskeletal Concept

It is not greatly surprising that the initial examination of cells using relatively primitive light microscopes led to the view of a membrane surrounding

KEY WORDS: muscle, cytoskeleton, texture, desmin, connectin, sarcolemma.
a structureless cytoplasm embedded in which were various organelles and inclusions. It became apparent, however, that the multitude of enzymatic processes taking place within a cell could not occur with the required frequency if substrate and enzyme were not ordered in some way. This, and the advent of better ways of looking at cells, led to a dynamic concept in which organized arrays of fibrous elements interact to form a highly integrated structural network. These interconnected members are collectively called the cytoskeleton. This term appears to have been introduced in the literature by Peters (1966) although fibrous networks of filaments were reported prior to this by early electron microscopists. It is of interest to note that Peters hypothesized the presence of a cytoskeleton without the benefit of direct microscopic evidence. Concerning the role of the cytoskeleton, he wrote "I felt forced to postulate the presence of a fluid anatomy in the geography of the cell, being some tenuous network by the action of which the cell's enzymic activities were coordinated."

This living framework called the cytoskeleton is now known to exist in all eukaryotic cells. It is composed of various fibrous elements which have been grouped into three major structural categories including microtubules, microfilaments and intermediate filaments. Various functions have been attributed to the cytoskeleton related to cell motilities, e.g. cytoplasmic streaming, organelle movement, cytokinesis, phagocytosis secretion and cell surface modulation (Brinkley, 1982). It is of importance to realize that the cytoskeleton is an active dynamic system involved with cell movement and changes in shape and not simply a passive network as its name might imply.

**Cytoskeleton of the Muscle Cell**

It will be appreciated that because of the unique architecture of the muscle cell, the function of a cytoskeleton would of necessity be different from that of nonmotile cells. As connective tissue serves as an extracellular source of support for the fiber so would the cytoskeleton be presumed to hold myofibrils in place and provide an ordering of the contractile mechanism.

The structural evidence for a muscle cell cytoskeleton would seem to have had its origin in early theories attempting to explain muscle elasticity. Hanson and Huxley (1955) proposed the presence of very thin but elastic "S-filaments" linking actin filaments and a similar proposal was advanced by Hoyle (1967) that featured "ultrathin filaments", independent of actin (Figure 1.1). A number of workers (Sjöstrand, 1962; Carlson et al., 1965; McNeill and Hoyle, 1967) used electron microscopy to demonstrate the actual occurrence of such filaments. Evidence was gathered using muscle tissue from which the contractile proteins had been extracted (Halcott and Ridgeway, 1967; dos Remedios and Gilmour, 1978) and from highly stretched muscle fibers (Locker and Leet, 1975, 1976). In all cases, very fine filaments persisted in the preparations (Figure 2). The names given to these structures and their exact spatial location have varied from worker to worker; the latter workers observed filaments occurring in the A-I gap in highly stretched fibers and used the name 'gap filaments', first conferred on them by Sjöstrand (1962). This name will be used in this review. Regarding location, it would seem reasonable to entertain the following possibilities for thin myofibrillar filaments running parallel to the fiber axis: 1) they could connect an A band to a Z-disc; 2) they could connect two A bands through a Z-disc; 3) they could connect two adjacent Z-discs; 4) or they could connect numerous Z-discs linking to transverse elements, and via these to the sarcolemma. The definitive answer to the exact location of gap filaments awaits further research.

Chemical characterization of the proposed elastic component of muscle was provided by Maruyama and coworkers (Maruyama et al., 1976, 1977) who obtained a rubbery, insoluble protein from extracted myofibrils, termed it "connectin" and concluded that it functions as an elastic component of muscle. The microscope showed the isolated protein to consist of thin filaments and antibody studies located the protein along the sarcomere except at the Z-discs (Maruyama et al., 1981). Connectin, alternatively called titin (see Wang and Ramirez-Mitchell, 1983), has also been identified in the sarcolemma where it might join the membrane and myofibrils.

**Figure 1.** Diagram showing proposed model for muscle including a very thin elastic filament (T) extending between Z-discs (Z) and parallel to the A band (A) and I band (I). Source: Hoyle (1967); used with permission.
Figure 2. Transmission electron micrograph of ox sterno-mandibularis muscle glycinated at twice the excised length and incubated in calcium-activated factor and calcium ions. Most of the Z-disc material has been removed and numerous small filaments can be seen spanning the A-I gap. Source: Locker et al. (1977); used with permission.

Figure 3. Typical force-deformation curve obtained from applying tensile force to uncooked beef muscle. A more pronounced effect may be seen in Locker and Wild (1982b). Source: Currie and Wolfe (1980); used with permission.

Another line of evidence for the presence of thin filaments located parallel to the fiber is based on studies of the tensile properties of muscle. In these experiments force is applied to muscle strips that acts to pull sarcomeres apart longitudinally and the resulting force-elongation curve recorded. Locker and his group (Locker and Carse, 1976; Locker et al., 1977; Locker and Wild, 1982a) have reported that gap filaments may make a significant contribution to the tensile strength of muscle. Upon the application of tensile force in an Instron testing machine, the muscle strips exhibit an initial yield (e.g. Figure 3). This appears to be the same as the "yield point" obtained in a more pronounced way with simpler methodology by Locker and Wild (1982b). This point occurs at a low extension where the connective tissue network is still slack, thus suggesting that the failure takes place in the myofilaments. The location of this yield point remains quantitatively constant even up to the point where the myofibril is becoming "cooked". Locker and Wild (1982a) concluded that the yield point is due to I-filaments snapping at the Z-line or pulling. On the basis of these ultrastructural and chemical observations, it would seem reasonable to conclude that gap filaments, composed of connectin, function as the axial element of the muscle cytoskeleton and are related to elasticity and strength.

Another possible cytoskeletal element was suggested by the observation...
that sarcomeres exist in axial register and exhibit lateral organization leading to the characteristic striated appearance of muscle tissue. Lazarides (1980) has reviewed this subject and cites work demonstrating that the fibrous elements that have been observed to link myofibrils laterally and to link them to the sarcolemma are so-called "intermediate filaments". This term refers to an ubiquitous class of filaments distinguished morphologically by an average diameter of 10 nm and found in many types of cells. Intermediate filaments are particularly abundant in adult smooth muscle. Exhaustive extraction of smooth muscle cells to remove contractile proteins results in a residual cytoskeleton and subsequent electrophoretic separation led to the isolation of a 50-55,000 dalton protein termed "desmin" by Lazarides and Hubbard (1976). This protein has now been prepared from adult mammalian skeletal muscle (O'Shea et al., 1981). These authors also reported the reconstitution of 10 nm filaments from purified desmin. In skeletal muscle desmin, alternatively called skeletal, has been identified in the periphery of the Z-disc in filamentous form (Robson et al., 1981). The unique honeycomb structure of desmin in interconnected Z-discs (Figure 4) leads to the conclusion that this protein, along with actin and α-actinin, comprise this structure. The latter protein gives a fluorescence pattern that is complementary to that of desmin, indicating their intimate association. Possible roles for desmin in muscle were proposed by Lazarides (1980, 1982) including the linking of Z-discs leading to an integration and alignment of contractile elements as well as functions during biogenesis. Figure 5 shows how desmin might function as a part of the muscle cell cytoskeleton. The evidence gathered to date on desmin strongly supports its inclusion as a major component of the muscle cell cytoskeleton.

Figure 5. Diagram of desmin location in skeletal muscle. Source: Lazarides (1980); used with permission.

Thus far two components of the muscle cell cytoskeleton, a gap filament composed of the protein connectin and an intermediate filament composed of the protein desmin, have been identified. Table 1 summarizes their characteristics. Certainly other elements may exist and be demonstrated in the future; new myofibrillar proteins continue to be reported (Greaser et al., 1981). With what is already known, however, an integrated muscle cell cytoskeleton can be envisaged that consists of a three-dimensional framework made up of lateral components (intermediate filaments) linked to axial components (gap filaments) at the level of the Z-disc. Whether the two elements interact directly or through a third constituent such as α-actinin is, at present, unknown but see Wang and Ramirez-Mitchell (1983).

It will be noted that most of the direct evidence gathered thus far to support the existence of a cytoskeleton in muscle cells has been gathered by transmission electron microscopy and protein isolation techniques followed by immunofluorescence. It is always more convincing if parallel proof can be gained by a different approach and, considering the three-dimensional information that can be obtained using the scanning electron microscope (SEM), this would appear to be a useful tool in the study of cytoskeletal elements. The
problem, of course, is one of inadequate resolution. Ip and Fischman (1979) have advanced this goal through the use of high resolution SEM to study isolated and in situ cytoskeletal elements from several sources (Figure 6). These authors conclude that with suitable specimen preparation it is possible to resolve and identify various elements of the cytoskeleton and to obtain direct three-dimensional information on their organization in situ by using the SEM.

Possible Relation of the Cytoskeleton to Meat Texture

In the following discussion it will be assumed that gap filaments and intermediate filaments do exist in muscle cells and that they approximate the structural functions outlined in the previous section. To postulate a role for these elements in meat texture, one must investigate how they are stabilized in situ, the influence of the post-mortem environment and the effect of cooking.

Evidence for cytoskeletal stabilizers is scarce. McCollester and Semente (1966) reported the nucleotide pyrophosphate flavin adenine dinucleotide (FAD) effective in this role but these findings have been questioned (see next section). While this seems the only direct observation recorded, one can speculate that since the former workers found calcium ions and incubation necessary for cytoskeletal breakdown an endogenous enzyme calcium-activated neutral protease (CANP seems a likely candidate) is involved in degradation. Whether this enzyme would hydrolyze filaments or their attachments to other structures is not known.

Post-mortem muscle tissue is degraded by proteolytic enzymes (Penny, 1980; Dayton et al., 1981) and it has been found that both desmin (Robson et al., 1981) and connectin (Takahashi and Saito, 1979) are susceptible to proteolysis. The latter authors reported connectin levels fell to zero within one day in chicken breast muscle and within 7 days in rabbit muscle; Robson et al. (1982) stated 80% of the desmin present in adult mammalian muscle was degraded in seven days at cold room temperature. Thus, whatever contribution these structures may have made to the physical properties of fresh muscle would be diminished as a result of conditioning and variations in tenderness may be related to the degree of cytoskeletal breakdown. Less is known about the effect of heat on the cytoskeleton. Gap filaments from unaged tissue are reported to survive cooking (Locker et al., 1977) but little is known of its influence on intermediate filaments.

It would seem, therefore, that both the known cytoskeletal components have the capacity to be qualitatively related to meat tenderness and the physical properties of muscle tissue. Evidence, or even speculation, for their direct involvement in these processes is limited, however. The existing literature may be summarized as follows:

Gap filaments

A) The disappearance of connectin correlates with the loss of about 30% of muscle elasticity (Takahashi and Saito, 1979).
Based on tensile properties and electron micrographs Locker and Wild (1962c) concluded that only gap filaments and collagen define the tensile strength of cooked muscle.

Intermediate filaments

A) Structural changes occur in the Z-disc, the location of desmin, as a result of conditioning which are reflected in increased tenderness (see review by Penny, 1980).

B) There may be some relation between post-mortem alterations in desmin and water holding capacity (Robson et al., 1981).

It is possible to postulate events in the post-mortem muscle cell that would make the breakdown of the cytoskeleton a major factor in tenderness. If the result of endogenous proteolysis was disconnection of the previously integrated units and not their total breakdown, the cytoskeleton would lose its functional role without any great change in its structure. Regarding this point, Locker et al. (1977) observed that the changes produced in gap filaments by muscle proteases were not evident in uncooked tissue but that heating caused their disappearance. Thus, if the function of the cytoskeleton is lost, the myofibrils may disengage and present less resistance to masticatory forces.

Relation of the Muscle Cell Cytoskeleton to Sarcolemma Isolation

General evidence for the presence of a cytoskeleton in muscle cells resulted from efforts to isolate their sarcolemmas. Reports by McCollister (McCollester, 1963; McCollester and Semente, 1964) described a procedure to obtain skeletal muscle cell membranes based upon the extraction of intracellular contents by water. Dissolution of the cell contents could only be accomplished following homogenization in the presence of Ca++ ions and an incubation step at 37°C. This led the authors to invoke the concept of a cytoskeleton which must be disrupted before the cell contents could be solubilized. The yield of membranes was used to monitor the extent to which the cytoskeleton had been broken down and this reaction was attributed to endogenous enzymes.

Further experimentation by this group (McCollester and Semente, 1966) resulted in the conclusion that nucleotide pyrophosphates, especially FAD, stabilized the cytoskeleton since it inhibited the emptying reaction. This finding was later challenged (Stanley et al., 1968). With our present knowledge of muscle proteolytic enzymes, it seems quite possible that the enzyme responsible for cytoskeletal breakdown is CANP which is located in the Z-disc and in or near the sarcolemma (Dayton et al., 1981). While this enzyme has been reported to be responsible for Z-disc breakdown post-mortem, and thus be of importance in tenderness, it is of interest to note that the yield and properties of a-actinin are unaltered by conditioning (see Penny, 1980). On the other hand, desmin, another major component of the Z-disc, is susceptible to proteolysis. Thus, it may be proposed that an action of the proteolytic enzymes active during conditioning of meat is to attack the cytoskeleton. If this is the case, it should be possible to measure this process indirectly through the yield of sarcolemmas since this reflects cytoskeletal breakdown. The subsequent section describes the results of experiments aimed at testing this supposition.

Preparation of Sarcolemmas from Beef Muscle and its Relation to Post Mortem Conditioning

Procedure

Two procedures were employed to obtain sarcolemmas from beef muscle. The first was adapted from McCollester (1962) and involved homogenization of 20 g of tissue (sternomandibularis muscle from steer carcasses obtained at the time of slaughter and held for 6-7 days at 0-5°C) in distilled, deionized water with 200 mL of cold (5°C) 50 mM CaCl₂ in an Osterizer-type blender fitted with a Polytron Model BEM cutter (Will Scientific, Inc., Rochester, N.Y.) for three bursts of 15 sec. The homogenate was strained through cheesecloth to remove fibrous connective tissue and the muscle cell segments recovered by centrifugation at room temperature for 30 sec in a clinical centrifuge set at full speed. The cells, still retained in four 50 mL centrifuge tubes, were then washed three times in a solution of 25 mM NaCl, 2.5 mM histidine buffered to pH 7.4 with Tris by gently resuspending the lightly packed cells in approximately 10 mL of solution and centrifuging as before. Following the washing steps, the cells were resuspended in the same solution and incubated at 37°C for 30 min. The cells were then centrifuged, washed once in the solution and the cloudy supernatant discarded. The residue was taken up in distilled, deionized water adjusted to pH 7.4 with Tris, centrifuged, and the sediment resuspended in Tris-buffered...
Figure 7. Interference light microscopy of bovine *sternomandibularis* muscle cell segments. a. Following homogenization in 50 mM CaCl$_2$; Note presence of crimp in large cell; b,c. Unemptied cells following extraction procedure; d,e. Partially emptied cell. Note presence of transverse striations in unemptied portions; f. Empty cell showing adhering collagen fibril.

Figure 8. Scanning electron microscopy of unemptied cell. Note presence of transverse striations in b.

The tubes were then shaken vigorously and the contents added to approximately 800 mL of Tris-buffered water stirred with a magnetic bar. The preparation was held overnight at 0-5°C and the degree of emptying quantitated by examination with a light microscope. Three slides with coverslips were prepared using drops of the stirred preparation and counts were made of full or partially emptied cells versus totally empty cells by scanning the entire area of each coverslip at 400x. Toluidine Blue O dye may be used to help differentiate full and empty cells, although with some experience this is not difficult. Data are reported as the percentage of empty cells.

The second method used to prepare empty cell segments was similar to that of Westort and Hultin (1966) and differing only in incubation temperature. The preparation was held at room temperature (ca. 20°C) instead of 37°C. In both cases, controls were added in which the tissue was homogenized in water but otherwise carried through the rest of the standard procedure.

**Results**

The purpose of these series of experiments was to investigate the role of post-mortem conditioning, Ca$^{++}$ ions and high temperature incubation in the production of beef skeletal muscle sarcolemmata since all of these parameters have been implicated in cytoskeletal breakdown. Thus, the methods described above were applied to fresh beef muscle and tissue that had been conditioned for up to 6 days at 0-5°C. The data from this work (Table 2) show the absolute requirement for Ca$^{++}$ ions. They also show that emptying did not occur for

<table>
<thead>
<tr>
<th>Post-mortem conditioning (days)</th>
<th>Homogenizing media</th>
<th>Temp. of incubation</th>
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<td></td>
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<td>50 mM CaCl$_2$</td>
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<td>Room Temp.</td>
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<td>81</td>
</tr>
<tr>
<td>9</td>
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</tr>
</tbody>
</table>

Table 2. Yield of sarcolemmata from beef skeletal muscle. Effect of post-mortem conditioning and procedural variation on percentage of empty cells. (Data are average of two animals).
rigor muscle or tissue kept only 3 days at 0-5°C, but occurred readily after 6 days of conditioning. Incubation temperature did not produce a significant effect under these conditions.

Micrographs were taken of the cells at various stages of the emptying process. Figure 7a shows a typical field of the homogenized tissue under low light microscope magnification. Note that several of the cell segments indicate the presence of crimp. As mentioned, the muscle tissue used in these experiments was removed from the carcass immediately post-mortem and thus went into rigor unrestrained which may explain their contracted appearance. It was observed that these bands were no longer apparent in unemptied cells at the end of the procedure. In Figures 7b and c may be seen two cell segments that have undergone the complete procedure but which did not empty. It will be observed that in both cases the cell contents have ballooned out, in 7b from the cut end and in 7c in the middle of the cell segment. Scanning electron micrographs of a similar cell gathered on a 45 μm Millipore filter and subsequently critical point dried and coated (Figure 8) show what appears to be a constricting sarcolemmal envelope holding in the cell contents; where these have escaped from the envelope characteristic transverse bandings of myofibrils can be observed which indicates that in unemptied cells Z-discs remain intact. The ballooning effect was more prevalent as conditioning time increased, suggesting that it results from a failure of transverse structures. Presumably it is this failure of transverse elements impinging upon the sarcolemma that makes emptying possible.

Partially emptied cells are shown in Figures 7d and e. The unempted portions may show transverse banding and the emptied tube is characterized by what may be interpreted as collagen fibers. Figure 7f, an emptied cell segment, displays in the lower left corner, a typical fiber thought to be an adhering strand of collagen. An isolated cell membrane was photographed at higher magnification using three different planes of focus (Figure 9). Presumed collagen fibers may be observed in apparently random orientation.

Conclusions

It is not difficult to reconcile the results of these experiments with what is known of the muscle cell cytoskeleton. The requirement for Ca++ ions and six days of post-mortem conditioning are consistent with a hypothesis that ascribes muscle cell emptying to a breakdown of the cytoskeleton by a calcium activated endogenous proteolytic enzyme. Of particular interest is that, in this system, emptying only occurs following a conditioning period which normally produces significant tenderizing in beef muscle (Stanley, 1983). While much more research is required in this area, these preliminary results indicate that muscle cell emptying may be a simple, albeit indirect, measure of breakdown of at least the transverse elements of the cytoskeleton which, in turn, may reflect the tenderization phenomenon occurring in conditioned meat.
MUSCLE CELL CYTOSKELETON AND TEXTURE

Acknowledgements

The contributions of Drs. H. J. Swatland, C. L. Duitschaever and Mrs. Alexandria Smith to this work are acknowledged with gratitude. The original work described herein was supported by the Natural Sciences and Engineering Research Council of Canada and the Ontario Ministry of Agriculture and Food.

References


Discussion with Reviewers

S.H. Cohen: Would the author comment on the conclusion made by Ullrick et al. (Ullrick W C, Tosell P A, Chase D and Dasse K. (1977). Are there extensions of thick filaments to the Z-line in vertebrate and invertebrate striated muscle? J. Ultrastruct. Res. 60: 263-271) that "extension of the myosin-containing filaments to the Z-line are present in invertebrate flight muscle, but that we find no evidence to suggest that they are present in vertebrate muscle"?

Author: Although the existence of 'gap filaments' has been challenged by the above authors, there is a compelling body of both experimental and theoretical evidence dating from the mid-50's (see text) that lead to the conclusion that, in fact, this proposal must be taken seriously.

S.H. Cohen: Does the author have any experimental evidence on the integrity of the sarcolemmas as indicated by the retention of enzymatic activity?

Author: No, our experiments thus far have centered on determining the percentage of empty tubes produced by various experimental conditions through microscopic rather than chemical means. It should be noted that it has been shown that isolated sarcolemmas exhibit ATPase activity but several glycolytic enzymes were not found (McCollester D L and Randle P J (1961). Isolation and some enzymatic activities of muscle cell membranes. Biochem. J. 28: 27 pl.
P.B. Bell: Can the author relate the results obtained by the "sarcolemmal emptying" assay to more direct ways of examining the cytoskeleton of muscle fibers?

Author: Work is presently underway in this laboratory to attempt to determine the relationship between the emptying reaction and both chemical and structural analyses.

S.H. Cohen: The author considers only CANP as contributing to the breakdown of the cytoskeleton. Could any other enzymes be involved?

Author: After reviewing the available evidence, it was concluded (Penny, 1980) that "CANP has clearly been shown to be the enzyme most likely to be involved (in conditioning) because its activation by Ca++ ions explains the accelerating effects of Ca++ ions on conditioning and it can mimic the changes observed during conditioning." It remains to be established if other proteolytic enzymes found in muscle cells such as cathepsins B and D have a role in breakdown of the cytoskeleton.

R.H. Locker: Since the paper begins on the theme of meat texture and tenderness, it should have paid more attention to cooked meat. Tenderness is assessed on cooked meat, where the relative significance of filaments is totally changed. I have attempted to discuss this in detail for myofibrillar filaments in papers presented at the Reciprocal and European Meat Conferences in 1982. The G-filaments have proved to be the survivors, even on extreme cooking.

The fate of the transverse "honeycomb" of desmin on cooking has had little attention. However in 1976 Davey, Niederer and Graafhuis (J. Sci. Fd. Agric. 27, 251) showed that links between the Z-lines of adjacent myofibrils survived cooking (40 min., 80°C). This evidence of course says nothing about strength.

The questions of whether the critical junctions between axial and transverse elements deform under tension to provide axial strength also remain unanswered.

Author: I agree with you that these are important considerations for future work in this area.

J.M. Squire: Are micrographs, such as those shown, thought to represent hard evidence about the state of a particular cell? Would it not be possible to compare pelleted and soluble mass under the various conditions used, to get good quantitative data on the emptying of cells?

Author: The answer to the first part is yes. Work is ongoing regarding the second part and we hope to report on it in near future.
MYOFIBRILLAR CHARACTERISTICS OF PORCINE STRESS SYNDROME

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Abstract

Porcine Stress Syndrome (PSS) is a genetic trait causing considerable economic loss to the swine industry through stress related death and the poor quality meat known as pale, soft, exudative (PSE) pork. A scanning and transmission electron microscopic examination of muscle biopsies from stress susceptible pigs revealed contracture bands, wide separation of myofibers and focal distortion and dissolution of myofibrils. The changes affecting myofibrillar characteristics and intra and intercellular accumulation of material suspected to be myoplasmin fluid in biopsies of halothane reactors suggest that the myopathic alterations presaging the carcass deterioration into pale, soft, exudative pork are integrants of this syndrome and that the PSE trait may not be a postmortem change triggered by the environmental factors just prior to or during slaughter.

Introduction

Porcine Stress Syndrome (PSS) is one of the major economic threats to modern day swine industry. The economic impact from this syndrome is two pronged with a major loss inflicted on the breeder through death in response to environmental stress, and a substantial loss to the meat industry and consumers from the inferior carcass quality of the pigs which survive until slaughter time. The undesirable carcass changes include the development of pale, soft, exudative (PSE) pork or dark, firm, dry (DFD) meat, depending upon the glycogen level in the muscles at the time of slaughter (Briskey, 1964). Previous investigations on stress susceptible pigs have shown that the trait occurs at a high frequency in some breeds and that it has a genetic etiology (Sybesma and Eikelenboom, 1969; Williams et al, 1977; Webb, 1980). The incidence is higher in breeds which exhibit rapid growth rate, high feed efficiency and heavy muscling (Nelson, 1973). The adverse and often fatal reaction is elicited by a variety of forms of stress including transport, crowding, fighting, restraint, temperature fluctuation, and exposure to drugs including halothane (Williams, 1977; Britt, 1972). The clinical symptoms of stress reaction include an increase in heart rate, muscle tone, cyanosis and body temperature (Britt, 1972; Grenert, 1980). The stress syndrome in pigs is similar to the condition recognized as malignant hyperthermia in man (Williams, 1977) and in both conditions, muscle enzymes including creatine kinase (CK) leak out into the circulation because of a defect in muscle cell membrane (Britt, 1972; 1974). It has been postulated that cells other than muscle (such as erythrocytes) may have defective membranes (Harrison and Verburg, 1973) and that the syndrome in man and pigs exhibits varying degrees of myopathy (Buxton, 1980).

The objectives of our investigation were to examine the scanning and transmission microscopic images of muscle biopsies in order to determine whether or not myopathic alterations related to the "watery pork" characteristic are detectable in biopsies of boars which were previously classified as either stress susceptible or normal on the basis of their response to halothane challenge.

KEY WORDS: Porcine Stress Syndrome; Contracture Band; Myofibrillar Distortions; Pale, soft, exudative (PSE) Pork; Malignant Hyperthermia; Watery Pork; Carcass Quality.
Materials and Methods

Halothane Test
For the halothane test, the boars were restrained on their back in a V-shaped backboard and were allowed to breathe halothane (Somnothane®) in oxygen through a tight-fitting face mask for five minutes. Halothane in oxygen was administered by a Narcovet Veterinary Anesthesia Machine at a concentration of 4.0% for the first three minutes and 2.0% for the last two minutes. During the administration of halothane, the boars were carefully observed for signs of muscular rigidity, and the flexibility of the limbs was tested frequently. Boars that showed muscular relaxation during anesthesia were classified as normal or nonreactors to halothane, the boars were carefully observed for signs of muscular rigidity, and the flexibility of the limbs was tested frequently. Boars that showed muscular relaxation during anesthesia were classified as normal or nonreactors to halothane (Eikelenboom and Minke, 1974). A positive reaction to halothane was recorded if muscular spasm and progressive rigidity were observed and when the hindlimbs became stiff and extended. Additional signs of a reactor to halothane includedlaboured, open-mouthed breathing and blotchy cyanosis of the skin (Eikelenboom and Minkema, 1974; Webb, 1980).

All halothane reactors were removed from the challengeing agent and subjected to surface cooling by water spray from a hose.

Collection and Preparation of Muscle
The animals included in this study were halothane tested boars belonging to the Yorkshire and Landrace breeds (Table 1). They consisted of six normal and six halothane reactors which were revived and maintained for approximately four months prior to subjecting them to the biopsy procedure.

Skeletal muscle samples were obtained from the medial portion of the gracilis muscle. For the biopsy, the nonreactors were anesthetized with halothane in oxygen and the reactors were anesthetized by intravenous administration of thiopental sodium (Pentothal®).

Muscle samples were placed in Hank's balanced salt solution (HBSS) immediately after collection. Pieces of muscle, approximately 10 mm in length and 4 mm in cross section were dissected out and placed in such a way that the myofibers were in longitudinal orientation when the specimen was tied at each end with a surgical silk thread prior to fixing on to a cork to prevent contraction. The specimens were immersed in fixative consisting of 5.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 hours. Some of the samples were then cut into 1 mm pieces, washed in phosphate buffer several times, and post-fixed in phosphate buffered 1.0% osmium tetroxide for one hour. The tissue pieces were then rinsed in phosphate buffer, dehydrated in a graded series of ethanol, infiltrated in Epon via propylene oxide, flat-embedded in Epon in aluminium weigh boats and polymerized at 50°C for 48 hours.

Epon embedded specimens were cut with a jeweller's saw and affixed to blank Epon blocks with the five minute epoxy glue and 1.0 µm sections were cut with a Reichert OMU2 ultramicrotome for light microscopy. Areas of interest were selected for ultrathin sections (60-150 nm) which were mounted on uncoated 300 mesh grids and were contrasted in 2.0% ethanolic uranyl acetate and lead citrate and examined in a JEOl 100S transmission electron microscope.

Samples from glutaraldehyde fixed muscle specimens were cut obliquely with a clean razor blade and varying levels of the cut surface of the muscle cells were exposed by gently detaching the cut ends, prior to processing the samples for scanning electron microscopy according to procedures outlined previously (Basrur and Basrur, 1977).

Results
The ultrastructural features of muscle from nonreactor pigs were generally similar to those reported for normal mammalian muscle. Muscle samples from halothane reactors showed a variety of changes including hypercontraction, widened interfibrillar spaces, alterations in the Z-bands, disorientation and dissolution of myofibrils, and bizarre orientation of myofibrillar remnants at right angles to the long axis of the muscle cell (Figs 1 to 5). Scanning electron microscopic studies showed that the surface contour of the normal muscle (from halothane nonreactors) is relatively even and that in the fractured regions the myofibrils were stacked in very close apposition to each other (Fig. 6). In halothane reactor muscle samples, individual muscle fibers were noted in hyper-contrasted state adjacent to relatively normal muscle fibers (Fig. 7). Aggregates of material probably including myoplasm that has segregated and leaked out of the muscle cells (Fig. 8) were noted in the intercellular space and within individual muscle fibers. The myofibrils toward the periphery of individual muscle cells were often noted to branch and stretch towards those of adjacent muscle fibers (Fig. 9) and myofibrillar bundles exhibited varying diameters and were separated from each other (Fig. 10).

Fig. 3. A muscle fiber from a halothane reactor boar showing separation (S) of the fibrillar components and myoplasm (MP) almost devoid of organelles. Note the nucleus (N) that has moved towards the centre from its characteristic position on the periphery of the muscle.

Fig. 4. Muscle fibers of a halothane reactor boar showing bulged segment of a narrow muscle strap (BS) displaying varying degrees of myofibrillar alterations including stretching, separation and dissolution. Note the relatively normal mitochondria (M) and a lysosome-like body (L) in the myoplasm (MP) devoid of contractile elements.

Fig. 5. Peripheral region of a muscle fiber from a halothane reactor boar showing bizarre organization of myofibrils (MF). Note the electron dense central region(CR) and the disoriented Z-bands (Z) on myofibrils radiating in all directions from the dense structure.

Fig. 6. Scanning electron microscopic view of a muscle biopsy from a normal (halothane nonreactor) boar. Note the smooth contour of four myofibrils (1 to 4) and the two levels of the muscle surface exposed with (1 to 3) and without (4) basement membrane (arrow). Note also the myofiber (S) displaying the closely packed nature of the myofibrillar bundles (MF).
Fig. 1. A muscle biopsy sample from a halothane reactor boar showing contracture bands (CB) in a few myofibrillar bundles adjacent to apparently normal bundles (MF). Note the focal nature of the contracture involving 13 to 14 bands, and the signs of detachment of the sarcomere (open arrows).

Fig. 2. A muscle biopsy sample from a reactor boar showing dissolution of contractile elements and the remnants of contractile elements (arrow) towards the plasma membrane (PM). Note the remnants of the T-system (arrowhead) and the wide space (S) between myofibrillar bundles.
Fig. 7. Surface view of muscle cells from a halothane reactor boar showing a myofiber exhibiting hypercontraction (HC), among apparently normal myofibers.

Fig. 8. An exposed view of the muscle from a halothane reactor boar. Note the bulged areas running almost parallel to the fibers, probably representing myoplasm (MP) that has segregated or leaked out of the muscle.

The ultrastructural changes observed in skeletal muscle samples of halothane reactor (stress susceptible) boars and boars which exhibited normal reaction to halothane are summarized in Table 1. Evidence of myofibrillar dissolution and the presence of vacuoles of varying size and number, were strikingly more prominent in the reactors although one of the boars classified as normal (4L; NR) also exhibited these features. Hypercontraction of individual muscle fibers, however, was noted only in the reactors (Table 1).

Fig. 9. Myofibrillar disorganization in a muscle fiber of a halothane reactor boar. Note the fibrillar branching (MF) and extension to the neighbouring myofibers (arrows).

Fig. 10. Myofibrillar bundles from a halothane reactor boar showing wide space (S) between individual myofibrillar bundles. Note the contracture bands (CB) in these myofibrils.

It is worthy of note that the muscle biopsies of all reactors included in this study exhibited two or more of these alterations (Table 1). In addition to these, muscle biopsies of halothane reactors also exhibited the presence of strap-like fibers, displacement of myonuclei from their peripheral location towards the center, accumulation of glycogen granules and the presence of lamellated bodies in varying proportions of the muscle fibers.
Elevation of sarcoplasmic calcium levels. These events are attributable to accelerated postmortem glycolysis including tissue acidity, and that the degree of muscle hydration is secondary to the other metabolic changes in muscle. Investigations also demonstrated that heat production occurs through futile cycling in muscle cells whereas the presence of strap-like interfibrillar space and the disorientation of myofilaments are suggestive of degeneration in gross appearance or histological features at the time of slaughter, in muscles which ultimately become PSE. These investigators believe that the PSE condition is attributable to accelerated postmortem glycolysis and that the degree of muscle hydration is related to the extent of rigor mortis. However, Williams, et al. (1977) have noted that excess heat generation occurs in PSE muscle 14 minutes before rigor mortis develops and have suggested that rigor, which also can cause heat production is secondary to the other metabolic changes including tissue acidity, ATP depletion and the elevation of sarcoplasmic calcium levels. These investigators also demonstrated that heat production occurs during futile cycling in PSS muscle before slaughter and that the alteration in muscle cell components and the development of PSE muscle postmortem may be related to this phenomenon (Williams et al., 1977).

Histological and ultrastructural changes during postmortem autolysis of muscle samples from normal and stress reactor pigs have been examined by various investigators (Briskey, 1964; Dutson et al., 1974). The most striking postmortem difference between PSE muscle and normal muscle was in the extent of derangement in mitochondria and sarcoplasmic reticulum and in the disruption of myofibrillar organization (Dutson et al., 1974). Myofibrillar disruption and a tendency for longitudinal splitting were generally more pronounced in the white fiber type as compared to the red fibers in muscle samples subjected to postmortem autolysis (Abbott et al., 1977). A recent scanning and transmission electron microscopic study on cryofractured longissimus dorsi muscle samples collected immediately after slaughter from pigs genetically predisposed to different degrees of stress, revealed changes similar to those described in this report (Cloke et al., 1981). These investigators also noted that the connective tissue investment of individual muscle fiber is detached and that the plasma membrane is disrupted in stress susceptible pigs and postulated that the extent of plasma membrane disruption noted in stress susceptible pigs may be indicative of the relative fragility of their muscle and connective tissue components. All these studies on PSS pigs (Cloke et al., 1981; Dutson et al., 1974; Briskey, 1964; Venable, 1973) relate to the state of muscle samples at the time of slaughter or at different intervals after slaughter. Our studies were conducted on biopsies which represent the status of muscle in the live animal with the exception that they were exposed to anesthetics during biopsy collection. The reactors were anesthetized with sodium pentothal instead of halothane in order to avoid a stress reaction from halothane exposure at the time of biopsy collection. The difference noted between the muscle samples of halothane reactors and non-reactors cannot be attributed to the difference in the anesthetics used since sodium pentothal or sodium thiamylal does not trigger stress reaction in pigs (Jones et al., 1972). Furthermore, the biopsy collection was accomplished within 20 minutes of induction, at which time no difference was noted in our pilot study on muscle samples collected from normal pigs anesthetized with halothane, sodium pentothal or sodium thiamylal. The similarity between the ultrastructural alterations in biopsies of stress susceptible pigs in the present study and those reported in pigs subjected to fatal stress reaction crisis (Venable, 1973) further supports our view that the myofibrillar alterations reported here represent a real difference between stress susceptible and normal pigs.

It has been hypothesized previously (Briskey, 1964; Swatland, 1980) that the PSE trait is precipitated by environmental factors that induce stress in a genetically predisposed animal. The environmental factors that

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**Table 1. Comparison of Skeletal Muscle Alterations in Landrace and Yorkshire Boars Identified as Reactors and Nonreactors to Halothane Challenge.**

<table>
<thead>
<tr>
<th>Boar # and Status</th>
<th>Hyper-Contraction</th>
<th>Vacoulation of Myoplasm</th>
<th>Myofibrillar Dissolution</th>
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</tr>
<tr>
<td>12L;R</td>
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Y: Yorkshire NR: Nonreactor to Halothane L: Landrace R: Reactor to Halothane

**Discussion**

Our transmission and scanning electron microscopic studies show that the biopsy specimens from stress susceptible boars exhibit features which may be causally related to the tendency of these animals to develop pale, soft, exudative (PSE) pork. The characteristic features relevant to this phenomenon are the abundance of non-myofibrillar material accumulated between individual myofibrillar bundles, hypercontraction, degeneration and dissolution of myofibrils, and the accumulation of material resembling myoplasm within and between muscle cells. The widened interfibrillar space and the disorientation of individual bundles of myofibrils are probably related to the fluid segregation in muscle cells whereas the presence of strap-like fibers and the displacement of myonuclei may be indicative of the regenerative process taking place in response to the degeneration of individual muscle fibers in stress susceptible boars.

Briskey (1964) and Briskey and Wismer-Pedersen (1961) reported that there is no change of degeneration in gross appearance or in histological features at the time of slaughter, in muscles which ultimately become PSE. These investigators believe that the PSE condition is attributable to accelerated postmortem glycolysis and that the degree of muscle hydration is related to the extent of rigor mortis. However, Williams, et al. (1977) have noted that excess heat generation occurs in PSS muscle 14 minutes before rigor mortis develops and have suggested that rigor, which also can cause heat production is secondary to the other metabolic changes including tissue acidity, ATP depletion and the elevation of sarcoplasmic calcium levels. These investigators also demonstrated that heat production occurs during futile cycling in PSS muscle before slaughter and that the alteration in muscle cell components and the development of PSE muscle postmortem may be related to this phenomenon (Williams et al., 1977).

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It has been hypothesized previously (Briskey, 1964; Swatland, 1980) that the PSE trait is precipitated by environmental factors that induce stress in a genetically predisposed animal. The environmental factors that
aggravate stress susceptibility include trucking, fighting and crowding trauma related to transportation to the slaughterhouse. The variable expression of PSE trait in pigs previously identified as stress susceptible has been attributed to the variability in the degree of pre-slaughter trauma and postmortem environmental factors (Swatland, 1980; Cassens et al., 1963; Barton-Gade, 1981). Our observations on muscle biopsies indicate that alterations relevant to the PSE trait are already present in PSS muscle in vivo, and that evidences of a membrane defect and myopathy postulated in porcine stress syndrome and that evidenced for halothane testing the pigs used in this study. The technical assistance of Mr. C. A. Ackerley, Mr. Ed. Reyes, Mrs. Helen Randall and Mrs. Mary John are gratefully acknowledged. This investigation was supported by Natural Sciences and Engineering Research Council of Canada, Ontario Ministry of Agriculture and Food and Agriculture Canada research contract.

Acknowledgements

Our sincere thanks are extended to Dr. Dave Seeler for halothane testing the pigs used in this study. The technical assistance of Mr. C. A. Ackerley, Mr. Ed. Reyes, Mrs. Helen Randall and Mrs. Mary John are gratefully acknowledged. This investigation was supported by Natural Sciences and Engineering Research Council of Canada, Ontario Ministry of Agriculture and Food and Agriculture Canada research contract.

References


Discussion with Reviewers

M. Ashraf: Please define myoplasm as discussed in Figure 8. the"myoplasm" appears to be a contracted blood vessel.
Authors: The term myoplasm is used in Figure 8 to describe the SEM image of the intracellular material similar to the TEM images free of myofibrils shown in Figures 2 and 3. Images similar to that identified as myoplasm in Figure 8 were noted scattered irregularly within and between
MYOFIBRILLAR CHARACTERISTICS OF PORCINE STRESS SYNDROME

Muscle fibers in our material and in the illustrations of muscle sample from stress susceptible pigs of the Pietrain breed examined by Cloke et al (1981). It is possible that the area marked as myoplasm in Figure 2 contains interfibrillar mitochondria and remnants of sarcoplasmic reticulum (as shown in Figure 2). However, we believe that the bulk of what is marked as myoplasm represents intracellular fluid (myoplasm) with or without disintegrated sarcotubular system.

We do not believe that it is a blood vessel since erythrocytes or leukocytes were never detected in areas where these "structures" appear to be disrupted or discontinuous.

Reviewer 4: The muscle samples of the reactors were taken after a malignant hyperthermia reaction, rather than before. Could it be that some or all of the changes observed were due to the reaction itself rather than being present genetically prior to the drug administration?

Authors: The biopsy samples were obtained from the pig approximately 4 months after they were subjected to halothane challenge. We believe that the interval between the halothane test and biopsy collection is long enough to ensure that the abnormalities are not the direct results of a halothane-induced stress reaction. At the time of biopsy collection no clinical sign of malignant hyperthermia was detected in the reactors. The anesthetic drug (sodium pentothal) used for biopsy collection from the reactors, does not trigger a stress reaction in stress susceptible pigs (Lucke et al, 1979; Jones et al, 1972).

M. Ashraf: Are the changes discussed specific to Porcine Stress Syndrome: How are they related to other known skeletal muscle myopathies?

Authors: Some of the changes noted in muscle samples of stress susceptible pigs, such as myofibrillar disintegration, hypercontraction and displacement of nuclei are also noted in a variety of human myopathies (Buxton, 1980). Williams (1977) had postulated that myopathy may be an integral part of malignant hyperthermia in man and stress susceptibility in pigs. Our biopsy material which showed myopathic changes in all stress susceptible pigs emphasizes the point that these changes may be the physical basis, or the forerunners, of the PSE trait which is generally considered to be the result of postmortem deterioration of muscle in certain genotypes.

K. Lundström: Have you checked pigs that do not react to halothane but are carriers of the halothane gene (heterozygotes)?

Authors: In our ongoing studies we have examined the biopsies from a few halothane nonreactors which were subsequently challenged with succinylcholine. Biopsies from boars which were nonreactors to halothane and succinylcholine were generally free of the severe ultrastructural alterations. More notably, we detected occasionally some disparity between the animal's response to halothane and the ultrastructural features of muscle sample from that animal. In one instance, the biopsy sample from a halothane nonreactor boar showed severe myopathic alterations similar to those reported here. This boar later reacted severely and fatally while being moved to another pen, exhibiting all the clinical signs of malignant hyperthermia. We are not sure whether this boar was a "heterozygote" for the halothane gene.

C.A. Voyle: Did the authors attempt to classify the muscle fibers, i.e. red, white or intermediate, which showed altered appearance in the halothane reactors?

Authors: We did not undertake fiber typing in this investigation. The muscle sample used in this study was obtained from gracilis which is mainly of the white muscle type and consequently a majority of the muscle fibers showing ultrastructural alterations were of the white type fibers. However, disintegration of myofibrils were also noted in thinner fibers which according to the Z-line criteria used by Dutson et al (1974) may well be red fibers. We are not sure however, whether these thin fibers are red fibers or newly formed strap-like cells resulting from the regeneration of white fibers.

C.A. Voyle: What proportion of the fibers in the muscle samples were affected?

Authors: The proportion of fibers detected with alterations in muscle biopsy samples varied depending upon the optical system used for examination. For example, light microscopic examination of semithin sections showed fibers exhibiting hypercontraction, displacement of nuclei and vacuolation in 5 to 10% of cells only. At the ultrastructural level, myofibrillar alterations, mitochondrial distortions and the presence of lamellated structures reminiscent of muscle degeneration were noted in 15 to 30% of the fibers. One of the striking features of these biopsies was that all samples from halothane reactors contained some fibers showing unequivocal degenerative changes although these were generally surrounded by apparent unaltered normal muscle cells.

K. Lundström: Which biopsy procedure did you use? Is it a surgical procedure with samples cut out with a scalpel or did you use some sort of biopsy needle?

Authors: We used the surgical procedures using scalpels to cut out muscle samples. The procedure introduced by Lundström et al (Swedish J. Agric. Res. 3:211-213, 1973) was not used in our studies since we needed larger samples to carry out our ultrastructural studies and some of our other investigations not reported here, including in vivo caffeine contracture test.

K. Lundström: You state that the incidence of stress susceptible pigs is higher in breeds which exhibit rapid growth rate and heavy muscling. I fully agree on heavy muscling, but I cannot remember any evidence of higher growth rate in stress susceptible pigs. The contrary is, however, well known in pigs of the Pietrain breed.

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Various investigators have reported that halothane reactor (stress susceptible) pigs exhibit increased muscle growth and increased feed efficiency and growth rate relative to non-stress susceptible pigs. However in a recent report, Webb (1980) states that the stress susceptible pigs are better than normal pigs on food conversion ratio and lean content criteria, while the former group performs less well in terms of daily food intake and growth rate. Even though these two statements would appear to be contradictory, it is possible that the lack of appetite of stress susceptible pigs combined with their genetic predisposition to be muscular, is responsible for the apparent superiority in food conversion ratio and carcass traits reported in stress susceptible pigs.
These abstracts are compiled by Dr. Kalab with the help of Dr. K. Saio. If you wish to submit abstracts for FOOD MICROSTRUCTURE, please contact Dr. Kalab.

MILK, DAIRY PRODUCTS

INORGANIC FOULING OF MEMBRANES DURING ULTRAFILTRATION OF CASEIN WHEY


The removal of microorganisms, material that accumulates and fouls the surface of membranes used in the ultrafiltration (UF) of casein whey at 2 New Zealand dairy factories has been measured at intervals during one season at one factory and 2 seasons at the other. Microorganisms containing S, P, Cl, Na, K, Ca, and Mg were deposited in significant amounts during the daily U.F. operation. The daily 'cleaning in place' (CIP) washing procedure was found to remove most of the deposit. However, some CIP-resistant deposit remained, and its slow incrustation was found to be principally caused by an increasing deposition of Fe. Data from an electron probe microanalyzer in an SEM was consistent with some Fe existing as a form of FeO.

THE MICROCRYSTALLINE INCLUSIONS IN GRANA CHEESE AND THEIR X-RAY MICROANALYSIS


The distribution, structure and chem. compn. of microcrystalline inclusions in Grana cheese were studied by SEM X-ray microanalysis. During the ripening of Grana cheese, 2 types of crystals were found. One of them was crystalline inclusions, 10-20 μm in diam., with a compact structure. They were found to consist of Ca phosphate. These crystals were abundant in cheese 48 h old and older. The other type of microcrystalline inclusions were needle-like crystals 20-50 μm long. These were found to have small quantities in ripened cheese; X-ray microanalysis showed that these inclusions were composed of Ca phosphate. The crystals were found to penetrate bacterial colonies in Grana cheese 8 months old.

IMMUNOLOGICAL APPROACH TO LOCATION OF γ-CASEIN IN THE CASEIN MICELLE BY ELECTRON MICROGRAPHY


The colloidal stability of skim milk is due to γ-casein in casein micelles, thus, the location of this protein within these micelles has been the subject of speculation. Various models have placed γ-casein at the surface, in the interior, or distributed uniformly in the casein micelle. By a ferri­tenia-stained antibody reaction coupled with immuno­localization, the location of γ-casein has been related to casein micelle size. Casein micelles with γ-casein located predominantly at the periphery of the micelle have diameters of 140-220 A, whereas micelles with γ-casein located uniformly throughout have diameters of 920-220 A. These results are in accord with the inverse relationship between micelle size and γ-casein content and support the model of Slattery (Slattery CM, Evard R. (1973). Biochin. Biophys. Acta 317, 529).

MICROSTRUCTURE OF YOGURT STABILIZED WITH MILK PROTEINS.


Skim milk yogurts were stabilized with a variety of casein- and whey-protein-based ingredients. Exptl. yogurts contd. 1.5% added protein, and compared to a reference yogurt prep. with 0.5% gelatin (225 Bloom strength). SEM and TEM revealed the presence of casein crystals. The structure of the casein crystals in the casein yogurts was dependent on the casein prep. with additional casein. yogurts prep. with skim milk powder and milk protein conc. were composed of casein mi­celles containing protein aggregates. Skim milk and renneted casein-based yogurts in that casein micelles were individual in nature with intermicellar spaces spanned by flocculated protein.

INFLUENCE OF THE COMPOSITION OF THE MILK FAT GLOBULE MEMBRANE ON THE RHEOLOGICAL PROPERTIES OF ACID MILK GELS.


Acid milk gels were made by acidification of skim milk with suspended fat globules to pH 4.6 at 4°C, followed by heating up at a rate of 0.3 K/min to 30°C. Washed natural milk fat globules were used as particles for which cross-linking between the membrane proteins and the casein gel matrix was not expected. As interacting particles recombined milk fat globules were used, prepared at 40°C by pre­mixing anhydrous milk fat with skim milk in an Ultra Tur­rax mixer and then homogenizing twice at 1 MPa and at ca. 0.25 MPa, respectively. In the latter sample, cross linking between the casein and the casein forming the gel matrix can be expected. It was checked by microscopic evaluation that homogenization clusters were absent or very small. It is suggested to describe matrices composed of interacting and non-interacting components.

CYTOPLASMIC REMNANTS IN MILK OF CERTAIN SPECIES.


The quantity of cytoplasmic remnants was detd. by fluores­cence microscopy in the milk of various species (human, rabbit, rat, pig, cow, sheep, and goat) and was found to be about the order of 10⁴-10⁵/mL of the milk. This result practically eliminates apocrine secretion as a mechanism in milk synthesis.

VERGLEICHENDE UNTERSUCHUNGEN VON BOHMILCHLITTERERÄSSEN MIT UND OHNE WACHAUHERZ. [Comparative analyses of raw milk Tilsit cheese with and without secondary fermentation]


Various methods have been applied to analyze throughout one year of raw milk Tilsit cheese with and without secondary fermentation. Data from an electrophoresis analysis of raw milk Tilsit cheeses of good quality and 54 cheeses with secondary fermentation (Unilever Sharnbrooke, UK) were partly differences in water and protein levels. From the variations in the enzyme patterns it can be concluded that the 2 cheese types must have different microfloras. SEM micrographs of the cheese protein matrices are shown.

RECENT ADVANCES IN THE DESTABILIZATION OF DAIRY EMULSIONS


Destabilization of emulsion droplets is described in terms of flocculation, coalescence, and adsorption processes, and then discussed with reference to various technological aspects of dairy emulsions. A semi-quantitative measurement of homogenization-induced flocculation of cream fats and defects described using measurements on the time-dependent decrease in viscosity that occurs during shear. The whipping of cream is discussed in terms of the adsorption of these droplets to air bubbles and the formation of a fat particle network with the fat directly in contact with air. The whipping of homogenized dairy creams is shown to be dependent upon the structure of the newly formed milk fat globule membrane. Since the major protein component of the membrane in casein, any change in casein micelle stability is reflected in the whisking properties of the cream. The profile of crystallinity of casein micelles and the casein angle between the fat crystals is described using measurements on the time-dependent decrease in viscosity that occurs during shear. The whipping of cream is described using measurements on the time-dependent decrease in viscosity that occurs during shear. The whipping of cream is described using measurements on the time-dependent decrease in viscosity that occurs during shear. The whipping of cream is described using measurements on the time-dependent decrease in viscosity that occurs during shear. The whipping of cream is described using measurements on the time-dependent decrease in viscosity that occurs during shear. The whipping of cream is described using measurements on the time-dependent decrease in viscosity that occurs during shear.
ASSOCIATION OF CASEINS AND CASEIN MICELLE STRUCTURE.


A review with 139 refs. using EM to illustrate the compn. and properties of casein micelles.

A MORPHOLOGICAL STUDY OF SOME LACTIC STREPTOCOCCAL BACTERIOPHAGES ISOLATED FROM NORWEGIAN CULTURED MILK.


Fermentation failures in Norwegian cultured milk products are in some cases caused by bacteriophages. These are phages homologous to certain species of Streptococcus involved in mixed starters. Sixteen phages have been isolated and their morphol. features studied by EM and optical diffraction. Four different morphol. groups of phages were observed. Two phage types had isometric heads and differed only in the presence and absence of a collar. The 2 other phage groups had prolate heads; they also differed in the presence and absence of collars. The group without collar, however, had a significantly longer tail. The 4 groups can all be classified in Bradley's group B and Tikhonenko's group IV.

PHYSICOCHEMICAL TRANSFORMATION OF MILK COMPONENTS AND RELATIONSHIP OF ONE-SIDED AND TWO-SIDED BUTTER VIRUS.

Blackwell JH, McKercher RC. 1983. Journal of Dairy Research 50(1), 17-25. (Plum Island Anim. Dis. Center, U.S. Dept. of Agriculture, 2500 Independence Ave., Ichaca, NY). Possible mechanisms for protective roles of milk components on foot-and-mouth disease virus present in the milk of infected cows. Light scattering bands collected from Ficoll-sucrose gradient fractions of skim milk contained membrane-limited structures but these were non-infectious for the virus. Infectivity titres in butter milk higher than those of the original cream or butter suggested assocn. of virus with milk fat globules. Increased infectivity in skim milk after treatment with SDS suggested release of virus particles from disoaced casein micelle subunits. Cheating agents, de-emasifying agents, and trepsin, which alter the structure of the individual milk components casein, lipid, and milk fat globule membrane were without effect on infectivity titres.

ZUR KINETIK DER BUTTERKORNGBILDUNG. [Kinetics of the butter grain formation.]


Under the action of constant shearing forces cream attains various steady and reversible states. They are conditioned by cluster-like aggregation of fat globules, which disintegrate after exceeding the actuality limit, the state transitions are irreversible. Here separation of the phases is announced by strong pulsations of the milk. On submersion of the system oscillations 2 quasi stable states. Probably by shearing of the clusters hydrophobic sections are set free, which are covered again and melt as well at last separation or fat separation under certain conditions the system fluctuates. In this case after exceeding the stability limit semp. of phases does not occur, but the clusters fall back to the initial state of the fat globules.

REVIEWS ON THE PROGRESS OF DAIRY SCIENCE: THE BOVINE MILK FAT GLOBULE MEMBRANE-ITS FORMATION, COMPOSITION, STRUCTURE AND BEHAVIOUR IN MILK AND DAIRY PRODUCTS.


A review with 157 refs. A proposed model for the bovine milk fat globule membrane is discussed.

AGE GELATION OF STERILIZED MILKS.


A review with 142 refs. and 6 TEM micrographs illustrating changes in the microstructure of ultra-high-temp. sterilized coned. skim milk stored for up to 17 weeks.

MILK GEL STRUCTURE. XIII. ROTARY SHADING OF CASEIN MICELLES FOR ELECTRON MICROSCOPY.


Casein micelles were fixed in a glutaraldehyde solution, attached to freshly cleaned mica sheets pretreated with poly-L-lysine, dehydrated in a gradeous, critical-point dried from CO2, and shadowed with Pt at 85 laser for the specimen. The method was reinforced with C, floated on the surface of a 3% Na hypochlorite solution, washed with water, and examined by TEM. In unheated milk, casein micelles were uniformly distributed on the mica support. At high magnifications, the submicellar composition of micellar surfaces was clearly visible. In heated milk, minute corpuscles ("spikes") were attached to micellar surfaces. Micrographs of ovine and caprine milk samples revealed large quantities of submicelles. Casein micelles in a heated (95°C for 10 min) suspension of nonfat dry milk (30% total solids) were linked to each other by short fibres and formed aggregates. Maximum benefits of the technique were derived by studying casein micelles and submicelles at magnifications in excess of 50,000 X.

FOODS OF PLANT ORIGIN

THE PREPARATION OF LEAF SURFACES FOR SCANNING ELECTRON MICROSCOPY: A COMPARATIVE STUDY.


Cryopreservation is the superior technique for viewing leaf surfaces in the SEM. Epidermal cells become distorted and lose their papilla-like epitypic and epi­cuticular wax structures. The latter are largely lost during fixation with glutardialdehyde. Nevertheless, the appearance of surface structures after subjecting them to each drying method is valuable in interpreting the features observed by cryopreservation.

SCANNING ELECTRON MICROSCOPY OF INFECTION SITES AND LESION DEVELOPMENT ON TOMATO FRUIT INFECTED WITH PSEUDO-MONAS SYRINGAE PV. TOMATO.


Tomato ovary and fruit surfaces inoculated with P. syringae pv. tomato (= P. tomato) were examined by SEM to observe possible infection sites and to follow lesion development. Bacteria were detected on both glandular and nonglandular trichomes present on ovaries during anthesis. Following anthesis, trichomes were gradually lost, leaving openings through the epidermis. Young fruits developed into epidermis that resembled trichome bases were filled with bac­teria, suggesting that trichome bases may serve as fruit infection sites. Mature lesions were either sunken or raised, and masses of bacteria were extruded from cracks in the lesion surface.

EFFECT OF ACETIC ACID ON SUBCELLULAR STRUCTURES OF COCOA BEAN COTYLEDONS.


Characteristic post-mortem changes in subcellular structures are described which are caused by the penetration of acetic acid (AA) during incubation of cocoa seeds in aqueous media. In the storage cells, lipid is agglomerated and separates from hydrophilic portions in proportion to the AA concentration and pH. This effect is less pronounced at 50°C than at 40°C. In the absence of AA or with very low concentration of AA, other substructural characteristics dominate in most of the cells. At 50°C, post-mortem changes do not induce lipid agglomeration. At 40°C, the intact protein vacuole swells and its matrix structure becomes spongy as a response to in vivo water ab­sorption. Finally, it is shown that a concentration gra­dient persists in whole seeds for most of the time required for fermentation, because of the slow diffusion of AA. The results are compared with temperature effects on subcellu­lar structures and are discussed in relation to their sig­nificance for protolysis in cocoa seeds during fermentation. (Cornforth, 1982. Food Chemistry, 0022-2624/82/1100-1101 $02.00).
ULTRASTRUCTURE OF SENATURED POTATO PROTEINS.

(Dept. Agric. Res., Israel Institute of Plant Research, The Volcani Center, P.O.Box 6, Bet Dagan 50250, Israel).

The structure of potato protein precipitates formed by heat treatment or acidification of potato juice was investigated by scanning electron microscopy. A precipitate formed by heating (pH 10, temperature 95°C) consists of electron-dense particles of diameter 300 nm, segregated into 3-dimensional open networks. Precipitates formed by precipitation to pH 0 and boiling the potato juice at room temp. consist of network-like aggregates of electron-dense particles embedded in thin films which hinder the formation of a dense sediment. This structure explains the high sediment vol. which are typical of this kind of precipitate. By warming the acidified potato juice to 40°C, rapid flocculation and sedimentation of the suspended insol. protein are observed within a few min and a dense sediment is finally obtained. This sediment consists mainly of the network-like aggregates of electron-dense particles and material of low electron density formed by the collapse of the films. Protein films have also been observed in precipitates of heat-denatured bovine serum albumin (BSA). High-methoxyl pectin promotes the formation of protein films on heat deorientation of potato proteins or BSA.

ASPARAGUS TISSUE CHANGES BY COOKING METHODS AND DIGESTION IN RATS AS OBSERVED BY SCANNING ELECTRON MICROSCOPY.

SEM was used to observe fresh asparagus and the changes in tissue structure when it is cooked by boiling and microwave. Boiling distorted parenchymal tissues; microwave cooking produced cracks and distorted adjacent tissues. Microscopic observations of asparagus remnants in rat gastrointestinal contents showed that the only cellular rupture in the upper tract was cellular separation by stomach HCl. Boiled and microwaved asparagus digestion began in the ileum and was completed in the cecum. Raw asparagus digestion started after remnants reached the cecum and continued into the large intestine. Any differences that may have existed because of cooking methods could not be detected in digestive patterns of cellular tissues.

ON ULTRASTRUCTURAL AND NUTRITIONAL ASPECTS OF SOME TROPICAL TUBER CROPS.

Type A starches (Manihot utilissima, Dioscorea dumetorum, and Colocasia antiquorum) and type B starches (Canna edulis, Dioscorea alata, and Dioscorea cayennensis) are investigated. Results show that the degradation of the pure tuber starch as compared with that of feeds made from a particular starch is similar in the rat. Type A starches were more rapidly degraded by bacterial amylase. The susceptibility to attack is compared to the nutritional efficacy of the starches using a model of bovine starches. Comparison of the degree of degradation of the starch granules of the different starches is made by SEM. Type A starches show always a higher susceptibility to amylase attack. The feed efficiency test and the susceptibility of the starch granules to amylase in the sheep rumen and in the chicken crop increase in the ease of degradation by bacterial amylase (in vitro) increases.

ISOLATION AND PARTIAL CHARACTERIZATION OF BLACK GRAM (PHASEOLUS MUNGUS L.) STARCH.

Black gram (P. mungus L.) starch was isolated. The starch yield was 30% and the weight-average molecular size ranged from 7.3-28.5 um (length) to 7.3-27.0 um (width). Hulum length ranged from 23 to 100% of the starch granule length. Amylose was 26.65% (starch basis). Gelatinization temperature range for the starch was 71.5-74°C. Unlike several legume starches, black gram starch is more susceptible to gelatinization as indicated by Brunsvik Viscoemlograph. The starch viscosity was dependent on pH and ionic strength. The raw as well as cooked starch was resistant to hog pancreatic amylase hydrolysis in vitro.

STRUCTURAL CHANGES IN STARCH GRANULES OF LOW MOISTURE CONTENT DURING HEATING.

Thermal behaviour of the starch structure was determined under low moisture conditions by differential thermal analysis (DTA). Three kinds of starch (potato, kuzu, and corn) were used. Microscopic observation of structural changes in the starches was compared with the results of DTA thermograms, X-ray diffraction, and solubility.

PROPERTIES OF YUBA-LIKE FILM FROM SOY PROTEIN ISOLATE AND FORMATION OF A MIDDLE TEXTURED LAYER.

The physical nature and microstructure of Yuba-like films prepared from soybean isolate and other foodstuffs were investigated. Films obtained from mixtures of soybean protein isolate and glycerol, ascorbic acid, and egg white were superior to other films. The Yuba film from soy milk or soy protein consisted of 2 layers but films prepared from soy protein and the above ingredients showed a clear middle textured layer.

CHARACTERIZATION OF A POTATO STARCH-DIGESTING BACTERIUM AND ITS PRODUCTION OF AMYLASE.
Taniguchi H, Oshima F, Igarashi M. 1982. Agricultural and Biological Chemistry 46(8), 2107-2115. (Dept. Agric. Chem., Univ. of Tokyo, Bunkyo-ku, Tokyo, Japan)

A bacterium which can utilize potato starch granules as a sole source of carbon was isolated and identified as Bacillus circulans. SEM observation of potato starch granules revealed that the granules were degraded gradually from their surface which resulted in elongated granules with layered structures. Amylase of this bacterium has a unique property in that it produces only maltolhexose from gelatinized starch at an early stage of the reaction.

AN L-ARABINAN FROM APPLE-juice CONCENTRATES.

The title arabinan consisted of L-arabinofuranosyl residues only, which were (1→3) linked in an essentially linear chain. EM (pos. and neg staining) showed that the purified crystallized arabinan consisted of large ellipsoidal aggregates, on the average 1700 and 840 nm along the major and minor axes, resp. X-ray crystallographic analysis data are also presented.

BETWEEN-SPECIES DIFFERENCES IN FRACTURABILITY LOSS: MICROSCOPIC AND CHEMICAL COMPARISON OF POTATO AND CEDGEDE WATERCRESS.

Potato and watercress tissues were used as exptl. materials for comparing between-species differences in heat sensitivity. Rate of temperature change, SEM was employed to compare cellular and subcellular changes with corresponding fracturability losses during cooking of potato and Chinese watercress by 4 cooking methods. SEM observations strongly suggest that cell wall adhesion and its heat resistance play a major role in fracturability changes during heating. Intracellular substances such as starch and minerals may slightly alter the cell wall strength but do not substantially affect fracturability changes. In addition, differences in the gross chem. comm. between species were inadequate for explaining fracturability changes of the 2 vegetable tissues during heating.

FORCED AIR DRYING OF PARTIALLY FREEZE-DRIED COMPRESSED CARROTS BASE.

The quality of compressed carrot bars produced by combining freeze-drying with air-drying was investigated. Quality parameters measured were color, texture, rehydration ratio, carotene, ascorbic acid, a-tocopherol, and sensory acceptance. It was found that a high quality compressed carrot bar could be obtained by freeze-drying with air-drying as the last drying step, equilibrating with microwave energy, compressing, then air-drying at 60°C. SEM proved useful in delineating reasons for differences in texture and rehydration.

Literature Abstracts
INVESTIGATION OF THE PHYSICAL AND CHEMICAL PROPERTIES OF BANANA STARCHES.


Banana (Musa sp.) starch was isolated after steeping in 0.05 N NaOH. The starch had a granule size of 20-60 μm, a gelatinization temperature range of 74-83°C, and a type X-ray diffraction pattern. The Brabender viscoamylography of the swelling pattern indicated a fairly restricted-swelling starch. The starch exhibited single stage swelling and low solubility patterns. The extensive solubility in dimethyl sulfoxide may be attributed to heterogeneous bonding forces within the granule. The amyloytic susceptibility of native and gela­ tinized starch with amyloitic enzymes has been studied. The starch was found to be non-ionic.

HEAT AND MASS TRANSPORT IN THE FREEZING OF APPLE TISSUE.


A newly developed cold-stage SEM was used to examine ice morphology in frozen apple tissue as a function of freezing rate. The morphological structure of the ice was analyzed using theories of cellular water transport and solution solidification. The transition from intracellular to extracellular ice occurred in apple tissue at a critical rate approx. 1 k/min. The dendritic spacing of the ice was proportional to the inverse square root of the cooling rate. This behaviour can be rationalized through an analysis of the dependence of the formation of dendrites upon solute mass transfer. (Copyright 1982 Blackwell Scientific Publications, 0022 - 1163/82/1000 - 0615 $02.00)

PHYSICAL PROPERTIES OF STARCH FROM CAVENDISH BANANA FRUIT.


Starch was isolated from green Cavendish bananas after NaOH treatment, and its physical properties as it affected acceptance as a food ingredient were measured and compared with those of corn, wax corn, corn diphosphate, acetylated wax corn diphosphate, potato, and tapioca starches. Banana starch granules had a moisture content of 15.5%, an amylose content of 19.3% on a dry weight basis, and were highly irregular in shape and size, with the mode of characteristic length falling at 15 μm. The gelatinization range was 70.1 to 74.6°C. SEM showed that in water the granules underwent surface cracking at 61°C and progressively greater swelling, deformation, and erosion between 70 and 90°C. At 95°C, 6% banana starch paste in a Brabender Amylograph had a viscosity four times that of corn starch of the same concentration, and viscosity decreased rather slowly with stirring. The paste was much stronger than that of corn starch. New granules precipitated much more rapidly than tapioca starch paste. Gelled banana starch was nearly as strong as corn starch, and also was nearly as opaque and reflective.

ULTRASTRUCTURAL CHANGES DURING SENESCENCE OF PEAR FRUIT CELLS DURING AUXIN STARVATION.


Some of the variations in the hormonal balance involved in plant senescence are due to auxin limitation. The resulting ultrastructural changes were studied using pear (Passe Crassane) cells cultured in a system designed to permit medium renewal without withdrawal of living cells. Senescence as functions began to appear at an advanced stage of aging. An initial period (13 days) was characterized by the maintenance of cell structure. The first ultrastructural sign of senescence was the thickening of the plasmalemma reticulum and a loss of hyloplasm density, followed by plasmalemma modifications. No significant change in mitochondria was noted over the aging period. However, after 8 days of culture, the stroma of the mitochondria were clearing with a diffuse inner membrane. In advanced senescence, the organelles were vacuolized and surrounded by many clear vesicles. Interestingly, all these modifications can not be seen or are delayed in a medium enriched with 2,4-D. The data lend support to the important role of auxin starvation in ultrastructural changes in pear and other plant senescence. (Copyright 1983 by The Faculty Press, Cambridge, Great Britain)

STUDIES ON ISOLATION AND PHYSICOCHEMICAL PROPERTIES OF STARCH FROM MOTH BEAN (PHASEOLUS ACONITIFOLIUS).


The yield of starch isolated from whole seeds of the title legume was approx. 33.5%. The amylose content of the starch was 60.6%. Gelatinization of 74-72°C in the starch exhibited single stage swelling and low solubility patterns. The extensive solubility in dimethyl sulfoxide may be attributed to heterogeneous bonding forces within the granule. The amyloytic susceptibility of native and gela­ tinized starch with amyloitic enzymes has been studied. The starch was found to be non-ionic.

ENZYMIC DEGRADATION OF STARCH GRANULES.


Starch granules from roots and tubers were, in general, more resistant to the action of amylases than starch granules from cereals. However, tare starch granules were highly susceptible and starch granules from banana, high-amyllose corn, and gingko were rather resis­ tant to the action of pancreatin compared to starch granules from normal corn. The susceptibility depends not only on the source of the starch but also on the source of amylase. SEM of starch granules susceptible to amylases showed polygonal shapes whereas starch granules resistant to amylases had smooth surfaces and were of round or oval shapes.

FORMATION OF OLEOSOMES IN MATURING SAFFLOWER SEEDS.


The accumulation of oil in maturing safflower seeds was studied by EM. It was indicated that the types of oleosomes. Some evidence was obtained that oleosomes originate in the endoplasmic reticulum. A hypothesis has been proposed that triglycerols are ini­ tially synthesized by the new-like clusters to form a protein-oil complex and are concentrated in the center of the clusters as their formation proceeds.

CEREALS NONLINEAR MODEL OF WHEAT STARCH GRANULE DISTRIBUTION AT SEVERAL STAGES OF DEVELOPMENT.


Wheat starch granule size distribution can be satisfactorily described by sets of intersecting hyperolas on a volume-cumulative number plane. Distinct peaks arise from the inter­ sections of the hyperolas when the sets are arranged for the appropriate. The intersections can be ded by formula after the hyperolas are transformed to straight lines graphically or by regres­sion. The total mass within a given vol. range or the per­centage of mass contributed by a given vol. can be calcd. by formulas derived from integration or differentiation. The intersections could demarcate more than 2 sizes of granules. This suggestion is illustrated by data for growth of wheat starch granules in 2 cultivars during 3 seasons. The possi­ble physiol. significance of these findings is discussed, and alternative interpretations of the A-type granule peak are outlined.

SORGHUM PERICARP THICKNESS AND ITS RELATION TO DECORICATION IN A WOODEN MORTAR AND PESTLE.


Traditionally, sorghum in Mali is decoricated by pounding the grain in a wooden mortar with a wooden pestle. This procedure was used to decoricate sorghum kernels of 3 dif­ferent pericarp types; very thin pericarp (Bio-Florso), sorghum and thick and thin pericarp Guineense sorghums. Pericarp thickness was delayed between 10 and 20% in pericarp sorghums required at least 25% more decorication time than thick pericarp sorghums. When the decorication time was held constant, the amyloin yield increased with the increased pericarp thickness.
The effects of commercial levels of flour chlorination on some physical-chemical characteristics of the flour were studied, LM being one of the methods. At temperatures of 90°C and above, swelling power and solubility of the high-starch fractions from the air classifier increased as a result of chlorination. Although chlorination did not alter the differential scanning calorimetry gelatinization phase transition in either simple (flour-water) or complex batter systems compared to unchlorinated counterparts, at 90°C starch granules in batters of range of 3-500 sec-1 exhibited a greater loss of birefringence and swelled more than starch granules in batters made with untreated flour. Flours having X-ray nuclear magnetic resonance transition-strand stretchings differing slightly from the same moisture content, the characteristics of the T2 relaxation curves were affected by chloride dose. Chlorination levels of 2 or 4 oz/cwt (i.e. 56.7 or 113.4 g/45.36 kg) altered the distribution of molecular species of water in flours having a moisture content of 16%. Water activity of batters made with unchlorinated and chlorinated flours were identical until the temperature reached 80°C. At that temperature, batters containing chlorinated flour exhibited higher water activity than batters containing untreated flour. Greater mobility of water in systems containing chlorinated flour might account for the greater starch gelatinization in those samples.
HIGH-TEMPERATURE SHORT-TERM EXTRUSION COOKING OF WHEAT STARCH AND FLOUR. I. EFFECT OF MOISTURE AND FLOUR TYPE ON EXTRUDATE PROPERTIES.


The role of wheat flour components in the extrusion cooking of wheat starch and flour was evaluated. Feed materials, varying in water content and flour type were extruded and the products analyzed for texture, expansion, and ultrastructure. High-temperature extrusion increased initial moisture content, expanded the expansion and weakened the texture of both starch and flour. Starch showed the greatest sensitivity to differences in moisture content. Analysis by SEM showed that extruded starch and extruded flour had complex but individually different ultrastructures. The most obvious difference noted was the presence in extruded flour of roughened cell walls with frequent failures. Hard (11% protein) and soft (9% protein) wheat flours extrudates were similar in expansion and ultrastructure, whereas the extrudate of a high-protein (15%) flour differed in all 3 characteristics. Supplementing the flour and did not always reflect expansion with high-protein gluten showed that differences among flours were due primarily to gluten quantity rather than to source.

HIGH-TEMPERATURE SHORT-TERM EXTRUSION COOKING OF WHEAT STARCH AND FLOUR. II. EFFECT OF PROTEIN AND LIPID ON EXTRUDATE PROPERTIES.


Effects caused by protein depended on protein type and concentration. When added to wheat starch at concentrations up to 11%, wheat gluten increased expansion and texture. The ultrastructure of starch-plus-gluten extrudates (as shown by SEM) changed gradually from starch to flour-like as the gluten content increased. A specific gluten concentration could be identified as the border between starchlike and flourlike structures. At equivalent concentrations, soy protein isolate increased expansion and texture. Textural measures increased even after expansion began to decrease at the highest soy concentration tested. At and above 5% soy protein isolate, starch extruded to produce ultrastructure of wheat flour. At 10%, wheat flour and highly extruded flour lipids increased extruded flour texture and expansion. Adding flour lipids to all materials tested resulted in decreased extrudate texture. The magnitude of the changes depended on the material extruded. Changes in ultrastructure caused by adding or removing lipids were not always reflected in the texture changes observed in the same sample.

THE BOTANICAL CONSTITUENTS OF WHEAT AND WHEAT MILLING FRACTIONS. I. QUANTITATIVE BY AUTOFLUORESCENCE.

Jensen L, Martens H. 1982. Cereal Chemistry 59(6), 477-484. (Carlsberg Research Laboratory, Amager, Denmark)

A method is described for quantifying pericarp, aleurone, and endosperm fractions of milling flours. Fluorescence characteristics. Fluorescence data from wheat fractions are evaluated by a statistical model initially calibrated against manually dissected botanical parts and synthesized mixtures with known compositions. The components of mixtures of pure botanical components could be quantified by their autofluorescence. The method was tested on dejector fractions from a winter wheat milled in an abrasive decorticator. A high correlation existed between pericarp and fiber and between endosperm and starch, whereas a significantly lower correlation existed between aleurone and ash. Autofluorescent quantification of the botanical components of wheat flour streams may be more relevant than standard chem. analyses such as ash for monitoring the phys. semen. of these components in wheat milling processes.

STARCH GELATINATION: A MORPHOLOGICAL STUDY OF TRITICEAE AND OTHER STARCHES.


SEM has been used to study the swelling properties of potato, maize, rye, barley, field bean, and oat starch in terms of morphological changes during heating in aqueous suspension. SEM has shown that the development of the Triticeae (wheat, barley, and rye) studied here share a common mechanism of swelling which differs from the other starches examined.

BUCKWHEAT (FAGOPYRUM ESCULENTUM) STARCH - PHYSICO-CHEMICAL PROPERTIES AND FUNCTIONAL CHARACTERISTICS.


Starch was isolated from buckwheat (F. esculentum) for determination of physico-chemical properties and functional characteristics. Feed materials showed that extruded starch to be round or polygonal in shape and smaller than wheat starch granules in average particle size. X-Ray diffraction showed a pattern typical of that of cereal starches. Determination of swelling power, solubility, water binding, enzyme susceptibility, gelatinization temperature, and Amylographic consistency of untreated, defatted, and enzyme-inactivated buckwheat starch produced values which were within the range of those reported in the literature for cereal starches. Buckwheat starch performed satisfactorily in fillings, but did not produce cakes of acceptable quality.

EFFECT OF THE ENZYMATIC REMOVAL OF ENDOSPERM CELL WALL ON THE GELATINIZATION PROPERTIES OF AGED AND UNAGED RICE FLOORS.


Endosperm cell walls of the milled rice flour derived from aged and unaged rice were degraded and removed selectively using several commercial enzymes. Amylography of these cell wall-degraded rice flours showed that the difference between the gelatinization properties of aged rice flour and unaged rice flour had disappeared after the removal of the cell walls. The possible role of "structure-maintaining components" in the formation of the characteristic cooking properties of the aged rice during storage is discussed based on these results and also on the microscopic observation of the gelatinization process of the flour.

ELEKTRONENMIKROSKOPISCHE UNTERSUCHUNGEN DES GERSTENHELDENTOFEN- AUS (ERYSIPHE GRAMinis DC F.SP. MORDEI MARCHAL) NACH RESISTENZINDUKTION MIT MIKROBIIllen STOFFWECHSELPRODUKten.

(Electron microscopical studies on mildew in barley (Erysiphe graminis DC f. sp. hordei Marchal) after induced resistance with products of microbial metabolism)


Ultrastruetural studies of the infection process of barley by E. graminis after induction of resistance with products of microbial metabolism. The development was affected through inducer-activated defence mechanisms. The formation of papillae-like structures and a accumulation of electron dense material below the host cell wall at the penetration site appeared to be associated with reduced penetration of the pathogen. The haustoria were altered in the extrahaustorial membrane and electron dense material accumulated at the cell wall of haustorial body and neck, thus impeding functioning and allowing only limited growth of the pathogen. (Copyright 1983 by Verlag Paul Parey, Berlin & Hamburg)

ENZYMIC MODIFICATION OF SORGHUM ENDOSPERM DURING SEEDLING GROWTH AND MELTING.

Aisen AO. 1982. Journal of the Science of Food and Agriculture 33(8), 734-739. (Dept. Bioclim, Univ. of Benin, Benin City, Nigeria)

Modification in the sorghum grain endosperm during seedling growth and maelting was found to be associated mainly with increased activities of a-amylase, endo-β-glucanase, limit dextrinase, and endopeptidase. The major starch-degrading enzyme was a-amylase. The activities of endo-β-glucanase, limit dextrinase, and endopeptidase were comparatively higher in the endosperm than in the embryo during seedling growth. Endo-β-glucanase activity appeared to be relatively low during seedling growth. The low activity of this enzyme might be partially responsible for the limited degradation of the cell wall and starch observed in this study. SEM micrographs of the maelly endosperm are presented. (Copyrigh 1982 by the Society of Chemical Industry. 0022-5142/82/08080-075A $02.00)
USE OF SCANNING ELECTRON MICROSCOPY TO DEMONSTRATE MICROBIAL ATTACHMENT TO BEEF AND BEEF CONTACT SURFACES. 
Microorganisms present on fresh beef for stew were enumerated by Aerobic Plate Count (APC) using both rinse and immersion methods. Results showed greatly increased recovery of microorganisms with maceration than with rinse procedures. 
SEM was used to examine the surface of the beef for attachment sites. After inoculation and before and after rinsing, sterile stainless steel chips (6 mm x 6 mm) were placed on both the unrinse and rinsed beef surfaces. Contacting surfaces of the stainless steel chips were examined by SEM. Transfer of microorganisms from the beef surface was seen in all cases with the organisms demonstrating clearly defined attachment fibrils. A reference experiment, using the same beef for stew and a culture of Pseudomonas fragi (ATCC 4793) was also carried out. Similar attachment fibrils were observed with the pseudomonads.

ULTRASTRUCTURAL STUDY OF SKELETAL FISH MUSCLE AFTER FREEZING AT DIFFERENT TEMPERATURES. 

Goldfish skeletal muscle was examined with EM to investigate ultrastructural changes produced by freezing. Aquatic fish were used because they are more closely associated with catching and killing large fish. Muscle tissue was frozen slowly in a refrigerator or quickly in liquid nitrogen. The results of these experiments and conclusions in the literature that fast freezing produces many small ice crystals which cause minimum disruption of ultrastructural components, whereas slow freezing generates fewer ice crystals which distort cells and crush myofibrils. These results reveal the ultrastructural counterpart to those alterations which are visible in LM using much simpler equipment and methodology (Bello RA et al. 1981, J. Food Sci. 46, 733).

EFFECT OF CHOPPING TEMPERATURE ON THE MICROSTRUCTURE OF MEAT EMULSIONS. 

A standard frankfurter formulation (25% fat) was used to evaluate the effect of 4 endpoint chopping temperatures (12°C, 22°C, and 28°C) on the ultrastructure of meat emulsions produced with a grind-mix-emulsify production system. SEM was used to evaluate the changes that occur in the ultrastructure of a meat emulsion, a partially processed frankfurter from each of the 4 endpoint chopping temperature treatments were fractured, fixed, dehydrated, critical-point dried, and coated with Au/Pd before being examined. Micrographs suggest that maximal emulsion stability is attained with a tradeoff between the first function that is related to the interfacial protein film thickness and the second function is related to the integrity and cohesion of the fat globules. Both functions appear to be directly related to the fat-holding and water-binding abilities of the meat emulsion or batter.

GEL CHARACTERISTICS - STRUCTURE AS RELATED TO TEXTURE AND WATERBINDING OF BLOOD PLASMA GELS. 

Heat-induced denaturation and aggregation of plasma protein solutions were studied by low-shear viscometry and turbidity measurements. The microstructure of blood plasma gels was elucidated by SEM. Relationships between gel structure, texture, and water-binding properties of blood plasma gels prepared under various conditions such as different heating temperatures, pH, and protein concentrations were investigated. Generally, it was found that the degree of elasticity and water-binding properties decreased with an increasing degree of random aggregation of the protein gel network. The degree of aggregation increased with increasing protein concentrations and decreasing heating temperatures from 77°C to 92°C. With increasing heating temperature from 77°C to 92°C, a partial disruption of the gel structure due to local aggregation phenomena was demonstrated by SEM micrographs.
faecalis var. liguefaciens caused the muscle was estimated by laser diffraction and oil immersion microscopy. SF-02150 at CLupea harengus membras) were studied by assaying the activities of lysosomal COIIID Only and mitochondrial cytochrome c oxidase. The different between methods whether macerated muscle samples or ed neutrophils were rich in myeloperoxidase which was extd. detected in the more slowly sedimenting fractions. Freezing length on those estimated by laser diffraction individual muscle fibres were measured. However, for macer­ated samples regression of optically determined sarcomere length those estimated by laser diffraction gave slopes and intercepts which differed from 1 and 0, respectively (P<0.05). It was concluded that laser diffraction was only accurate when individual muscle fibres were measured.

**CHANGES IN MUSCLE SUBCELLULAR FRACTIONS OF BALTIC HERRING (Clupea harengus membras) DURING COLD AND FROZEN STORAGE.** Karvinen V-P, Bamford DH, Grenroth B. 1982. Journal of the Science of Food and Agriculture 33(8), 763-770. (Tech. Res. Cent. of Food Res. Finland; V-P, Biologinkuja 1, SF-02150 Espoo 15, Finland.)

The autolytic enzymic degradation of the muscle of Baltic herring (Clupea harengus membras) was studied investigating the muscle fractions after 2 different types of freeze commonly used in fish handling, i.e. freezing and storage at -18°C and -23°C. Comparing them with muscle fractions of fresh fish, Nuclear, mitochondrial, lysosomal, and microsomal as well as soluble fractions were compared by measuring the activities of lysosomal cathepsin D and mitochondrial cytochrome c oxidase. The morphology of sedimented fractions as revealed by EM compared with the enzymic activities. After storing at -18°C for 7 days no enzyme was detected in the more slowly sedimenting fractions. Freezing and thawing samples resulted in the lysys of mitochondrion and lysosomes and in a loose, disorganized muscle structure. (Copyright 1982 by Society of Chemical Industry; 0022-5142/82/0800-0763 $02.00)

**LIPID DESTRUCTION INITIATED BY PHAGOCYTIC CELLS IN MUSCLE FIBRES. POST-MORTEM DAMAGE BY MYELOPEROXIDASE-HYDROGEN PEROXIDE-HALIDE SYSTEM.** Kanner J, Kinsella JE. 1983. Journal of Agricultural and Food Chemistry 31, 370-376. (Inst. Food Sci., Cornell Univ., Ithaca, NY 14853, U.S.A.) In fresh muscle foods, phagocytic cells may conceivably initiate and accelerate lipid oxidation. Fish leukocytes were obtained by density gradient centrifugation. The isolated neutrophils were rich in myeloperoxidase which was excised from the leukocytes at pH 4.0 in the presence of 0.3 M sucrose. The crude enzyme showed a peroxidase activity of about 160 nmol of purpuragolin (mg or protein)-1 min-1. An oxidation system using discoloration of 3-carotene as an indicator and an electro chemiluminescent detector from fish leukocytes caused rapid discoloration of 3-carotene in the presence of hydrogen peroxide and iodide or bromide ions. The effect of myeloperoxidase from fish leukocytes on the discoloration of 3-carotene in the presence of Cl- was studied. No destruction of 3-carotene occurred when halogen ions, hydrogen peroxide, or the enzyme was omitted from the system. The data indicate that leukocytes may be a focus for the initiation of lipid peroxidation in biol. tissues. Leukocytes from rainbow trout blood were stained for microscopy with Wright's stain and with pyrogallol and hydrogen peroxide.

**ICE RECRYSTALLIZATION IN FROZEN BEEF.** Bevilacqua AE, Zaretsky ME. 1982. Journal of Food Science 47(5), 1410-1414. (Centro Invest. Desarrollo Cristal. Aliment. (CIDCA). Univ. Nacional de la Plata, 47 y 116 La Plata (1900). Prov. de Buenos Aires, Argentina.) Temperature fluctuations produced during storage and transport of beef lead to recrystallization of the ice in frozen beef was established considering that in this phenomenon the driving force is the tendency to a decrease in the size of the crystal of the phase and in the movement of the grain boundary is proportional to its cur­ve length. The heat treatment and submitted to higher storage temperatures. Histological analysis of tissues immediately after freezing and of samples stored at different conditions was not possible to measure evolution of ice crystal size and obtain kinetic constants for proposed recrystallization model. Mechanism for interpreting the phe­nomena were discussed, discussing that thawing of crystals occurs preferentially through the liquid phase.

**A FIXATIVE FOR USE IN MUSCLE HISTOCHEMISTRY.** Crocker BP, Bossen EH, Brinn NT, Hammond PA. 1983. The Journal of Histochimistry and Cytochemistry 31(1), 110-112. (Dept. Pathol., Box J-275, Univ. of Florida, Gaines­ville, FL 32610, U.S.A.) A fixative solution that preserves the activity of some relevant enzymes in muscle histochimistry is described: 5% of ammonium sulfite dissolved in distilled water of 0.02 M K3 citrate and 0.005 M bicarbonate buffer. pH was adjusted to 7.0. Muscle, kidney, and spleen were fixed for up to 3 days at room temperature, washed in the buffer free of ammonium sulfite and then immersed in isopentane cooled in liquid nitrogen.


**EGGS**


The ultrastructure of the eggshell of the domestic fowl is reviewed with regard to the influence that the various shell layers have on determining eggshell quality. Emphasis is placed on the importance of normal mammillary knob forma­tion. Eggshells having good eggshell quality have a regular distribution of mammil­lary knobs. The data indicate that leukocytes may be a focus for the initiation of lipid peroxidation in biol. tissues. Leukocytes from rainbow trout blood were stained for microscopy with Wright's stain and with pyrogallol and hydrogen peroxide.

**CHANGES IN THE ULTRASTRUCTURE OF EGG YOLK BY THE GROWTH OF S. FAECALIS.** Gravani RB, Vadodra DV, Hood LF, Baker RC. 1982. Journal of Food Science 47(5), 1433-1437. (Inst. Food Science. Cornell Univ., Ithaca, NY 14853, U.S.A.) The growth of S. faecalis var. liquefaciens caused the separation of egg yolk into upper and lower layers. Ultra­structure studies using TEM showed that the upper layer was mostly low-density fraction (LDF) with few electron­dense particles. In contrast, the lower layer was mostly high-density fraction (HDF). The separation factor caused an increase in the thickness of the eggshell, and the loss of the matrix. The extent of incubation had a pronounced effect on the ultrastructure. Freeze-thaw treatment produced greater aggregation and disruption of the LDF matrix, particularly in the lower layer.

**STUDIES OF COLLOIDAL PROPERTIES OF WHOLE EGG MAGMA.** Torton J, Eisenberg H. 1982. Journal of Food Science 47 (5), 1423-1428. (Polymer Dept., The Weizmann Inst. of Science, Rehovot, Israel 76100.) Alterations in colloidal properties resulting from heat treatment and/or freezing of whole egg magmas were studied. Both heating and freezing led to increase in apparent vis­cosity and in surface tension. All viscosities are non­Newtonian. Increasing rates of shear followed immediately by decreasing rates resulted in open hysteresis loops; repeated cycles showed only one decrease in viscosity. Polyacrylamide gel electrophoresis demonstrated changes induced by heat treatment at 60°C, 3.5 min; 64.4°C, 2.5 min; and 68°C, 1 min. SEM pointed out morphological modifications caused by heating and/or freezing. Sucrose and D-glucose preserved lower viscosity during frozen storage. A preliminary postulation of colloidal structure in the whole egg is presented.

AN EVALUATION OF METHODS FOR PREPARING EASILY DAMAGED CUTICULAR SURFACES OF PLANTS FOR SCANNING ELECTRON MICROSCOPY.


Techniques were tested to ascertain the best method for preparing the surfaces of delicate plant cuticles for microscopy at mag. not exceeding 10,000. Using SEM, comparative exams. were made of cuticles of fresh material, ambient dried, freeze dried, critical-point dried, and frozen material kept at low temp. Micrographs were compared with material exam. using LM which acted as a control at low mag. Cuticles of the leaves of runner bean, Coleus, and the petals of Nicotiana showed best surface preservation and least wrinkling when frozen, held at low temp., and examined on cryo-stage. (Copyright 1983. The Royal Microscopical Society).

IMIDAZOLE-BUFFERED OSMIUM TETROXIDE: AN EXCELLENT STAIN FOR VISUALIZATION OF LIPIDS IN TRANSMISSION ELECTRON MICROSCOPY.


Tissues were fixed with glutaraldehyde and postfixed with Oso4-imidazole and the appearance of lipid droplets was compared with that after postfixation in unbuffered Oso4 or an Oso4 soln. buffered otherwise. Prominent electron-opaque staining of lipid droplets and of lipoprotein particl.-es was noted only in postfixed solns. buffered with imidazole, pH 7.5, for 30 min. Test spots on filter paper revealed that unsaturated fatty acids, particularly linolenic and linoleic acids reacted more strongly with Oso4 than with Oso4. (Copyright 1982. Chapman and Hall Ltd.)

A SCANNING ULTRASTRUCTURAL HISTOCHEMICAL PROCEDURE FOR THE LOCALIZATION OF POLYSACCHARIDE.


Basidiocarps of Sphaerobolus stellaris were fixed at 4°C for 1 h in 4% glutaraldehyde and postfixed for 2 h in 1% osmium tetroxide (pH 7.2). Following a 15 min rinse in a 0.1 M Na cacodylate buffer (pH 7.2), the samples were treated with 1% periodic acid for 45 min, rinsed in 2 changes of water for 10 min each, placed in 1% thioserylcarbazide in 10% acetic acid for 10 min, rinsed in 2 changes of 10% acetic acid (10 min each), rinsed in water (5 min), stained in 1% silver proteinate in the dark (30 min), and rinsed in water (2 x 5 min). The samples were mounted on C plats using C cement and were examined by SEM using a dual annular photolithographic disc back-scattered electron detector. Polysaccharides in the basidioles and the hyphal cells were visible using backscattered electron imaging and wavelength dispersive X-ray analysis confirmed that the light areas were due to silver deposits.
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FOOD MICROSTRUCTURE 1984

FOOD MICROSTRUCTURE will be the subject of a program scheduled during April 15-20, 1984 at the Adam’s Mark Hotel, in Philadelphia, PA. Two special programs – one on Meat Foods and one on Plant Foods, as well as several tutorials are being planned.

All types of foods, including vegetables, grains, sea foods, meat, dairy products and others will be covered. Papers on the fundamental aspects of food microstructure such as the molecular and colloidal forces which determine it, and the practical relationship between food microstructure and processing, ingredient changes, shelf life, consumer acceptability, and other food-related areas are invited. Techniques may include transmission electron microscopy, scanning electron microscopy, light microscopy, x-ray microanalysis or related microscopy/microanalytical methods.


Papers offered for this program will be published in the semi-annual Journal, “FOOD MICROSTRUCTURE”. The editors of this Journal are Dr. S.H. Cohen, U.S. Army R&D Command-Food Research Laboratory, Natick, Mass. (Telephone 617-651-4578); Prof. E.A. Davis, Food Science Department, University of Minnesota, St. Paul (Telephone 612-373-1158); Dr. D.N. Holcomb, Kraft R&D, Glenview, IL (Telephone 312-998-3724); and Dr. M. Kalab, Food Research Institute, Agriculture Canada, Ottawa (Telephone 613-995-3700 Ext. 272).

The Food Microstructure program in Philadelphia will be held in conjunction with the SCANNING ELECTRON MICROSCOPY/1984 meetings from April 15-20, 1984. Registrants at the Food Microstructure program will be able to attend all of the activities of the SEM meeting at no additional charge. Of particular interest should be: several tutorials, and programs on Analytical Electron Microscopy (including STEM), Microprobe Surface Analytical Techniques, Particulate Characterization, Plants, and many areas of physical, biological and biomedical application. A comprehensive equipment exhibition will take place during the meetings also. Complete details of SEM meetings are available on request.

The registration fee for this program is $40.00, if paid before Jan. 31, 1984, and $55.00 after that. For subscription to the journal include an additional $50.00 (for U.S. delivery) and $55.00 for elsewhere.

A Call for Papers, Registration Form, details of travel support and Hotel information are available on request. For more information on this program and the Journal, “Food Microstructure” contact Dr. Om Johari or one of the editors.
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