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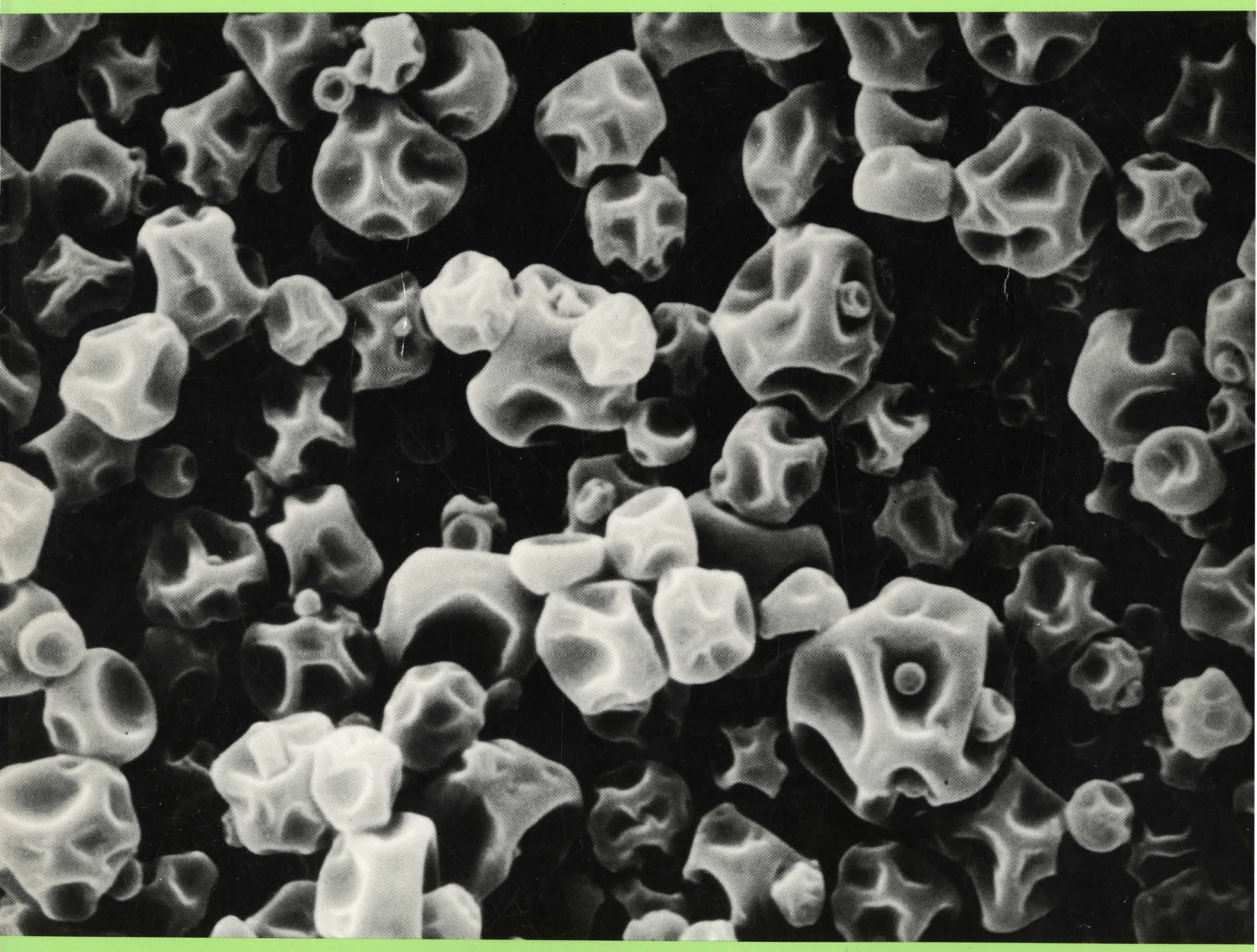

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THE EFFECTS OF GAMMA IRRADIATION AND CALCIUM TREATMENT ON THE ULTRASTRUCTURE OF APPLES AND PEARS

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Abstract

The post-storage quality of fruits depends on the ultrastructure of the outer tissues and on the fruit mineral composition (mostly on calcium). Pectins and their bonds with calcium play a central role in the tissues during ripening.

During senescence the middle lamella disappears from the cell walls, the fruits become soft and cell wall reticulation can be seen. There are changes within the cells too; plasmalemma separates from the wall, tonoplast from the cytoplasm, and plastids, mitochondria and nuclei are disorganized.

The ultrastructure of the skin and flesh of apples and pears were studied as a function of treatments (calcium, irradiation, calcium combined with irradiation), storage time and varieties. The texture and calcium content of the different parts of fruits were determined.

We established that low dose (1 kGy) irradiation induced softening in the fruit, dissolution of middle lamellae, wrinkling of cell membranes which generally remained intact, and retention of starch by plastids of the skin. Calcium treatment preserved the cell membranes and middle lamellae. The combined treatment preserved the cell compartments the best (with a lot of starch in the plastids; the cytoplasm remained essentially unchanged). At the same time this treatment could not prevent the breakdown of the middle lamellae in irradiated tissue.

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Introduction

A major limiting factor of the crop food supply in the world is loss due to spoilage in the production, transportation and storage of fresh commodities.

One of the possibilities to extend the shelf-life of fresh fruits is to use irradiation in postharvest handling (Akamine and Moy 1983; Dennison and Ahmed 1966; Dharkar and Sreenivasan, 1966;Maxie and Abdel-Kader, 1967; Al-Jasim et al., 1968). The limiting factor in the use of irradiation to extend shelf-life of the fruits is textural change (softening) which is the result of the breakdown of cell wall constituents (pectin, cellulose and hemicellulose) (Kertész et al.,1964; Romani, 1964; Maxie et al., 1966; Massey and Faust, 1969; Bramlage and Covey, 1975) The degradation of pectic and cellulosic materials resulted in architectural weakening in tissue and damage in the semipermeability of the cell membranes leading to loss of turgor (Massey, 1969). The loss in the firmness is shown to be associated with the activity of cell wall degrading enzymes (Pilnik and Voragen, 1970; Lieberman, 1983; Gross and Wallner, 1979), particularly polygalacturonase (Grierson et al., 1981), although a role has been suggested for cellulase (Pesis et al., 1978; Shomer et al., 1984).

Lewis (1986) reported that pectinase treatment had a major effect on cell walls, causing layering and leading to separation of cells in potato. Use of externally applied polygalacturonase and cellulase had a widely different effect on the cell walls of apple, pear tissue (Ben-Arie and Kislev, 1979) and tomatoes Crookes and Grierson, 1983).

In apples, polygalacturonase treatment, without cellulase activity resulted in similar, though somewhat more extensive dissolution of the middle lamella, than that occuring in soft, mealy fruit. Treatment with both enzymes caused cell wall disintegration far in excess of that occuring naturally even in overripe fruit

(Ben-Arie and Kislev, 1979). Characteristic of middle lamella dissolution in ripe fruit was the frequent occurrence of vesicles appearing usually in the area close to intercellular spaces. Vesicles were usually observed in areas adjacent to the plasmodesmata-wall complex. A similar "reticulate structure" was described in apples by Fuller (1976), and it was concluded that this was a symptom of cell wall breakdown appearing after the wall digestion by polygalacturonase (Ben-Arie and Kislev, 1979). Plasmodesmata are resistant to the action of pectinase and cellulase (Jones, 1976). Crookes and Grierson (1983) investigated the degree of structural organization retained by ripening tomato fruit. The pericarp of mature green tomato fruit was found to be composed of large isodiametric parenchymous cells. Cytoplasm contains many normal organelles including mitochondria, chloroplasts, endoplasmic reticulum and crystalloid-containing microbodies. The cell wall consists of fibrils in an electrontranslucent matrix. Middle lamella is visible as an electrondense region between walls of adjacent cells. Dissolution of the middle lamella begins after the onset of ethylene production. Plastids are chromoplasts containing angular lycopene crystalloid remnants. The cytoplasm remains relatively intact with normal mitochondria and endoplasmic reticulum. Polygalacturonase activity was first detectable 2 or 3 days after the onset of ethylene production, at about the same time as the plastid transformation and pigment changes.

Calcium has been found to play an important role (Bangerth, 1974a,b; Conway and Sams, 1984) in maintaining fruit quality and prolonging storage life. The post-storage quality of apple was found to be related to the ultrastructure of the outer tissue and the fruit mineral composition (Marinos, 1962; Mahanty and Fineran, 1975). Fuller (1976, 1980) established that many intact mitochondria could be observed in cells from both high- and low-calcium apples following storage. The shelf-life of apples and pears was increased by irradiation (1 kGy) combined with calcium treatment (Kovács et al., 1985). The objective of this study was to elucidate the effect of low dose irradiation (1 kGy) alone or combined with calcium treatment on the microstructure of apples and pears.

Materials and Methods

Raw materials and treatments

Apples and pears were harvested in an orchard of Micsurin Coop., Dánszentmiklós. The following varieties were tested: - apples - Gloster 69 (Glochenapfel Richared x Delicious); Idared (Jonathan x Wagnerapfel) Mutsu (Golden Delicious x Indo); Starking (NM 104 starking clone); - pears - Hardenpont (Pyrus ussuriensis Maxin cv. king-pai-li). Harvest time of apples and pears was 130-140 days after full blossoming, when starch iodine pattern index value of apple was 3-4 (Reid et al., 1982) After picking, fruits were treated with calcium chloride solution and irradiated on the next day. Samples were irradiated at the Institute of Isotopes of Hungarian Academy of Sciences by a Co-60 radiation source (total activity 3.7 PBq). Irradiation dose rate was 1 kGy . h^{-1}. After treatments all samples were stored at 1°C, 95-97 % RH. Investigations were carried out as a function of storage time. Microstructure of fruits was determined just after picking (control samples) and after 3 months storage (all samples).

Determination of calcium and texture

Ca^{2+} was determined by atomic absorption spectrometry (PYE UNICAM SP 2900). Preparation of sample was carried out after Borusné-Böszörményi and Kovács (1976).

Texture was measured by Penetrometer (Labor MIM, Hungary). The head (500 g) penetrated into the fruit for 5 sec. The softness value was expressed as penetration in 0.1 mm.

Examination of ultrastructure

SEM Samples taken from the fruit were fixed in cold 2 % glutaraldehyde dissolved in 0.14 M cacodylate buffer (pH 7) for 24h. After washing with the buffer, they were postfixed in cacodylate buffered 1 % OsO_4 for 2 h, dehydrated in a series of ethanol and amyl acetate and dried through liquid CO_2 in a Dupont-Sorwall critical point drying apparatus. The samples were then coated with gold in a Zeiss HBA vacuum evaporator and examined in a JEOL-50A type scanning electron microscope at a 20 kV accelerating voltage.

TEM Fixation was carried out in 6% (v/v) glutaraldehyde (in 0.035 M K-Na phosphate buffer, pH 7.2) for 2 h at 4°C. After thorough washing in the above buffer, samples were postfixed in 1 % (w/v) OsO_4 for 1.5 h, dehydrated in an acetone series and embedded in Spurr's resin. Using flat molds, samples from the skin could be oriented so that they were always sectioned transversely. Sections were made with a Porter-Blum ultramicrotome equipped with an LKB glass knife, and after contrast staining with uranyl acetate and lead citrate, were examined in a Tesla BS 500 electron microscope operated at 60 kV. Fig.1 represents different parts of fruit which were investigated.

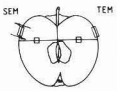


Fig. 1. Sampling from apple fruit

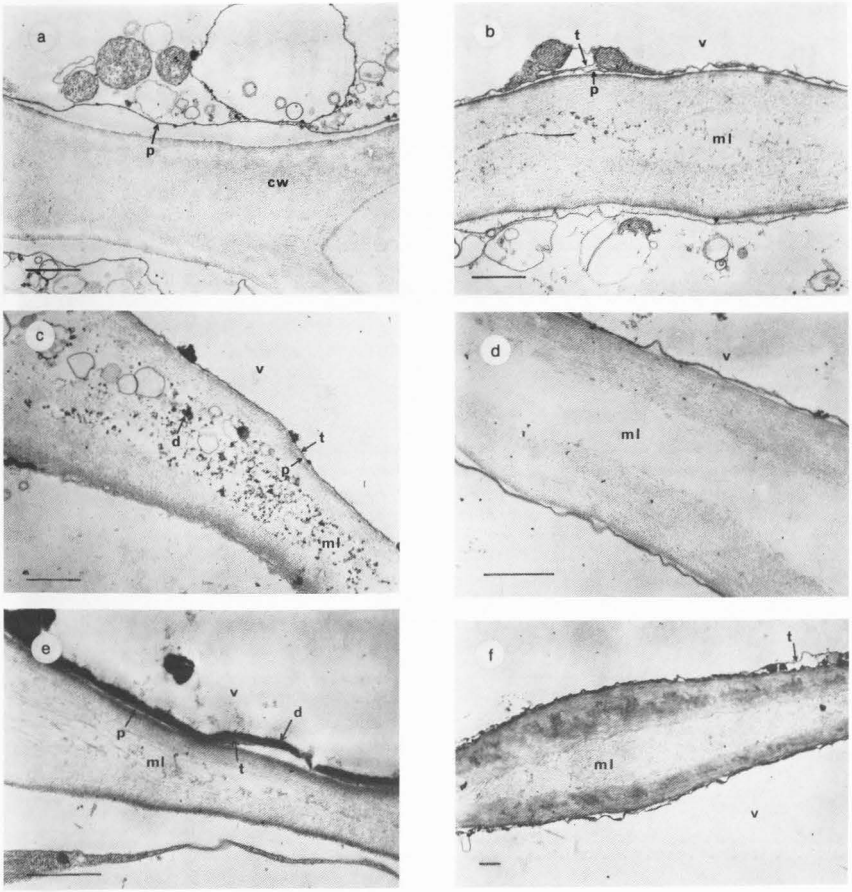


Fig. 2. Ultrastructure (TEM) of parenchymous tissue (flesh) in Hardenpont pear (a-e) as a function of treatments (a: fresh control; b: stored control; c: calcium treated then stored; d: irradiated then stored; e: calcium treatment combined with irradiation then stored). For comparison, f: stored fruit flesh of Golden Delicious apple; cw=cell wall; ml=middle lamella; d=deposits; p=plasmalemma; t=tonoplast; v=vacuoles; Bars= 1 µm

Results and Discussion

The fruit flesh

Fig. 2 shows the effect of different treatments (irradiation, calcium and calcium combined with irradiation) on the ultrastructure (TEM) of the cell walls of pear and apple fruit flesh. Cell walls of the fresh control are intact (Fig.2a), but in the stored control (Fig. 2b) cell wall reticulation and fibrils adjacent to a dilated middle lamella can be seen. In the cell wall and vac-

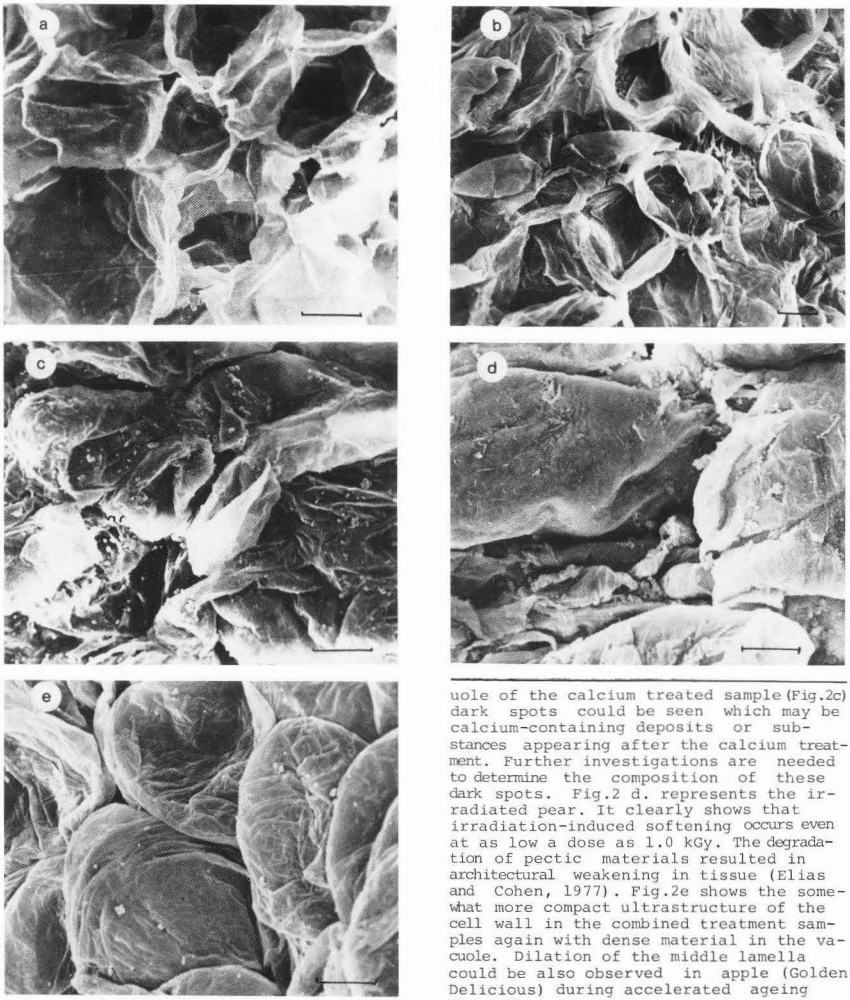
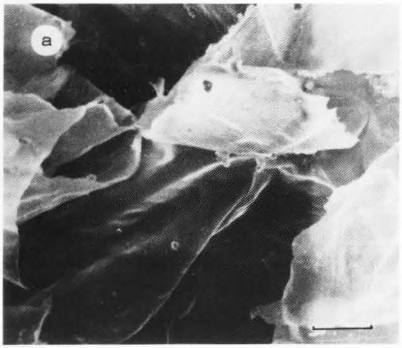
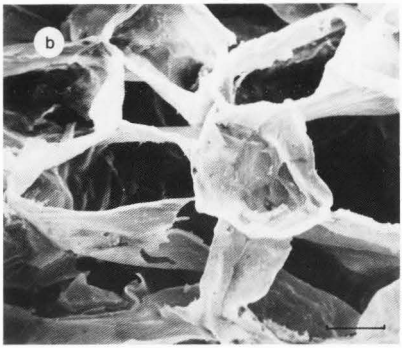


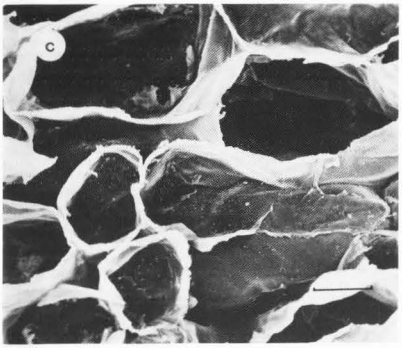
Fig. 3. Ultrastructure (SEM) of parenchymous tissue (flesh) in Hardenpont pear (a: fresh control; b: stored control; c: calcium treated; d: irradiated; e: calcium treatment combined with irradiation then stored). Bars = 50 µm

uole of the calcium treated sample (Fig.2c) dark spots could be seen which may be calcium-containing deposits or substances appearing after the calcium treatment. Further investigations are needed to determine the composition of these dark spots. Fig.2 d. represents the irradiated pear. It clearly shows that irradiation-induced softening occurs even at as low a dose as 1.0 kGy. The degradation of pectic materials resulted in architectural weakening in tissue (Elias and Cohen, 1977). Fig.2e shows the somewhat more compact ultrastructure of the cell wall in the combined treatment samples again with dense material in the vacuole. Dilation of the middle lamella could be also observed in apple (Golden Delicious) during accelerated ageing Fig. 2f).

In Figs. 3-4 the SEM morphology of parenchymous cells of pear and apple is demonstrated. The changes are the same in pear and apple. Cells in the fresh control are opened up by the preparation (Figs 3a, 4a) presumably as a consequence of their firmly attached cell walls and turges-







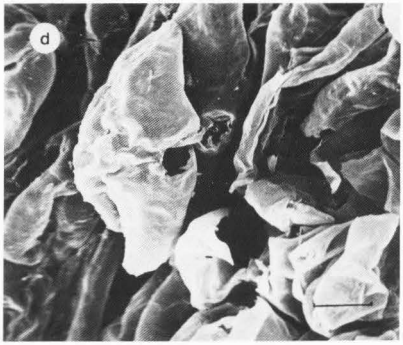


Fig. 4. Ultrastructure (SEM) of parenchymous tissue (flesh) in Mutsu apple (a:fresh control; b:stored control; c:calcium treated; d: irradiated; e:calcium treatment combined with irradiation then stored) Bars = 50 µm

cence. This applies more or less also to the stored control (Figs. 3b and 4b) and to the calcium treated samples (Figs. 3c and 4c). Cells of the irradiated (Fig. 3d, Fig. 4d) and combined treatment (Fig. 3e, Fig. 4e) samples generally are not cut through, indicating that the middle lamella is dissolved and the cells could separate from each other. The irradiation and the combined treatment led to similar changes in fruit tissues independently of the fruit varieties (Kovács et al., 1985).

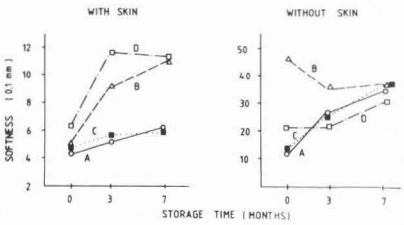


Fig. 5. The effect of irradiation and calcium treatment on the texture of Gloster apple (A=control; B= irradiated; C=calcium treated; D=calcium treatment combined with irradiation).

Table 1

Analysis of variance of the softness of apple

Source of variance		
With skin	df	MS
Storage time	2	13.73 x
Treatment	3	15.20 x
Error	6	1.99
Without skin		
Storage time	2	146.44
Treatment	3	146.76
Error	6	61.07

Significance at the level 0.5 %, (x), MS = mean of squares, df = degree of freedom, $SD_{5\%}$ = 4.87 (with skin). n = 40

In Table 1 the results of analysis of variance are summarized. The differences are significant among the storage times and treatments with skin but insignificant when the skin was removed.

In Fig 5. the results of texture analyses (Gloster) can be seen. It is shown that irradiation immediately induced softening in the flesh when the skin was removed and later when the skin was not removed.
The differences were significant (95 %) between control sample and irradiated one, if 0 day data of the flesh were separately analysed.

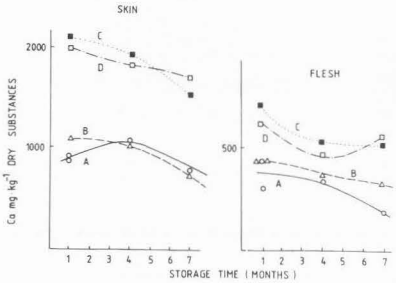


Fig. 6. Distribution of calcium in different parts of Gloster apple as a function of treatments and storage time (A=control; B=irradiated; C=calcium treated; D=calcium treatment combined with irradiation).

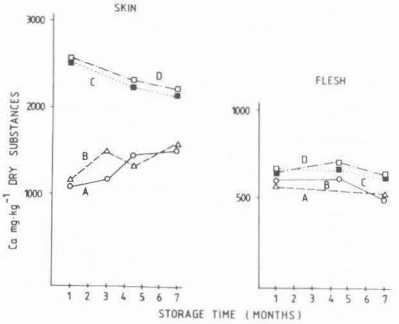


Fig. 7. Distribution of calcium in different parts of Mutsu apple as a function of treatments and storage time (A=control; B=irradiated;C=calcium treated; D=calcium treatment combined with irradiation).

The fruit skin

In Figs. 6-7 the uptake and distribution of the calcium can be seen as a function of treatment, storage time and variety (Mutsu, Gloster). It was established that the calcium content was higher in Mutsu than in Gloster (Table 2). Mutsu absorbed much more calcium in the skin than Gloster. We think the state of the calcium uptake depends partly on cuticle and partly it is (Korban and Swiadar, 1984) genetically regulated. Glenn et al.,(1985) established that cracks and other breaks in the surface of the cuticle may have an important effect on calcium penetration. Although the lenticel is reported to play a significant role in the uptake of calcium sprays (Lewis and Martin, 1973; Link 1974), Reed and Tukey (1982) reported that differences in the permeability of plant cuticles are more closely correlated with the cuticular thickness and percentage of wax content (Greene and Smith, 1979).

Our unpublished SEM data showed, that cuticles of different apple, or pear varieties are very different in the irregularities and cracks on the surface of skin.

The changing of the calcium content during storage depends on the varieties (Figs. 6-7). The calcium content decreases in the Gloster skin and flesh as a function of storage time, but only minor changes occur in Mutsu.

In the skin of the irradiated samples the level of the calcium is significantly higher than the control (Table 3.) Our results are in line with those of other authors who established that irradiation induced the mobilization of calcium in the tissues (Skou, 1963; Shah, 1966); Maynard and Gentile, 1963.

Echandi and Massey (1970) established that of the elements detected in the leakage, calcium was most susceptible to radiation-induced leaking from irradiated carrot tissues. An increase of 60 % over the controls was found at the 1.0 kGy level.

We agree with Christensen and Foy (1979) who demonstrated that the primary function of calcium in plants appears to be that of membrane stabilization. Calcium may indeed bind together components of the cell membrane. The negatively-charged head groups of the phospholipids project from the membrance surface and readily bind cations such as calcium. These cations may also bind to proteins that are exposed on the membrane surface. Divalent cations may thereby crosslink membrane phospholipids and exert great effects on the topography on the membrane surface, on the availability of surface groups for binding by other molecules (e.g., enzymes) and the functionality of membrane components (Bramlage et al.,1980). Our hypothesis is that the calcium cannot bind to pectin in irradiated tissue for the pectin is decomposed (SEM), but that it has an unknown role in the cell wall (TEM) and probably in the membranes too. It was established that organoleptically the combination treated samples were much better

Table 2

Analysis of variance of calcium data as a function of the storage time (3 and 7 months), treatments and varieties (Gloster, Mutsu)

Source of variance	df	MS
Skin		
Storage time (A)	1	91960.56 xx
Treatments (B)	3	1048736.00 xxx
Varieties (C)	1	735735.1o xxx
AxB	3	12621.90
AxC	1	95018.06 xx
BxC	3	14692.73 x
Error	3	1445.40
Flesh		
Storage time (A)	1	812.25
Treatments (B)	3	36040.42
Varieties (C)	1	104976.00 x
AxB	3	3517.42
AxC	1	1444.00
BxC	3	4321.83
Error	3	4156.17

Significance at the 0.1 % (xxx), 1 % (xx), 5 % (x) level, MS = mean of squares, df= degree of freedom

Table 3

Comparison of the differences (skin-flesh) of calcium concentration in the irradiated and non-irradiated apple (Mutsu, Gloster, Idared) by analysis of variance. (Apple was stored for 1 month)

Source of variance	df	MS
Varieties	2	55589 xxx
Treatments	1	74037 xxx
Error	34	4823

Significance at the 0.1 % (xxx)
MS = mean of squares, df= degree of freedom

than the irradiated or control ones (unpublished results). The reason for better quality could be that the permeability of the membranes or some essential membrane-associated calcium dependent biochemical processes are preserved.

The success of storage depends on the ultrastructure of the outer tissues and mineral composition of the fruits (calcium being the most important) (Mahanty and Fineran, 1975; Fuller, 1976; Platanova et al., 1981, Fukumoto and Nagai, 1983; Korban and Swiader, 1984; Bramlage et al., 1985; Zagorian et al., 1985; Stahly, 1986). Cellular profile analyses of apples exhibiting corking disorders related to calcium and potassium have been characterized and tissue analysis of the epidermis, hypodermis and fruit cortex revealed that physiological breakdown occured during the early formative changes in the development. Simons and Chu (1980; 1983) investigated anomalous tissues in relation to cork spot development on 'York Imperial' apples 45 days prior to fruit maturation by SEM. The following significant cellular characteristics were found throughout the cortex: cell proliferation, sclerified cell walls and supportive vascular tissue, pectin increase in cell walls, starch accumulation, thickening of walls and disintegration of cytoplasmic membranes. The potassium and calcium levels were determined by electron microprobe analyses for these tissues 30 days before maturation, and showed that both potassium and calcium levels were low throughout the affected tissues, the calcium content being proportionally lower than potassium.

Initiation of bitter pit in apples has been shown to occur as early as four to six weeks after anthesis. Development of the disorder was characterized by breakdown of the cuticle (fragmentation) and the changes in the cells.

The cuticle showed longitudinal ridging which extended into the outer cell walls of the epidermis. The hypodermis was found to be abnormal in transverse section and extended 20 to 30 cellular layers into the outer cortex. The hypodermal cells were characterized by thick walls and they contained cellular deposits which were not present in normal tissues.

In our experiment different apple varieties were investigated with respect to the physiological disorders. Irradiation generally reduced the disorders of the surface in apples. The calcium treatment was ineffective or it increased the deterioration. In all cases, the best effect occured in combined treatment (Table 4).

The results of statistical analysis showed (Table 5) that the Gloster was significantly worse than the other varieties. There are significant differences among the treatments. Control and Ca-treated samples are significantly worse than the irradiated and irradiated combined with calcium treatment.

Table 4

Deterioration of apple as a function of treatments (1°C, 90-95 % RH, 7 months)

Treatments	Deterioration (%)			
	Varieties			
	Gloster	Idared	Mutsu	Starking
Control	50.4	13.0	14.3	12.3
Irradiated	33.5	9.6	8.0	12.2
Ca-treated	56.2	8.6	28.7	13.6
Combined treatment	25.5	6.6	6.7	9.9

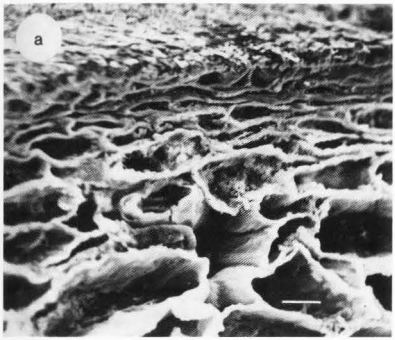
Table 5

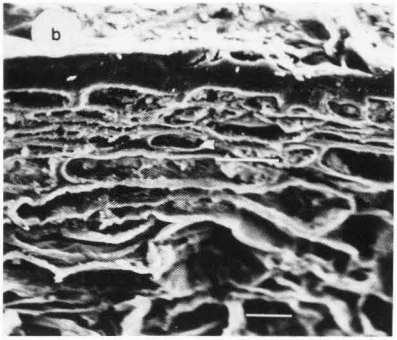
Evaluation of deterioration (%) with analysis of variance (calculation was carried out with transformed (arcsin) values)

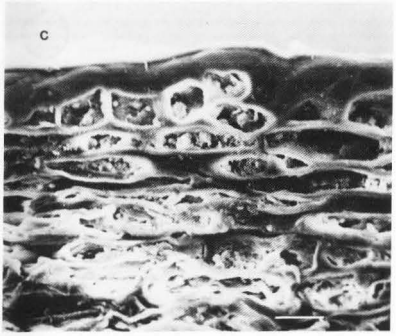
Source of variance	df	MS
Varieties (1,2,3,4)	3	412 xxx
Treatments (A,B,C,D)	3	86 x
Error	9	19

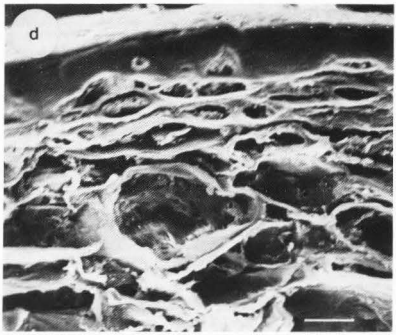
Significance at the 0.1 % (xxx), 5 % (x) level, MS= mean of squares
A=control, B=irradiated, C=calcium treated, D=calcium treated combined with irradiation, 1=Gloster, 2=Idared, 3=Mutsu, 4=Starking.
df= degree of freedom

The differences among the treatments are shown in SEM micrographs of apple skin (Fig. 8.). The fresh control and the calcium treated sample are rich in cell content (Figs. 8a and c). In the stored control, the irradiated and combined treated samples (Figs. 8b, and e) the cells of the hypodermis and outer cortex seem to be flattened.









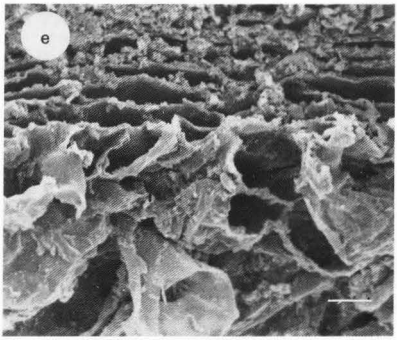
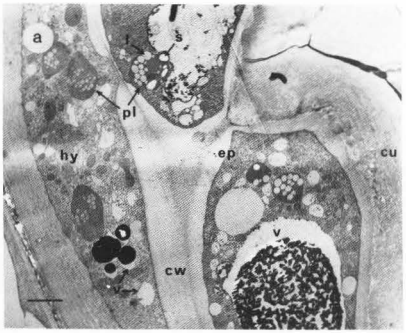
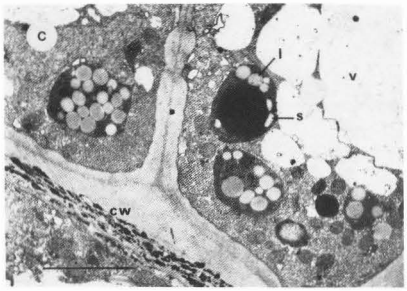
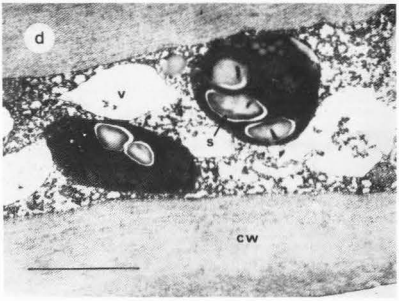


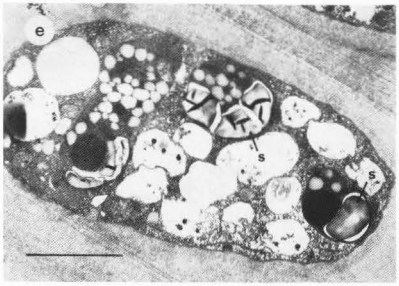
Fig. 8. Ultrastructure (SEM) of Mutsu apple skin as a function of treatments (a: fresh control; b: stored control; c: calcium treated; d: irradiated; e: calcium treatment combined with irradiation). Bars = 50 µm

TEM of the apple skin shows considerable ultrastructural differences between the control and treated samples. (Figs. 9-10). All three treatments maintained the basic compartmentation of the cytoplasm, the plastids, and the vacuoles, which was lost in the control (mainly in the epidermis) by the end of storage. Calcium treatment either alone or in combination resulted in a somewhat better preservation than irradiation alone. Irradiation and the combined treatment preserved or reformed starch; these samples contained more starch already 4 days after the treatment than the control and this effect lasted for at least 3 months. Moreover,









these treatments retarded cell wall loosening, which was apparent by the end of storage. None of the treatments preserved the electron dense vacuolar inclusions, either in the epidermis or in the hypodermis.

Irradiation influences biochemical processes responsible for the starch decomposition or synthesis. Sucrose was accumulated in irradiated sweet potato roots accompanied with the decrease in starch

Fig. 9. Ultrastructure (TEM) of Gloster apple epidermis (a,b,c) and hypodermis (a,d,e) as a function of treatments a: fresh control: b:stored control; c:calcium treated; d: irradiated; e:calcium treatment combined with irradiation (cu: cuticle; ep: epidermis; hy: hypodermis; cw: cell wall; pl: plastid; v: vacuole; s: starch; l:lipid globule. Bars=3 μm

content, which would suggest that gamma irradiation (2-3 kGy) triggered the conversion of starch into sucrose. The accumulation of sucrose caused by gamma irradiation was two or three times larger than that caused by cold storage. It was established that the activities of phosphorylase, sucrose synthase and sucrose phosphate synthase were significantly enhanced by irradiation and remained at a high level while sucrose was accumulated in irradiated tubers (Hayashi and Kawashima, 1983).

At storage temperatures below 5°C, starch in tubers is converted into sugars whereas at storage temperatures above 10°C, sugars are converted to starch (Rubin and Metlitsky, 1958).

Further investigations are needed to find out why the starch content is higher in irradiated samples than in the control in our case.

Conclusions

Food irradiation has great practical importance. It is well-known, that the most limiting factor in the use of irradiation to extend shelf life of the fruit is textural changes (softening) which are caused by the breakdown of cell wall constituents (pectin, cellulose and hemicellulose). Mobilization of the different ions in the tissues is induced by irradiation. Calcium is of fundamental importance in postharvest storage.

In our findings irradiation as low as 1 kGy induced softening in fruits; however, it preserved the cell compartments in a better state than that of the control. The combined treatment of calcium and irradiation had an even more advantageous effect. Although ultrastructural investigations proved that the calcium could not prevent the breakdown of the middle lamella in irradiated tissues, probably the calcium had a very positive effect on the cell membranes.

Our investigations show that some of the charges could not have been evaluated without ultrastructural studies. The ultrastructural changes are in good correlation with the changes in texture (cell wall decomposition) and in taste (cell membrane degradation) of fruits.

Acknowledgements

Miss K. Baló, Mr. K. Bóka and Miss. J.Bencze-Bócs are thanked for their participation in some parts of the investigations presented here. Mr. E. Zukál and Mr. I. Fehér are thanked for the statistical analysis.

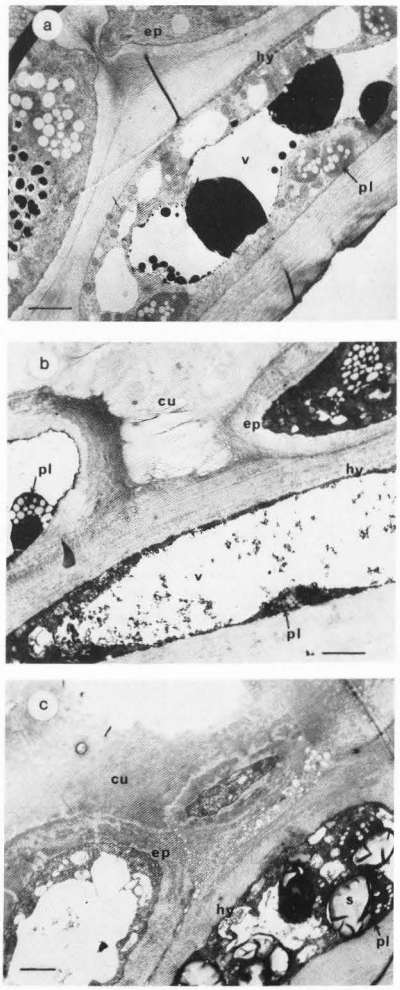


Fig. 10. Ultrastructure (TEM) of Mutsu apple skin as a function of treatments (a: fresh control; b: stored control; c: irradiated) (cu: cuticle; ep:epidermis; hy: hypodermis; cw: cell wall; pl:plastid; v: vacuole; s: starch; l: lipid globule). Bars = 3 µm

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Discussion with Reviewers

J.H. Moy: Why was only one dose (1 kGy) used in the authors' experiment? Would it not be more informative if several doses were used such as 0.5, 1.0, 1.25 and 1.5 kGy?

Authors: 1 kGy was choosen on the basis of preliminary experiments and practice.

J.H. Moy: Why was only one calcium salt used? Why were its concentration and the time and temperature for dipping not varied so that different effects can be seen?

Authors: $CaCl_2$ is commonly used in the practice. The concentration, time and temperature of dipping solution were optimized in preliminary experiments (details of this treatment are described by Hungarian Patent /A23 B7/00/1983.07.12). The aim of present work was to compare the nonirradiated, irradiated, Ca-treated and combined treated samples.

J.H. Moy: With the relative low dose rate of 1 kGy/hr., could some oxidation have occured to the fruit tissue which might affect the ultrastructure?

Authors: We have carried out experiments on the oxidation of unsaturated fatty acids and tocopherol at 0.2-1.0 kGy . h^{-1} dose rate and we have found no significant oxidative changes.

P.L. Irwin: The authors state "irradiation of plant tissue results in a loss of calcium ions" (in reference to Shah, 1966). Where are the ions lost from the internal Ca^{++} pool and/or the cell wall-bound Ca? Is it possible to distinguish the two? I ask because, indirectly via the radiolysis of H_2O, one of the major potential events during irradiation of fresh plant tissues (the cell wall and middle lamellar constituents alone have up to 50-100 %) is the production of localized volumes of relatively high H^+ . Could this be a potential mechanism of the observed Ca^{++} loss (e.g., titration of the carboxyl group) and the subsequent dissolution of middle lamella?

Authors: The calcium redistributes in apple during storage. Proportions of calcium in the peel rose in the early part of storage (till 80 days), and then declined rapidly although the decline was delayed in the peel of the fruit from the control trees (Perring, 1985. J.Sci. Food Agric., 36, 333-342). Just after irradiation the softening of apple increases, the middle lamellae are dissolved, the pectin polymer changes, and the quantity of the free calcium increases. Calcium could be lost partly from the cell wall and partly from the internal pool. These questions have not been investigated.

D.F. Lewis: There has recently been much interest in the role of glycoproteins in plant cell walls. Do you think that some of the calcium or irradiation effects could be due to changes in the cell wall glycoproteins?

Authors: Yes, we feel this possible. The permeability of some bacterial cell walls can be increased by irradiation, probably glycoproteins are changed.

D.F. Lewis: Comparing figures 3 and 4 with figure 8 shows that cells from the flesh of apples and pears tend to separate after irradiation whilst cells from near the skin of apple appear to break open even after irradiation. What differences in the cell wall structure or pectin composition do you think could account for this difference in behavior?

Authors: We think that pectin is responsible for changes in the fruit flesh. The different behaviour of the skin may be caused either by different dynamics of these changes or simply by the presence of the thick cuticle preventing nearby cells from slipping apart at preparation.

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THE MICROSTRUCTURE OF SPRAY-DRIED MICROCAPSULES

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Abstract

A newly developed technique for SEM sample preparation has been applied in the study of the effects of process parameters on the microstructure of gum arabic spray-dried microcapsules. The technique, that employs embedding in an apolar resin and partial polymerization, makes it possible to simultaneously observe the inner and outer structure of microcapsules. Our results show how the core material is organized in the solid wall matrix, existence of one or more internal voids in the microcapsule, indentation and caps in the exterior of the microcapsules, and how these microstructural features are affected by solids concentration in the sprayed emulsion, and by feed and drying air temperature.

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KEY WORDS: microcapsules, spray-drying, SEM, resin-embedding, powder particles, microstructure, gum arabic.

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Introduction

Microencapsulation is a packaging technology by which liquid droplets or solid particles are packed into continuous individual shells. The shells (or 'walls') are designed to protect the encapsulated material ('core') from factors that may cause its deterioration. In different applications, microcapsules are designed for controlled release of the core material under desired conditions, and at predetermined rate. Microencapsulation techniques in general, and in the food industry in particular, have been extensively reviewed, e.g. Bakan (1973), Balassa (1971), Graves (1972), Herbig (1970), McKernan (1972, 1973) and Puisieux and Benita (1984). In the food industry, the technique has been mainly used for the encapsulation of volatiles and oxygen-sensitive materials, using mostly spray-drying techniques (Todd, 1970, Graves, 1972, and Kirby and Law, 1987).

Successful microencapsulation is the result of a judicious choice of wall material composition for a given core material. The protection afforded by the wall and the flow properties of the encapsulated product depend on the inner and outer microstructure of the capsule, and on how the core material is organized within the microcapsule. These factors are readily studied by scanning electron microscopy (SEM) in the secondary electron imaging (SEI) mode, with great depth of field and sufficiently high resolution (Rosenberg et al., 1985). In this paper we describe the application of improved SEM techniques to study the effects of wall composition and drying conditions on the inner and outer structure of spray-dried microcapsules.

Materials and Methods

Microcapsules were prepared by

spray-drying emulsions of the core material in aqueous solutions of the wall material. As model core material we used ethylbutyrate, ethylcaproate, and ethylcaprylate (analytic grade from Frutarom, Israel). These were chosen to represent a wide range of solubilities in conjunction with retention and shelf life studies to be published separately by these authors. The wall material in all the experiments described here was gum arabic (technical grade from Sharon Laboratories, Israel). Emulsification was carried out using an X1020 micro-turrax homogenizer (Interlabs App. GmbH), and spray-drying in a mobile Minor Niro-atomizer spray dryer. Feed rate to the spray dryer was 15 g/min, and feed temperature was usually 20 °C (except for specific experiments described below). Experiments were performed at inlet air temperatures of 100, 150, and 250°C that give rise to outlet air temperatures of 70, 90 and 140°C, respectively. Atomizer speed was usually 30,000 rpm. Emulsion drop sizes were determined by light microscopy; emulsion viscosities were measured by a Brookfield viscometer at 25°C.

SEM specimen preparation procedures have been described in detail in previous publications (Rosenberg et al., 1984, 1985). To study their outer structure, microcapsules were attached to an SEM stub by a two-sided adhesive tape, and gold-coated (15 nm layer) in a Polaron E515 sputter-coater. To study the inner structure of the capsules, the powder was embedded in the apolar resin, Lowicryl HM-20 (Polaron, U.K.), and sectioned in an 820 rotary microtome (American Optical). A modification to the previously described embedding method is presented in the results section. The sectioned blocks were then mounted on SEM stubs, and gold coated. Specimens were examined in JEOL T-200 and T-300 SEMs in the secondary electron

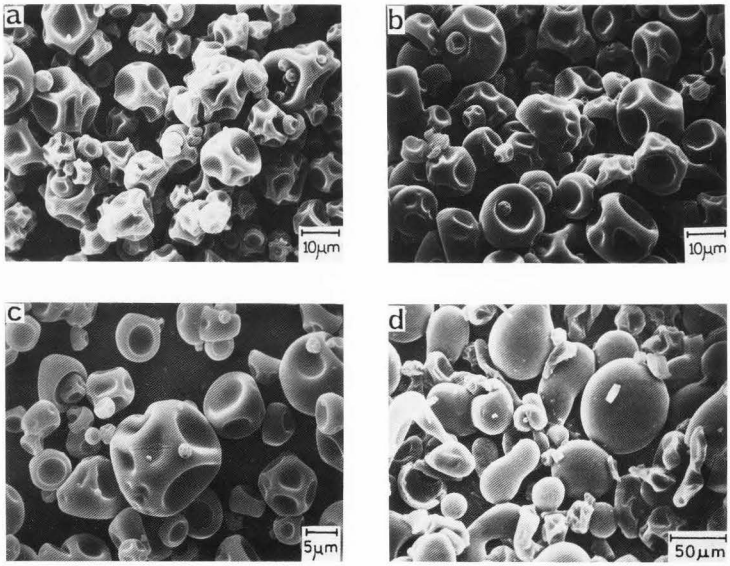

Figure 1: Gum arabic spray-dried microcapsules containing 20% caprylate on a dry basis (DB) with increasing solids concentration (w/w): (a) 10%; (b) 20%; (c) 30%, (d) 40%. Drying conditions: air inlet/outlet temperatures 150 °C/ 90°C, emulsion feed temperature 20°C.

imaging mode, at acceleration voltage of 25 kV. Microcapsule average size was determined from prints of SEM micrographs at magnifications of about 2000X. At least 100 particles were counted for each population.

Results

The effect of solids concentration in the continuous phase of the spray-dried emulsion is shown in Figures 1a, 1b, 1c, 1d from experiments in which the drying conditions were fixed: inlet air temperature of 150 °C, outlet air temperature 90 °C, and feed temperature of 20 °C. The core material (ethyl-caprylate content in these experiments was 20% on dry gum arabic basis ("20%DB"). Different magnifications were used in the various parts of the figure to show large microcapsule populations, or to emphasize detail.

At solids concentration of 10, 20 and 30% the produced microcapsules exhibited the same outer geometry of spherical particles with fairly deep indentations. These indentations were not present at the highest concentration checked of 40%. In all cases no cracks or pores could be seen in the microcapsule wall. The mean size of the particles increased with solids concentration: 10.5, 11.0 and 20.5 μm for 10, 20 and 30% solids, respectively. A wide particle size distribution was found in all experiments. This is attributed to the atomizer properties. At 40% solids, the microcapsules were much larger; many of them were not spherical, but rather elongated. This is probably linked to the viscosity of the continuous phase that increased sharply with solids concentration: 10, 23, 84 and 250 centipoise for the four concentrations examined. Solids concentrations might also affect the amount of shrinkage of the drying emulsion droplets.

Figure 2 illustrates the effect of feed and air temperature on the outer structure of the formed microcapsules. Figures 2a and 2b show the effect of raising feed temperature. The reduced temperature difference between feed and drying-air causes less shrinkage of the capsule. Another phenomenon, possibly due to slower cooling, but higher inside temperature, is the formation of caps inside some of the indentations (Figure 2b). A similar phenomenon is observed when the drying air is very hot, e.g., 250°C, as seen in Figure 2c. In this case, heating of the gas or vapor trapped within a capsule is very rapid and the outward growth of the cap is as fast as, or faster than the shrinkage due to water loss. The result is sometimes almost smooth spheres, or in some cases exploded ones (Figure 2d). At a lower drying air temperature, shrinkage is the dominant factor as can be seen in Figures 2a and 2e. The lower solid concentration of the microcapsules of Figure 2e caused more pronounced shrinkage than in Figure 2a. At a still lower drying-air temperature of 100 °C (Figure 2f), microcapsules with only shallow indentations were produced. This is a result of a lower rate of drying which causes uniform shrinkage of gum arabic in the walls. Microcapsule size is also affected by rate of drying. Many large capsules with diameters up to 40 μm are found in batches dried at 250 °C or at high feed temperature (75 °C), whereas at feed temperature of 20°C and drying air temperature of 150°C the mean diameter is only 10 μm. This value rises to 15 μm when the feed temperature is raised to 50 °C. This finding is also related to the increasing expansion of microcapsules at the above-mentioned conditions.

To study the inner structure of the microcapsules, they must be opened. The most efficient way to do this is to embed the microcapsule powder in a resin, polymerize it, and fracture the resulting block, so that the fracture surface includes open microcapsules. In previous publications (Rosenberg et al., 1984, 1985), we demonstrated the feasibility of embedding in Lowicryl HM-20, an apolar resin that does not damage the embedded capsules, and that is polymerized by UV radiation at room temperature or even much below it. But with this technique it is impossible to determine where the cutting plane passes through a capsule. This makes it ambiguous whether a capsule has a large or a small central void, and where that void is located within the capsule. To overcome these problems we modified our preparation technique. We found that at reduced polymerization time (20 minutes instead of 40 minutes at 10°C, using the manufacturer's recommended resin-to-crosslinker ratio, two 15W UV (360 nm) lamps at a bulb-to-sample distance of 35 cm) the center of the resin block is only partially polymerized. If the block is fractured through this region, the residual unpolymerized microcapsules-in-resin suspension can be removed using a needle, leaving behind a hole in the block. At the edges of this hole one can find many fractured capsules that are only partially embedded in the resin matrix. By tilting the microscope-stage, one can then observe simultaneously the inner and the outer structure of such a capsule, and thus determine where the capsule has been fractured. An example is given in Figures 3a, 3b, 3c, 3d which show a hole in the resin matrix and its edges at increasing magnifications.

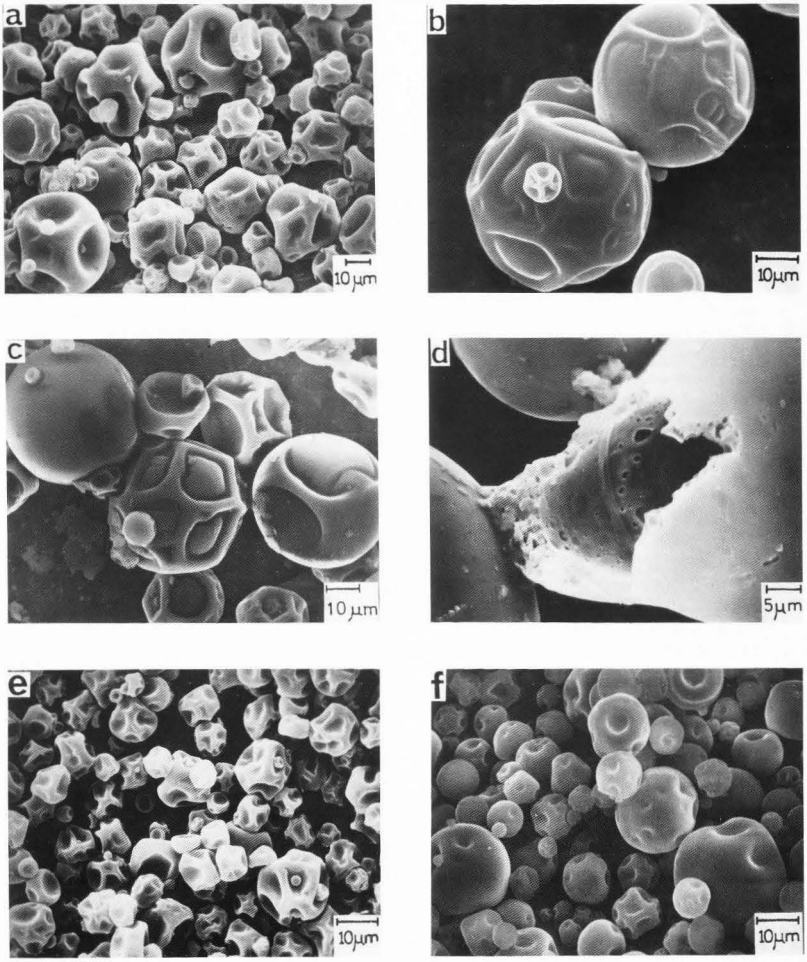

Figure 2: The effect of liquid feed and drying-air temperatures on spray-dried microcapsules. (a) 20% (w/w) gum arabic in emulsion (GA=20%); 20% ethylcaproate on dry basis (EC=20%), feed temperature, FT=20 C, air inlet and outlet temperatures, AT=150 C/90°C; (b) same as (a) but FT=75°C; (c) same as (a) but AT=250 °C; (d) same as (c) but GA=30%; (e) GA=10%, EC=20%, FT=20°C, AT=150°C/90°C; (f) same as (e) but AT=100°C/70°C.

Figures 3c, 3d show the inner and outer structure of a microcapsule that was fractured through its center. One can clearly see the position of the typical large central cavity, the limited effect on its shape by the outside 'dimples', and the distribution of the core material within the shell.

This new sample preparation method was used to determine the effect drying-air temperature has on the inner structure of the microcapsules. Figures 4a and 4b show microcapsules dried at 100°C. In most microcapsules no central voids were found. This was checked by examining many particles at the edges of holes in polymerized blocks, making sure that the cutting plane did not miss possible voids by passing above or below them. In all cases where microcapsules did have voids, these were small and located in the center of the capsule. The core material was found to be dispersed in the wall in small droplets, 0.5-1.5 μm in diameter. These droplets were well isolated from the outer surface of the microcapsules. No channels or cracks connected them to the outer surface. The deep indentations or 'dimples' that had been observed before from the outside can be clearly seen here in cross-section.

When the drying-air temperature was increased to 150°C, central voids were found in many more microcapsules (e.g., Figure 4c). In some cases, more than one void, up to four interconnected voids, were found (Figure 4d). The wall thickness of microcapsules with central voids varied from 5 to 10 μm depending on the size, shape, and location of the void within the microcapsule, and

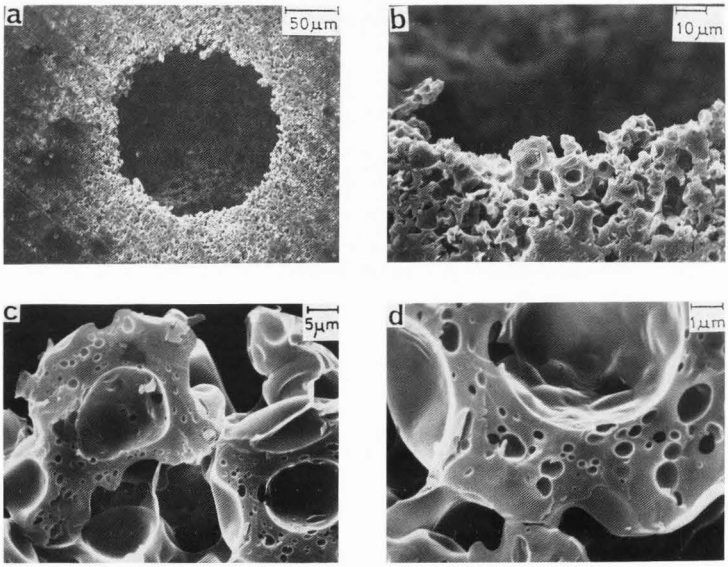

Figure 3: Fractured SEM specimen, of spray-dried gum arabic microcapsules containing 20% ethylbutyrate prepared by embedding in Lowicryl HM-20 resin and incomplete polymerization: (a) a low magnification image of the hole in the resin block; (b) a higher magnification micrograph showing the edge of the hole; (c) a higher magnification of (b) showing simultaneously the inner and outer structure of microcapsules; (d) magnified detail of (c); note the distribution of core material in the wall and the structure of the central void.

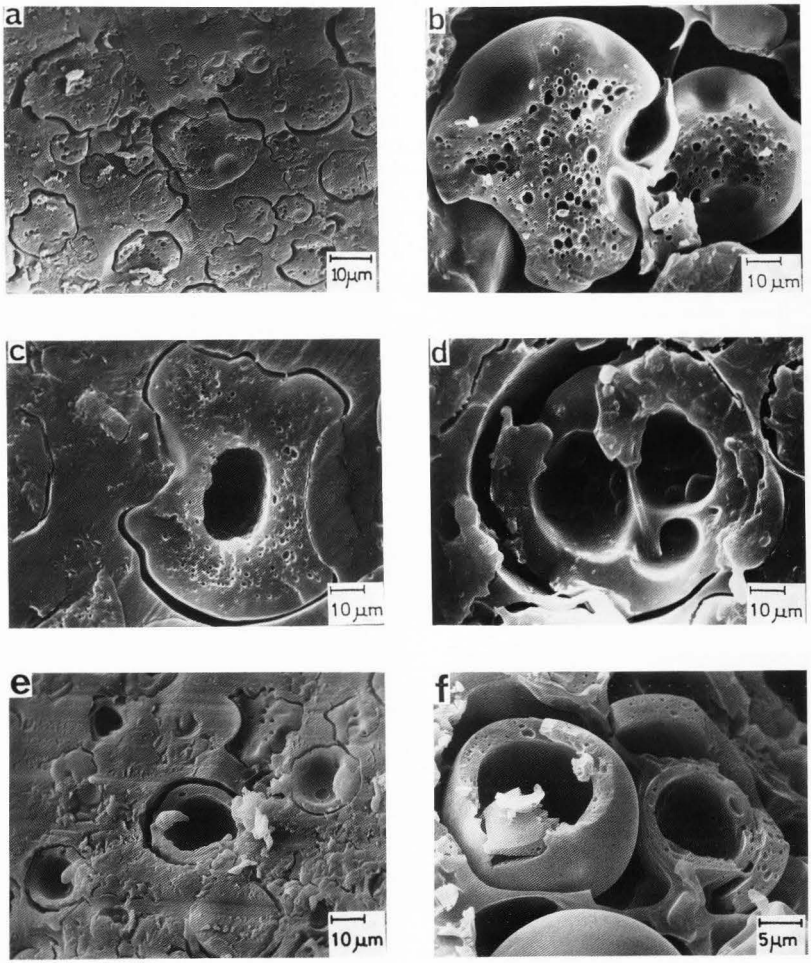

Figure 4: The effect of drying conditions and solids concentration on the inner structure of gum arabic spray-dried microcapsules: (a) GA=30%, ethylcaprylate concentration ECY=30%, FT=20°C, AT=100 °C/70 °C; (b) GA=20%, ECY=20%, FT=20°C, AT=100°C/70°C; (c) GA=20%, ECY=20%, FT=20°C, AT=150°C/90°C; (d) GA=20%, no core material, FT=20°C, AT=150°C/90°C; (e) GA=10%, EC=10%, FT=20°C, AT=250°C/140°C; (f) GA=20%, EC=20%, FT=20°C, AT=250°C/140°C.

whether deep indentations in the outer surface of the microcapsule were present. In some cases these indentations distorted the central void geometry. Drying with 250°C air produced smooth, indentation-free balloon-like microcapsules with very large central voids (Figures 4e and 4f). Here the central void occupied most of the microcapsule volume; the wall thickness in these cases was only 2-5 μm.

Discussion

The microstructure of spray-dried systems determines the product bulk density, porosity, and volatile core material retention. Despite the importance of microstructural information, very little of it has been available so far. Several theoretical and semi-empirical models describing the behavior of carbohydrate systems during spray-drying have been developed nevertheless, e.g., Menting et al., (1970), Kerkhof (1975) and Schoeber (1976), who assumed spherical drops with no internal voids, that shrink uniformly during drying. Van der Lijn (1976) suggested a mechanism for a drop expanding during spray-drying because of an expanding air bubble trapped in it. A similar mechanism of expansion was suggested by Verhey (1972a,b, 1973). Air may be trapped in the liquid during atomization, before drying. Charlesworth and Marshall (1960) attributed expansion of particles during spray-drying to the formation of steam bubbles within the liquid drops. In a more recent work, Greenwald (1980) claimed that the internal void within spray-dried particles is a result of air desorption from the liquid fed into the spray-dryer.

Our results, that provide direct inner and outer microstructural data on the microcapsules, indicate that the frequency of finding voids and their average size increase with increasing drying temperature under the same atomization conditions. The almost total absence of voids in microcapsules dried at 100 °C suggests that air bubble incorporation during atomization is negligible, and that the voids are the result of either air desorption or steam generation, or both. Higher drying temperatures cause these bubbles to expand considerably and offset, partially or totally, the shrinkage of the wall material due to loss of water. The overall result in the extreme cases are very thin walls, and occasionally exploded microcapsules.

The deep indentations or 'dimples' in the exterior of the microcapsules were found to occasionally also affect the structure of the inner voids. These indentations are probably the result of water loss from the drying drop, and are formed during the early stages of the process (Greenwald, 1980). This hypothesis is supported by our finding that at intermediate drying temperatures, "caps" develop within these dents, and that at high temperatures, the indentations disappear altogether. The expansion, therefore, must take place after dent formation, but when the wall material still contains enough water to be elastic and malleable. Conditions which favor slow drying rates were also found to favor the formation of smoother particles, since in that case, loss of water and shrinkage are more uniform. High solids concentrations give rise to viscous drops. These were found, as expected, to be larger than those formed from less viscous emulsions. Shrinkage in this case is slower than at low solids concentration because of smaller surface area-to-volume ratios, and because of higher viscosity. The drops also shrink less in this case because of less water to be lost from the system during spray-drying.

Under all the conditions studied, we found that the core material is dispersed as small droplets, 0.5-2.0 μm in diameter, in the solidified continuous wall material. This size range is identical to the size range of core material drops found in the emulsion fed into the spray-dryer. The micrographs of fractured microcapsules also showed that core material droplets are protected well by the matrix, and thus no losses are expected as long as the wall remains intact. It was found that at a given solids concentration and drying conditions, the increasing core material concentration led to thinner wall layers around each core drop.

The question of retention of the volatile core material during microencapsulation, i.e., how much of the emulsified material actually becomes microencapsulated, has not been addressed in the present paper; it will be discussed in a separate publication (Rosenberg et al., in preparation). Suffice here to say that our SEM work has revealed that substantial core material losses may occur during the initial stages of drying. This is demonstrated by Figure 5 in which there is no evidence of any ethylbutyrate remaining from an initial concentration of 10%. The loss must have occurred before complete solidification, since all the drops (or 'pools'), where the ester had been embedded in the matrix, disappeared.

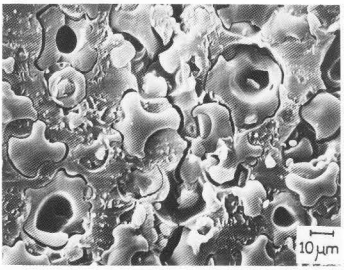

Figure 5: Spray-dried gum arabic microcapsules, GA=10%, FT=20°C, AT=150 C/90°C. The emulsion contained originally 10% (DB) ethylbutyrate which was lost during spray-drying, as evident from lack of small "pools" in the fractured microcapsules.

Acknowledgment

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Nomenclature

AT - air temperature: (inlet temperature)/(outlet temperature)
DB - concentration on a dry basis
EC - concentration of ethylcaproate on a dry basis
ECY - concentration of ethylcaprylate on a dry basis
FT - feed temperature
GA - concentration of gum arabic in emulsion

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Discussion with Reviewers

Reviewer II: Figures 2a and 2b are interesting. I am very surprised that higher infeed temperature produces larger particles. Is this supported in the literature?
Authors: The phenomenon was not observed. The magnification of Figure 2b is almost twice that of Figure 2a; also, in Figure 2b we show two large particles to emphasize detail.

Reviewer II: The difference in appearance of Figures 4a and 4b is not clear. The same for 4c and 4e and 4f. One part of the figure looks like clearly distinguished particles, and the other an embedded particle. Why are there such differences?
Authors: The differences stem from the variation of the embedding technique used: whereas Figure 4a is an example of fully embedded particles (in a fully polymerized resin block), Figure 4f is an example of partially embedded particles (close to the hole in the partially polymerized resin block; see text and Figure 3 for details), allowing views of both inside and outside of the microcapsules.

Reviewer III: Do the authors feel this method could be applied to other dried systems, like milk or corn sweeteners?
Authors: The method can be used with any dried system. The embedding resin should be selected according to the properties of the system.

Reviewer IV: What are the implications of using such high temperatures for the materials that are to be encapsulated? This would be especially of importance for volatile compounds.
Authors: The quantitative aspects of volatiles retention during microencapsulation, including temperature effects are discussed in detail in a paper by these authors, to be submitted for publication soon.

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FUNCTIONAL AND MICROSTRUCTURAL EFFECTS OF FILLERS IN COMMINUTED MEAT PRODUCTS

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Abstract

Fillers are used in comminuted meat products such as wieners to increase yield, improve stability, and modify textural properties. Light microscopy, scanning electron microscopy and transmission electron microscopy show that comminuted meat products are mechanical mixtures in which the microstructural features of starch and insoluble protein ingredients are largely retained. The water absorption and gelation properties of these ingredients contribute to the stability and textural firmness. Soluble proteins may improve stability through emulsion formation but the role of emulsion formation is clearly secondary to that of gelation. The characteristic springy gel structure of wieners is determined by the gelation of myofibrillar meat proteins. Provided the structure of the meat protein gel is not disrupted, fillers will generally increase both textural firmness and stability. Starch and protein fillers have been shown to increase the stability of wiener homogenates prepared at a higher (26°C) temperature than that which is normally used (16°C). Light microscopy revealed that the "all-meat" wieners had a higher degree of fat agglomeration than did the more stable wieners containing added starch fillers. Electron microscopy revealed that the starch granules participated in the process of physically entrapping the fat globules. Fat globules varied in size and shape, and were observed in environments ranging from low to high protein densities. In summary, comminuted meat products are shown to have a complex heterogeneous microstructure.

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KEY WORDS: Binder, Comminuted meat, Emulsion, Filler, Frankfurter, Functional effects, Microstructure, Protein matrix, Wiener.

Introduction

Fillers are used in a wide range of processed meat products. Comminuted meat products such as wieners, bologna, and luncheon meats are the largest and most complex class of such products. These products are often referred to as "fine emulsion products" to differentiate them from coarsely chopped sausage and patty products. A food emulsion is a two-phase system, *e.g.* oil in water, in which the dispersed droplets have diameters between 0.1 and 10 µm (Powrie and Tung, 1976). When applied to comminuted meat products, therefore, the term "emulsion" is a misnomer which unfortunately has encouraged food scientists to perform seemingly endless model system and functionality tests based on the emulsion concept. One reason why the concept has endured, and to some extent continues to endure, may be because the emulsifying capacity of a protein can be quantified accurately (Carpenter and Saffle, 1964).

Saffle (1964, 1966) established a bind value scale for meat ingredients based on emulsifying capacity data which is widely used in least-cost computer programs for formulating comminuted meat products. For undegraded skeletal muscle proteins, salt solubility and emulsifying capacity may correlate with textural and stability performance (Saffle and Galbreath, 1964; Schut, 1978). However, for other meat proteins and for non-meat proteins, these correlations do not hold as shown by Comer (1979) and Comer and Dempster (1981). The effective use of least-cost formulation programs involves refinement of ingredient bind values and the use of both analytical and ingredient constraints.

Comminuted meat products are complex food systems in which water absorption, gelation phenomena, and emulsion formation influence the stability and texture of the cooked product. The functional effects of fillers are best understood by taking each of these mechanisms into consideration.

The microstructure of comminuted meat products and its relationship to stability and texture have previously been examined and reviewed (Lee, 1985). Most of the previous work has involved "all-meat" systems. The purpose of this review is to re-examine the microstructural and functional data for "all-meat" systems, and to critically examine the role fillers play in comminuted meat product stability, texture, and microstructure. Data from our previous work are presented as well as new data

which examine the effects which starch and caseinate fillers have upon the stability, texture, and microstructure of wieners which have been homogenized at a normal temperature (16°C) and at a higher temperature (26°C).

"All Meat" Systems

Function of ingredients

To understand the effects of fillers, comparisons must be made with "all meat" systems. In the preparation of comminuted meat products, several non-meat ingredients are used which are not generally considered as fillers (Paquette, 1986). Salt is added at a 2-3% level to improve the functional performance of the meat ingredients and contribute to flavour. A curing system ("cure") of sodium nitrite (120 ppm) and sodium erythorbate or ascorbate (550 ppm) is used to produce the cured meat colour, contribute to flavour, prevent fat oxidation, and retard microbial growth. Spices, seasonings, and smoke flavouring are added at levels of 1 to 3% to provide flavour and colour. Water is added at levels of 20% to 40% to increase yield, improve the functional performance of the meat ingredients, and to modify the textural properties of the finished product. Like fillers, all of these non-meat ingredients increase yield, and, in the use of some seasonings and added water, this is clearly one of their designed functions. Mustard flour and yeast products are two seasonings which are also functional fillers. The major components of these products are proteins and polysaccharides which influence the stability and textural properties of meat systems principally through water binding mechanisms.

In "all meat" systems, water and salt contribute to the stability and textural properties through interactions with the myofibrillar proteins. In experiments which involved using both sodium chloride and alkaline phosphates, Trout and Schmidt (1983) showed that low ionic strength and low pH resulted in large losses of fat and water during cooking, and a soft product texture. Recent attempts to reduce the salt levels in comminuted meat products have also resulted in reduced stability and textural firmness at salt levels below 2% (Sofos, 1983a, 1983b; Whiting, 1984a, 1984b).

Trout and Schmidt (1986) found that high salt concentrations are required to maintain the water binding ability of comminuted meat products at a normal minimum cook temperature of 68°C. In the presence of salt and phosphates, myofibrillar proteins bind water and swell. During heat treatment at temperatures of 50°C to 70°C these proteins form an irreversible gel which provides the matrix that holds both water and fat in the meat system. Water released during heating by the shrinkage of collagen fibres and, to a lesser degree, by shrinkage of the myofibrillar proteins, affects the thermal stability. In the process of comminution, some of the proteins are solubilized. These proteins may interact with the fat particles, which have been reduced in size, to form protective membranes. Hansen (1960) presented photomicrographs which showed the reduction in fat globule size during chopping and the formation of fat globule membranes. The emulsion theory was born and soon given support by model system experiments which showed that salt-soluble meat proteins can form emulsions, (Hegarty *et al.*, 1963; Swift *et al.*, 1961; Swift and Sulzbacher, 1963).

Stability and microstructure

The emulsion theory focussed attention on the size of the fat globules and the protein-fat interface. Emulsion stability, in particular, the effect of chopping temperature on stability, has been investigated by a number of workers (Ambrosiadis and Wirth, 1984; Brown and Toledo, 1975; Carroll and Lee, 1981; Girard *et al.*, 1985; Hansen, 1960; Helmer and Saffle, 1963; Jones and Mandigo, 1982; Lee *et al.*, 1981; Townsend *et al.*, 1968, 1971). Chopping temperatures of 10° to 16°C produce the most stable products. Stability decreases rapidly in the temperature range of 18° to 30°C. Light microscopy has been used to show that fat agglomeration occurs at higher chopping temperatures (Hansen, 1960; Helmer and Saffle, 1963). Using differential thermal analysis (DTA), Townsend *et al.* (1968) showed that fat melting in the range of 18° to 30°C is the most likely cause of reduced cook stability at higher chopping temperatures. Myosin protein transitions are revealed by DTA at 42.0°, 49.5° and 60.5°C (Siegel and Schmidt, 1979).

Carroll and Lee (1981), Lee *et al.* (1981), and Jones and Mandigo (1982) showed that losses in stability at high chopping temperatures are accompanied by decreases in textural firmness. Both light microscopy (LM) and scanning electron microscopy (SEM) were used to reveal a protein matrix disrupted by fat channels.

A critical element of the emulsion theory is the presence of protein membranes around fat globules. In model systems, true emulsions of salt soluble meat proteins and oil are formed. Both light microscopy (Carpenter and Saffle, 1964; Hansen, 1960; Swift *et al.*, 1961; Tsai *et al.*, 1972) and transmission electron microscopy (TEM) (Acton *et al.*, 1982) have clearly shown complete membranes. Evidence presented in micrographs of meat products is less convincing. TEM and SEM evidence has been presented to show protein membranes in comminuted meat products (Borchert *et al.*, 1967; Jones and Mandigo, 1982; Theno and Schmidt, 1978). However, Swasdee *et al.* (1982) using TEM showed that even after extended chopping times, not all fat globules were uniformly surrounded with protein membranes. In a recent review, Lee (1985) has questioned the significance of protein membranes in meat product stability. Meyer *et al.* (1964) showed that emulsifiers such as mono- and diglycerides decrease the stability of sausage emulsions.

Light microscopy was used to support the explanation that the emulsifiers affected the matrix and prevented protein film formation. Based on light microscopy observations, van den Oord and Visser (1973) and Evans and Ranken (1975) concluded that the fatty tissue in comminuted meat products was not emulsified. Hamm (1973) also de-emphasized the role of emulsification by drawing attention to the importance of the water-holding capacity of meat proteins in producing stable meat products. Brown and Toledo (1975) concluded that mechanical entrapment of fat particles is responsible for the stability of comminuted meat batters. Hermansson (1986) reported that differential interference contrast light microscopy revealed some kind of

protein film around all fat particles in raw meat batters. However, after cooking, structural changes such as gel shrinkage may reduce the rate of film formation in determining fat stability.

Using SEM, Theno and Schmidt (1978) showed that commercial frankfurters vary widely in microstructure from a fine protein matrix structure to a very coarse matrix structure containing large fat globules and intact muscle pieces. It is clear that for many comminuted meat products, emulsion formation plays a minor role in determining product stability and texture. Based on SEM and TEM observations, Katsaras and Stenzel (1984) described frankfurter-type sausages as mechanical mixtures. This term seems appropriate to describe the mixture of fat particles and various meat proteins which have been produced by mechanical chopping and mixing action in the preparation of meat homogenates. Microstructural observations by Ray *et al.* (1979, 1981), Lewis (1979), Swasdee *et al.* (1982), and Comer *et al.* (1986) support this description. In a recent publication, Oelker (1988) described frankfurters, on the basis of TEM observations, as a structural multiphase system, or microflake structure, which emphasized the water binding properties of myofilaments.

Gelation

During cooking, meat proteins aggregate and form irreversible gels. Yasui *et al.* (1979); Ishioroshi *et al.* (1979); and Siegel and Schmidt (1979) examined myosin gels using SEM, and showed that myosin transitions occur stepwise in the temperature range of 30° to 60°C. Optimal gel strength for myosin gels occurs at 60° to 70°C (Foegeding *et al.*, 1986a; Ishioroshi *et al.*, 1979). Replacement of part of the lean meat with blood plasma in wiener formulations results in softer texture, whereas in retorted products (120°C cook) an increase in textural firmness may occur (Comer and Dempster, 1981). Foegeding *et al.* (1986a) have shown that albumin gels do not form until 95°C, although the gelling temperature could be lowered to 80°C by interactions with myosin (Foegeding *et al.*, 1986b). Using SEM, Siegel and Schmidt (1979) showed that in the absence of salt, myosin forms a spongy network which has a weak gel strength. Hermansson (1986) used SEM to show that salt caused a disintegration of the myofibrils and formation of an aggregated protein network. It is now clear that the characteristic textural properties of comminuted meat products are largely determined by the gelation of myosin. Other meat proteins and non-meat ingredients may modify the textural properties, but if an insufficient amount of myosin is present in the formulation, "mushy" or "cakey" texture is the result.

Investigations on the gelation of protein ingredients is an active research area (Asghar *et al.*, 1985; Beveridge *et al.*, 1983, 1984, 1985; Foegeding and Lanier, 1987; Hamann, 1987; Hermansson and Larsson, 1986; King, 1977; Montejano *et al.*, 1984a, 1984b; Sadowska and Sikorski, 1976). Unlike emulsifying capacity, gelation properties are difficult to quantify and are significantly influenced by protein interactions.

Effects of Fillers

Functional fillers

Milk-derived and plant-derived food ingredients are used as functional fillers in comminuted meat products. The milk ingredients include skim milk powder, buttermilk powder, sodium caseinate, calcium caseinate, whey protein concentrates, calcium-reduced milk powder, and whey powder. For each milk product it is the protein component which provides the functionality. The carbohydrate component, lactose, has a sweetening effect upon flavour but at the levels normally used appears to have little effect on either the texture or stability. The plant-derived ingredients include soy isolate, wheat gluten, soy concentrate, textured soy flour, inactive yeasts, mustard flour, cereal flours, legume flours, baked cereals, starches, and modified starches. In plant products, both protein and carbohydrate components provide functionality when incorporated into comminuted meat products.

Functional effects of adding fillers

The economic incentive for using fillers in meat products is to reduce ingredient costs. Yields for comminuted meat products are commonly expressed in terms of 100 parts of meat. For "all-meat" products, the addition of water, salt, cure, spices, and seasonings may increase the yield 10-30% depending on the strength of the meat block, product textural quality standards, and government regulations. The functional effects of adding protein and starch-based fillers to "all-meat" systems have been shown by Comer *et al.* (1986) to be increased yield, textural firmness, and stability (reduced processing "shrinks" and cooking losses). Table 1 summarizes previously published data (Comer *et al.*, 1986) showing the analytical and functional effects of adding various fillers to a comminuted meat formulation.

The results for the five fillers are compared with the "all-meat" control in row 1 which is labelled "Nil". Water and fat are lower with added filler simply because of dilution. The protein content is lower for the wieners containing starch, but is higher for the samples containing other fillers which have more than 11% protein found in the control. The yields are higher principally because of the direct effects of adding 7% filler; however a little more moisture was also retained than in the control. The stability data is a composite value determined by subtracting moisture losses in the smokehouse, and fat and moisture losses during cooking in boiling water (to simulate home preparation) from 100%. In every case, the added fillers reduced both processing and cooking losses for a 4% to 7% increase in stability. All fillers also increased textural firmness. These positive effects of fillers in meat products have often been obscured by comparisons with lean meat ingredients and by attempts to extend meat products beyond yields of 150%.

Standardized comminuted meat products in Canada and in the United States are not "extended" products in the sense that meat ingredients are the principal sources of protein. The meat protein content is generally in the range of 9.5% to 11%, which is sufficient to ensure a good gel structure. The major factors controlling the textural firmness and stability are the proportion of lean meat ingredients and the water/fat ratio. When fillers are added to this system, they bind some of the water which allows the meat proteins to form a firmer gel structure. This function of fillers is especially important for low fat products which have correspondingly higher moisture contents.

Functional effects of replacing meat proteins

Heat induced gelation of the myofibrillar muscle proteins, especially myosin, is responsible for the springy, chewy gel structure of comminuted meat products (Comer, 1979; Roberts, 1974b; Schut, 1978; Webb, 1974). Attempts to replace myofibrillar proteins with non-meat proteins have resulted in softer, less chewy products (Decker *et al.*, 1986; Comer and Dempster, 1981; Terrell, *et al.*, 1979a, 1979b). Although replacement of lean meat proteins with non-meat proteins results in loss of textural firmness, stability may be maintained or even enhanced by replacement (Comer, 1979; Randall *et al.*, 1976; Van Eerd, 1971). Table 2 summarizes previously published data (Comer, 1979; Comer and Dempster, 1981). In these experiments, 22% of the lean beef in the formula was replaced with 4.4% of filler solids (equivalent to the protein solids of the lean beef replaced). Moisture and fat were adjusted to be equivalent to the lean beef control. The skeletal muscle protein contents of the strong, medium, and weak meat systems were 8.0%, 4.5% and 1.8%, respectively, which is almost in direct proportion to the textural firmness values for the lean beef control. These data show that the springy gel structure is determined by the skeletal muscle proteins.

The fillers being compared are soy concentrate, sodium caseinate, textured soy flour, potato starch, wheat flour, and skim milk powder. With the exception of the skim milk powder, the fillers are composed principally of proteins and polysaccharides. The milk powder contains only 35% protein and the rest is non-binding lactose. This is a disadvantage from the stability viewpoint, as shown in Table 2 in the strong meat system, where only the skim milk powder performed more poorly than the lean beef. For the medium and weak meat blocks, the beef outperformed all fillers except caseinate. Caseinate is the only filler in this table which has a higher emulsifying capacity than lean beef (Comer, 1979). Commercial wieners contain medium to strong meat blocks and, therefore, based on the data in Table 2, replacement of meat protein with fillers has minimal effects upon yield and stability. Even in the weak system, wieners containing fillers were stable in the sense that they did not render out fat during processing. The lower stabilities reflect predominantly higher moisture losses.

The texture results in Table 2 reveal a significant effect of meat block strength upon the relative functional performance of fillers. In the medium and weak systems, the soy concentrate and textured soy flour clearly produced the firmest texture. In the strong system, however, starch and skim milk powder performed better. Both ingredients absorb less water than the soy proteins. This is desirable in a strong system, since the meat proteins require the water to form a strong gel. In the weak system, there is too much water available to the lean meat proteins to form a firm gel and, therefore, stronger absorbing fillers are desired.

Sodium caseinate is unique in that it does not gel but holds the moisture in an emulsion (Van den Hoven, 1987). Since the caseinate is soluble, there is a greater potential for interaction with the soluble meat proteins. This may be one of the causes of the softer gel structure revealed by the low texture values for the wieners containing caseinate (Table 2). The adverse effects of caseinate upon texture have also been reported by other workers (Oelker, 1988; Schut, 1976).

Several studies have shown that up to 50% of the meat protein in a comminuted meat system can be replaced by non-meat proteins (Randall *et al.*, 1976; Roberts, 1974a; Rongey and Bratzler, 1966); and simulated non-meat sausage products have been prepared from soy isolate (Frank and Circle, 1959). Non-meat protein and starch gels are much softer than myosin gels (Circle *et al.*, 1964), and, therefore, replacement of lean meat with fillers results in products having a softer and a less chewy texture. If the gel structure is weakened too severely, then the stability of the system is adversely affected as shown for the weak system in Table 2.

Correlation of functional properties and functional effects

The effects of specific fillers have been described in a recent review by Mittal and Usborne (1985), and in a comprehensive review of meat emulsions by Schut (1976). Most of the research done to date has involved protein ingredients. Initially, the emphasis was directed towards the emulsifying properties of non-meat proteins (Acton and Saffle, 1971; Crenwelge *et al.*, 1974; Pearson *et al.*, 1965; Puski, 1976; Saffle, 1968). However, Smith *et al.*, (1973) demonstrated that higher nitrogen solubility index (NSI) values for non-meat proteins did not correlate with stability performance in frankfurters. Many of the papers compare the effects of soy proteins with plant (Keeton *et al.*, 1984; Lin *et al.*, 1975; Patana-Anake and Foegeding, 1985; Rakosky, 1970; Terrell and Staniec, 1975; Thompson *et al.*, 1984) and milk proteins (Casella, 1983; Hermansson, 1975; Hermansson and Akesson, 1975a, 1975b; Lauck, 1975; Parks and Carpenter, 1987; Porteous and Quinn, 1979; Thomas *et al.*, 1973, 1976). It is difficult to deduce general conclusions on efficacy of filler ingredients from these papers because of differences in experimental design and purpose. Torgersen and Toledo (1977) concluded that fillers which have high water binding capacities and which form firm gels have better functionality in meat systems than those with high solubility. Comer (1979) proposed that the effects of fillers were best understood by considering comminuted meat products as gel systems rather than emulsions.

The stability and textural properties of the gels are influenced by competition for moisture between proteins and carbohydrates and by protein interactions with water, fat, and other proteins. It is not surprising that attempts to develop correlations between functional properties of meat and filler ingredients and functional effects in comminuted meat products have been of limited success (Comer and Dempster, 1981; Li-Chan *et al.*, 1987; Mittal and Usborne, 1986; Parks *et al.*, 1985; Porteous and Quinn, 1979). Nevertheless, we have found that the functional effects of ingredients can be rationalized and predicted by considering their water binding and gelation properties. Relative functional performance of ingredients is dependent upon the composition of the total system, *i.e.*, ingredients do not have absolute bind values.

Microstructural effects

There have been only a few papers in which the microstructural effects of fillers in meat products

Table 1. Effects of adding fillers (Wieners contain 7% filler[a])

Filler	Water (%)	Fat (%)	Protein (%)	Yield[b] (%)	Stability (%)	Texture[c] (kg)
Nil	59.4	25.9	11.0	125	79	1.1
Modified corn starch	55.5	23.4	10.4	135	86	2.0
Hard wheat flour	55.4	23.7	11.3	136	86	1.4
Skim milk powder	56.7	23.7	12.5	137	84	1.2
Soy concentrate	56.3	23.2	15.2	136	84	1.6
Vital wheat gluten	56.4	23.3	15.3	137	83	1.5

[a] Comer *et al.*, 1986.
[b] Yield is based on meat ingredients equal to 100%.
[c] Texture is determined by the comparison force required to rupture 2 cm segments of cooked wieners (Comer *et al.*, 1986).

Table 2. Effects of fillers relative to lean beef in strong, medium, and weak meat systems (Wieners contain 4.4% filler)[a]

Ingredient	Total stability (%)			Texture[b] (kg)		
	Strong	Medium	Weak	Strong	Medium	Weak
Lean beef	84	83	78	3.6	2.1	1.1
Soy concentrate	86	82	70	2.7	1.4	0.9
Sodium caseinate	88	85	81	1.9	1.0	0.4
Textured soy flour	84	81	62	2.2	1.3	0.8
Potato starch	84	80	74	2.9	0.8	0.4
Hard wheat flour	84	80	76	1.9	1.1	0.4
Skim milk powder	82	78	67	2.8	0.9	0.4

[a] Comer, 1979, Comer and Dempster, 1981.
[b] See footnote c in Table 1.

have been investigated. Flint and Pickering (1984) and Flint and Firth (1981) developed staining procedures which differentiate between a variety of ingredients found in comminuted meat products, including muscle cells, collagen, starch granules, wheat gluten, and soy protein. Cassens *et al.* (1975) used light microscopy to show the physical distribution of textured soy flour particles in frankfurters. Textured soy flour absorbs moisture but does not disperse. Irregularly shaped fat globules were sometimes observed next to particles of textured soy flour but there were no significant effects upon stability at usage levels of 3-4% filler. Lee (1985) used light microscopy to show that similar levels of soy protein isolate also had minor effects upon fat distribution. The soy isolate increased the viscosity of the raw meat batter. This may have been responsible for the observed slight increase in average fat globule size. Soy protein isolates are dispersible and form heat-set gels. Siegel *et al.* (1979a, 1979b) used SEM to study the gel structure of soy isolates and other non-meat proteins in model systems alone and in the presence of myosin. The non-meat protein gels were shown to have structures different from those of myosin gels and in several cases interactions with myosin were evident. Comer *et al.* (1986) used SEM and TEM to show that the gel features of wheat gluten and soy proteins were observable in comminuted meat products containing 6% filler. Both wheat gluten and soy proteins have limited dispersibility at the 5% salt concentration found in frankfurters. Therefore, it is difficult to observe interactions of these fillers with meat proteins. Skim milk powder is highly dispersible at this salt concentration, and it was shown that the micelle structure is lost and replaced by a distinctive granular protein density pattern possibly due to interactions of casein micelles and salt-soluble meat proteins (Comer *et al.*, 1986).

Kempton *et al.* (1982, 1983) applied a statistical evaluation procedure in analyzing photomicrographs to describe the microstructural effects of textured soy flour, soy protein concentrate and yeast fillers. They observed increased clumping of fat and protein ingredients in some fields of some slices. These observations are consistent with increased densities reported by Lee *et al.*, (1981) and Cassens *et al.*, (1975). However, whereas Lee *et al.*, (1981) reported a larger number of smaller fat globules, Kempton *et al.* (1982, 1983) reported a greater frequency of fat agglomerates due to vegetable proteins. The different effects may be due to differences in either chopping times or viscosities. Kempton (1983) observed that neither stability nor textural firmness were correlated with the degree of clumping. Similar observations were made by Comer *et al.* (1986). Schmidt *et al.*

(1982) used light microscopy to show that soy concentrate formed an integral part of the matrix to stabilize a high fat, canned luncheon meat formula.

Comer *et al.* (1986) used light microscopy, SEM, and TEM to show corn starch and wheat starch granules in wieners. In water, the starch granules lose their birefringence at about 60°C and start merging in the temperature range 60° to 70°C. However, in wieners cooked to 72°C (internal temperature), most of the starch granules were discrete and several possessed birefringence. It was shown that this is the result of the limited moisture environment in the comminuted meat products.

Lin and Zayas (1987a) used TEM to show that pre-emulsification of fat with corn germ protein produced a finer dispersion of fat globules in the meat batter than if the filler was added directly to the batter. Zayas (1985) prepared pre-emulsified fat with sodium caseinate and skim milk powder and also showed a more uniform dispersion of fat particles using TEM. Soy proteins and wheat gluten have also been used in preparing fat emulsions for use in comminuted products (Hand *et al.*, 1983). The pre-emulsions bind both fat and added water which may result in higher fat stability (Lin and Zayas, 1987b), and increased yield and softer texture because of higher water binding capacity (Zayas, 1985). Pre-emulsions are being used in Germany (Wirth, 1985). However, in Canadian comminuted meat formulations, fat stability is rarely a concern and the water holding capacity is generally sufficient to nullify the beneficial yield effects of pre-emulsion addition.

Summary of effects

In general, meat systems have the capacity to tolerate substantial amounts of filler ingredients, especially starches and flours where levels of 5% to 10% are currently used in Canadian formulations. Ingredients which interfere with the meat gel structure such as gums and soluble proteins must be used at lower levels. Beyond a certain point, more filler can be added to form a dry texture but the "springy" gel structure is transformed into a "cakey" structure such as is found in vegetable protein extended products.

Experimental design of present study

To further our knowledge on the effects of fillers, we have carried out experiments to determine the functional and microstructural effects of fillers in wieners at different homogenizing temperatures. Previous studies of the effects of chopping temperatures have involved "all-meat" systems. For comparison with "all-meat" systems, we selected two fillers: modified corn starch at a level of 7% to bind excess moisture and produce a firm texture, and sodium caseinate at a 1% level to increase the emulsifying capacity of the system. The homogenizing temperatures selected were 16°C, which is known to produce good stability and textural firmness, and 26°C, which has previously been shown to result in lower stability and textural firmness (Carroll and Lee, 1981). Yield, stability, and textural firmness of the wieners were determined and the microstructural features of the wieners were examined by light microscopy, SEM, and TEM. The meat formulation was selected to be typical of that used in manufacturing wieners in Canada.

Materials and Methods

Preparation and analysis of wieners and fresh homogenates

The meat ingredients were obtained from local meat processors and stored frozen. The modified corn starch was a crosslinked, derivatized waxy maize starch supplied by National Starch and Chemical Corp, Bridgewater, New Jersey. The sodium caseinate was supplied by Rovianda EMBH & Co., Engelsberg/Wiesmühl, Germany.

With the exception of the backfat, the meat ingredients were tempered overnight in a cooler at 3°C, ground twice through a 0.48 cm (3/16 inch) plate, and then preblended in a Hobart mixer for 5 min. The formulations are shown in Table 3. The preblended meat was divided into two parts. One-half was stored at ambient temperature (23°C) for 4 h prior to use in the preparation of batches 5 to 8 at the higher homogenizing temperature of 26°C. The other half was used directly from the cooler to prepare batches 1 to 4 at the homogenizing temperature of 16°C. In each case, all of the meat for the four batches was placed in a Hobart mixer, and salt, cure, seasoning, and one-half of the water (40°C) were added. After 5 min of mixing, the fat was added and mixing continued for 3 min. The mixture was divided into four parts, and for each batch, the remaining ingredients (one-half of water and filler) were added in a Hobart mixer. Mixing was continued for 3 min and then the mixture was homogenized through a laboratory Mincemaster™ colloid mill (The Griffith Laboratories, Ltd.). The batch size was 3 kg. The homogenizing temperature was controlled by varying the temperature of the water added. For batches 1 to 4, the meat temperature prior to addition of the second half of the water was 9°C. Water was added at 10°C and the temperature rise was about 3°C during mixing and 3°C during homogenizing.

For batches 5 to 8, water at 40°C was added to meat at 20°C to give a homogenate temperature of 26°C. The homogenates were stuffed into 22 mm diameter cellulose casings, linked in 12.7 cm sections and cooked in a smokehouse to an internal temperature of 72°C in a 1 hour cycle with 40% relative humidity for the last 30 minutes. The wieners were hung in a cooler for 16 hours, smokehouse losses were determined, and the wieners were vacuum packaged and stored in the cooler. The fresh homogenates remaining in the stuffer were used to determine the cook stability at 75°C.

The textural firmness and stability of the wieners were determined according to the methods reported by Comer *et al.* (1986). The textural value is a compression rupture force (kg); yield is the product weight (parts) per 100 parts meat block; stability takes into account both processing and home preparation cooking losses. These data are shown in Table 4. The presence of fat caps, indicative of partial homogenate breakdown during preparation of the wieners, is also shown in Table 4.

The cook stabilities of the fresh homogenates were determined by a modification of the method reported by Comer (1979). Samples (35 g) of homogenate were centrifuged at 1600 xg in 50 mL glass tubes. The tubes were placed in a 50°C water bath for 10 min and the bath temperature was then increased to 75°C over 45 min to complete the cook cycle. The released fat and moisture were weighed

Table 3. Formulation data (w/w) for experimental wieners
(Expressed in units of parts by weight)

Component	Formula number[a] 1	2	3	4	5	6	7	8
Meat block[b]	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Salt/seasoning/cure[c]	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Added water[d]	41.0	39.9	39.8	40.9	41.0	39.9	39.8	40.9
Modified corn starch	0	10.3	10.3	0	0	10.3	10.3	0
Sodium caseinate	0	0	1.4	1.4	0	0	1.4	1.4

[a] Formulae 1 to 4 homogenized at 16°C and formulae 5 to 8 homogenized at 26°C. The experimental design features two sets of the same four formulae, each set being homogenized at a different temperature.
[b] Lean beef chuck 20, lean pork trim 20, mechanically separated chicken meat 25, pork hearts 14.2, pork backfat 20.8.
[c] Salt 3.6, spice oleoresin blend 0.8, sodium erythorbate 0.075, sodium nitrite 0.03.
[d] Added water adjusted to take into account the moisture contribution of the fillers.

Table 4. Effects of fillers and homogenizing temperatures
(Wieners contain 7% starch, 1% caseinate, or both)[a]

No.[b]	Filler	Temp. (°C)	Water (%)	Fat (%)	Protein (%)	Yield (%)	Stab. (%)	Text.[c] (kg)	Fat caps
1	Nil	16	63.5	22.4	10.9	132	88	1.1	Nil
5	Nil	26	64.0	21.8	11.1	133	83	1.0	+++
2	Starch	16	59.8	19.3	10.1	141	90	2.0	Nil
6	Starch	26	59.5	19.4	10.3	141	90	1.7	Nil
3	Caseinate	16	59.1	18.8	11.4	134	89	1.6	Nil
7	Caseinate	26	58.3	19.5	11.2	135	88	1.3	+
4	Starch+cas.	16	63.0	21.7	11.6	143	90	2.1	Nil
8	Starch+cas.	26	62.9	21.7	11.8	143	90	2.1	Nil

[a] Wieners were cooked to 72°C internal temperature in a 1-hour smokehouse cycle.
[b] Formulae numbers grouped in pairs, *e.g.*, 1 and 5, have the same composition but were homogenized at a different temperature.
[c] Honest significant difference (Tukey's test, Steel and Torrie, 1960) at the 95% confidence level is 0.2 kg; see footnote *c* in Table 1.

and are reported in Table 5 as percentage values of the total fat and moisture in the sampler.

Fat, protein and moisture were determined by AOAC procedures as reported by Comer *et al.* (1986).

Microscopic Analysis of Wieners

Light microscopy

The methods previously described by Comer *et al.* (1986) were used. Wiener slices (2x2x0.4 cm) were soaked for 2 h in a gum sugar solution (water: sucrose:gum acacia, 100:100:35), frozen using liquid carbon dioxide, and 30 µm sections were obtained and fixed on glass slides by immersion for 6 h in a mixture of a saturated aqueous mercuric chloride solution and ethanol (1:1, v/v). Two staining procedures were used.

Fat/protein stain: Fat was stained orange to red by immersion in a 0.06% Sudan IV (Aldrich, C.I. 26105) solution (isopropanol:water, 3:2) for 30 min. After rinsing with water for 10 min, the protein was counterstained green by immersion in a 0.5% aqueous solution of Light Green (BDH., C.I. 42095) for 20 min.

Table 5.
Effects of fillers and homogenizing temperatures on 75°C cook stability of fresh homogenates
(Wieners contain 7% starch, 1% caseinate, or both)[a]

No.[b]	Filler	Temp. (°C)	Fat stability (%)	Moisture stab. (%)
1	Nil	16	98	81
5	Nil	26	77	56
2	Starch	16	100	94
6	Starch	26	88	77
3	Caseinate	16	99	90
7	Caseinate	26	82	60
4	Starch + caseinate	16	100	95
8	Starch + caseinate	26	91	86

[a] The homogenates were cooked in a water bath in a 55 min cycle at bath temperature starting at 50°C and finishing at 75°C.
[b] Formulae numbers grouped in pairs, *e.g.*, 1 and 5, have the same composition but were homogenized at a different temperature.

Carbohydrate/protein stain: Carbohydrate was stained red by first immersion in a 2% periodic acid solution for 5 min, followed by running water (5 min), then immersion in Schiff's reagent for 5 min, as described by Disbrey and Rack (1970). The protein was counterstained with Light Green, as described above.

All photomicrographs were taken at 100 X magnification using 35 mm colour slide film (100 ASA), and the field size of the photomicrographs was 540 µm x 812 µm.

Scanning and transmission electron microscopy

The basic methods previously described by Comer *et al.* (1986) were used. All samples were fixed in a 3.5% aqueous glutaraldehyde solution. For SEM, the fixed samples were dehydrated in a graded ethanol series, defatted with chloroform, freeze-fractured after freezing in Freon 12 cooled to its freezing point with liquid nitrogen, thawed in absolute ethanol, and critical point dried from carbon dioxide. For TEM, the fixed samples were postfixed in a veronal-acetate buffered (pH 6.75) 2% osmium tetroxide solution for 18 to 24 h at 22°C, dehydrated in a graded ethanol series for 30 min per step at 22°C, and embedded in Spurr's resin (Spurr, 1969).

For comparison purposes, two modifications of the basic sample preparation method were evaluated. The first modification was done in an effort to promote optimal retention of fat in the sample. For samples destined for SEM, the preliminary glutaraldehyde fixation was followed by postfixation in a buffered osmium tetroxide solution. The samples were then dehydrated through an acetone series at 6°C as described by Carroll and Lee (1981). The defatting step using chloroform was eliminated. Samples destined for TEM were treated as in the basic method except that acetone dehydration in the cold replaced the ethanol dehydration at 22°C.

The second modification was employed to see whether a significant improvement in the quality of fixation could be achieved with a more complicated fixative. A Karnovsky-type fixative (Karnovsky, 1965), containing glutaraldehyde and paraformaldehyde and, in our case, buffered with cacodylate, was used similar to the fixatives used by other researchers in studies on muscle and meat (Carroll and Lee, 1981; Jones *et al.* 1976; Katsaras and Stenzel 1984; Katsaras *et al.*, 1984). This fixation was followed by dehydration in an ethanol series at 22°C, or by acetone dehydration as described by Carroll and Lee (1981).

Discussion of Results

The data in Table 4, obtained from samples prepared at a homogenizing temperature of 16°C are in agreement with the data from our previous study (Comer *et al.*, 1986) and show that fillers increase yield, stability, and textural firmness. A direct comparison of the data for the "all meat", *i.e.*, labelled "Nil", and starch filler batches with the data in Table 1 from our previous study shows that the same texture values, but significantly higher yields and stabilities, were obtained in the current test. Based on a comparison of the analytical data, the meat block used in this test had a higher protein content which may explain the differences. Variations in the water binding capacities of the meat ingredients may also contribute to the differences.

Homogenizing at a higher temperature (26°C) did not have a significant effect upon yield, even in the case of the control which rendered, *i.e.*, produced fat caps. We have determined yield with the casings on and, therefore, the fat caps are included. However, it is important to note that the homogenates processed at 26°C were able to retain as much water as those processed at 16°C. In addition, the textural quality of the wieners was not altered by the higher homogenizing temperature. In only two cases were the textural firmness values significantly lower, but not low enough to change the eating quality.

The expected loss in stability as shown by the presence of fat caps and lower stability value was observed for the "all meat", "Nil" control. The value of 83% stability for formula 5 is probably higher than the actual value since some fat was retained by the casing during peeling and was not included in the weight prior to cooking. The batches containing fillers, on the other hand, showed a negligible loss in stability with the exception of the one containing caseinate alone which had a few fat caps. The data in Table 4 show that the effects of higher homogenizing temperatures upon wiener yield, stability and texture are less severe for formulae containing filler ingredients.

Although we observed some decreases in wiener stability and textural firmness at the higher homogenizing temperature, some workers have previously reported considerably more severe changes. Possible reasons for this discrepancy may be that we have used a Mincemaster™ colloid mill which generally gives a more uniform homogenate than a bowl chopper; and secondly, most of the previous studies have used a model system cook test to determine stability. We have shown previously (Comer and Dempster, 1981) that model system cook tests are unreliable indicators of actual performance in wieners. For comparison with the wiener stability data, samples of the fresh homogenates were subjected to a cook stability test which is analogous to a model system cook test.

The data in Table 5 show that the model system cook test does give lower stability values than those obtained for wieners, and differences between homogenates prepared at 16°C and 26°C are much more pronounced. As for the wieners, the fillers improved the stability with the starch and the starch and caseinate combination being the most effective. Caseinate alone was able to maintain high fat stability but could not bind the water as effectively as did the starch. These data emphasize that one must be cautious in extrapolating stability data from model system cook tests to performance in wieners. In some cases, such as for the starch plus caseinate binder in this study, the stability differences shown in Table 5 were not statistically significant. The moisture losses and surface drying which occur in the smokehouse play an important role in the stability of the wieners.

To the naked eye wieners are homogenous products. The major source of nonhomogeneity is the air pockets which are more prevalent in laboratory prepared wieners than in commercial products. Even low-powered light microscopy reveals that the cooked "homogenate" has a heterogeneous microstructure. It is not possible to obtain a photomicrograph which characterizes the microstructure even at a magnification of only 30X (Kempton *et al.*,

1982). Therefore, the procedure generally employed is to scan many fields and select a "representative field". Figures 1 and 2 are examples of representative fields to compare the microstructure of wieners prepared in a silent cutter (Figure 1) with those prepared using a Mincemaster™ colloid mill (Figure 2). Colloid mills have previously been shown to produce finer homogenates than do cutters (Danchev *et al.*, 1984).

Figures 1 and 2 (Comer, unpublished) are good examples which show the range of fat globule size that is found in commercial wieners. Although Figure 1 shows a higher frequency of larger fat globules, there were no significant differences in yield, stability, or textural firmness between the two samples. Provided the fat is broken down into globules of an approximate diameter of 100 µm or less, the texture and stability are largely determined by the nonfat matrix which consists principally of meat proteins. In Canadian wieners, fillers, especially starches and flours, are generally present in the amounts of 30% to 50% of the nonfat, nonsalt solids, and therefore contribute significantly to the matrix.

When the Light Green protein stain is used, it is difficult to see microstructural changes in the matrix caused by fillers. Protein fillers tend to show up as an overall darker green matrix, whereas ungelatinized starch fillers appear as lighter coloured oval-shaped particles. We have used a carbohydrate stain to reveal the hydrated starch granules (Figures 3 and 4). Representative fields were not selected but rather two regions were selected to show high starch density in contrasting environments. In Figure 3, the starch granules form the continuous matrix as there is very little protein. Many of the voids have a globular shape and are believed to be fat particles immobilized in the starch matrix. Lewis (1981) reported a similar microstructure for British meat pastes. Hermansson (1986) also showed an example of fat droplets in a meat batter surrounded by starch particles. Figure 4 shows the starch granules embedded in a continuous protein matrix with only a few voids which may contain fat or air. During smokehouse cooking, the starch granules absorb any excess moisture not bound by the protein. The hydrated starch granules have an average diameter of about 15 µm which is one-half the thickness of the section examined. This increased diameter is achieved by the granules absorbing 2 to 4-times their weight of water (Comer, unpublished results). The granules are largely discrete and what appears to be clumping may, in part, be due to overlapping.

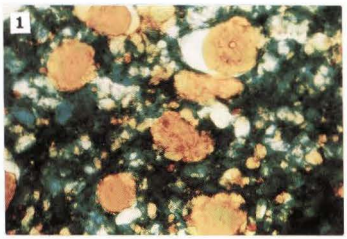
From the photomicrographs we conclude that the starch filler, like most protein fillers, contributes to stability by absorbing water, and by becoming a part of the continuous matrix which immobilizes the fat globules. The positive effects upon textural firmness cannot be directly deduced from the photomicrographs. From related studies (Comer, unpublished results) we observed that various starches and gums produce wieners with significantly different textural firmness values. Pregelatinized starches and nongelling gums produce a softer texture than hydrated but intact starch granules. The latter have an inherently firmer, nonflowing gel structure which reinforces the meat protein gel structure rather than weakening it. Therefore, we believe that gelation properties, rather than simply water binding capacity, are the primary determinant of firmness.

To examine the effects of homogenizing temperatures upon the microstructure, we found it most useful to use fat/protein staining and to select fields which have a high density of fat globules or agglomerates. Our microscopic analysis tends to support earlier conclusions that a homogenizing temperature of 26°C produces a greater incidence of fat agglomeration, and in extreme cases, the formation of fat channels (Carroll and Lee, 1981).

To illustrate the different degrees of fat agglomeration we have designated them as Types 1 to 4 as shown in Figures 5 to 8. We observed that both the nonfat solids level and the homogenizing temperature had significant effects upon the degree of fat agglomeration in the wieners. The "all meat" system homogenized at 16°C had many regions with a Type 2 pattern but some regions with both Type 1 and Type 3. The same system homogenized at 26°C had many regions with a Type 3 pattern with the occasional evidence of severe channelling, i.e. merging of fat agglomerates into continuous bands, as revealed by the Type 4 pattern in Figure 8.

The use of caseinate alone as a filler did not have a significant effect upon the fat agglomeration pattern at the 16°C treatment. However, there was no evidence of severe channelling at the 26°C treatment. The use of starch or the starch and caseinate combination had the greatest effect upon agglomeration. At the 16°C treatment, the wieners containing starch were almost free from fat agglomeration with the pattern shown in Figure 1. At the 26°C treatment regions of Type 1 and Type 2 agglomeration were observed but no evidence of channelling as shown in Figure 7 (Type 3) was observed. The effects of solids is in agreement with the observation by Lee (1985) that fat particle size increased with increased moisture content. The "all meat" control has too high a moisture content to produce a stable product with firm texture. The addition of filler solids physically stiffens the homogenate, which prevents fat agglomeration, and by binding excess moisture permits the meat proteins to form a firm gel during the smokehouse cooking.

Previous microstructural observations using electron microscopy have clearly shown that comminuted meat products such as wieners are mixtures in which the microstructural features of the individual meat protein and filler ingredients are still recognizable. Neither muscle and collagen fibers (Swasdee *et al.*, 1982) nor starch and hydrated nonmeat protein particles (Comer *et al.*, 1986) are completely destroyed by the comminuting processes. Meat fatty tissue is normally described as consisting of cells with connective tissue cell walls. The microstructure has been shown to vary from a highly regular, honeycomb pattern of cells found in soft fats (Katsaras *et al.*, 1984) to a pattern of irregular shaped cells with poorly organized collagen fibers found in highly crystalline hard fats (Lewis, 1979, 1981). During the comminution process the fat cells are separated and then broken down to release free fat which will form large amorphous regions unless emulsified or restrained in a matrix. Since the normal, regular shaped fat cell has a diameter of 70-80 µm, and since fat globules in wieners range in size from less than 20 µm to greater than 100 µm (van den Oord and Visser,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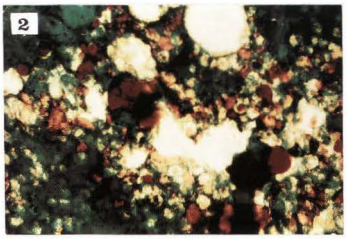
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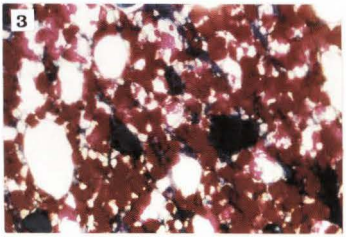
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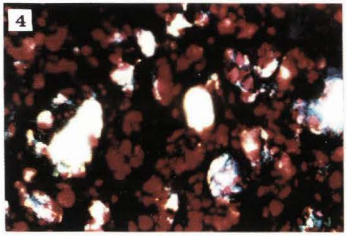
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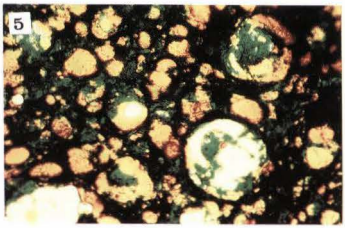
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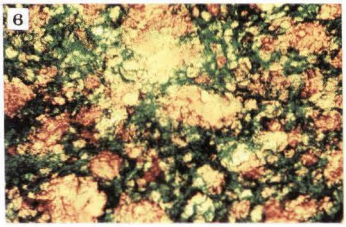
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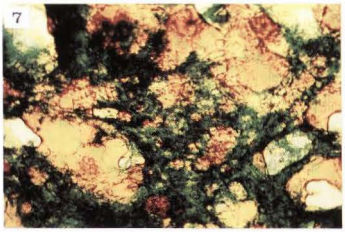
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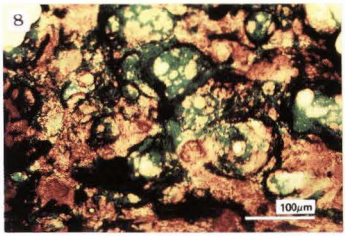
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Figure 1. Wiener from homogenate prepared in a laboratory silent cutter. Fat stained orange-red with Sudan IV; protein counterstained with Light Green. Representative field. Bar in Figure 8 represents 100 μm for Figures 1 to 8 (original magnification 100 X).
Figure 2. Commercial wiener from homogenate prepared in a Mincemaster™ colloid mill; staining as Figure 1. Representative field.
Figure 3. Wiener containing starch binder. Carbohydrate stained red with periodic acid-Schiff's reagent; protein counterstained with Light Green. Region of high starch density.
Figure 4. Wiener containing starch binder. Same staining as Figure 3. Region of high starch and high protein densities.
Figure 5. Type 1 fat agglomeration. Wiener section from formula 6. Same staining as Figure 1.
Figure 6. Type 2 fat agglomeration. Wiener section from formula 1. Same staining as Figure 1.
Figure 7. Type 3 fat agglomeration. Wiener section from formula 5. Same staining as Figure 1.
Figure 8. Type 4 fat agglomeration. Wiener section from formula 5. Same staining as Figure 1.

1973), it appears that few intact fat cells remain in the wiener. The fat cell membranes have been shown to have a lattice fibre structure by SEM (Katsaras *et al.*, 1984). However, in comminuted meat products the protein coverings may be from salt soluble myofibrillar proteins, non-meat proteins or cell membranes. It has not yet been possible to characterize the origins of the protein membranes using electron microscopy. Carroll and Lee (1981) concluded that nothing could be discerned about the protein-lipid interface in wieners using SEM. We used an acetone dehydration technique similar to the one used by Carroll and Lee (1981) to retain fat globules and obtained topographically low contrast SEM images in which the relationship between the fat globules and the protein matrix could not be easily seen.

Ethanol dehydration and chloroform defatting (without post fixation with osmium tetroxide) produced a much clearer view of the relationship between the fat and the protein matrix. The voids in the matrix left behind by the intentional extraction of fat using chloroform are believed to represent the original positions of fat globules, so a relationship between the fat globules and protein matrix could be seen.

In our comparison of sample preparation methods for electron microscopy we found no significant differences in the quality of specimen preservation between using either a glutaraldehyde solution or a Karnovsky-type fixative. Replacing ethanol with acetone in the dehydration step of the TEM sample preparation procedure produced some cases of poor embedding and poor staining. Ethanol dehydration followed by chloroform defatting was used in all the SEM micrographs shown.

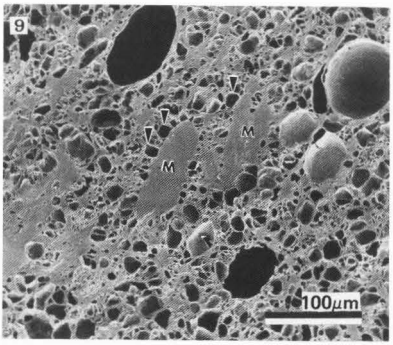
Electron microscopy of the wiener samples showed heterogeneity in the density of the protein matrix, and in the size, shape and environment of the fat globules. The protein matrix is best shown by low magnification (150-200X) SEM. Figure 9 is an example of a strong matrix where the majority of the fat globules are finely dispersed. Some large elongated pieces of muscle tissue are revealed in Figure 9. Most of the voids are believed to have contained fat prior to defatting, although some voids may be due to air or water pockets. The larger voids may be due to the presence of individual fat cells.

Figure 9 is at too low a magnification to identify starch granules in the matrix. Figures 10 and 11, however, show starch granules clearly. In Figure 10, one granule has been sliced and rests in a cavity. It has probably been shrunken during the dehydration step. The intact granule in Figure 10 is partly enclosed in muscle fibre and membrane material adjacent to a void. In Figure 11, the starch granule is in a similar environment except the membrane has taken the shape of the granule. The voids are believed to be due to fat globules.

The environments of the "fat globule" voids are more clearly shown through SEM at a higher magnification (Figures 10-12). Most of the voids are embedded in a protein matrix which varies from the spongy gel form of the salt-soluble proteins (Comer *et al.*, 1986) to pure muscle fiber. Some voids are, in part, adjacent to other voids with a membrane separating them. SEM reveals that the fat globules are not uniformly covered in membrane material but rather are dispersed in a matrix of variable protein density.

Figures 13 and 14 were taken at low magnification and show less homogeneous environments than does Figure 9, with a cluster of voids which may be due to regions of undegraded clumps of adipose tissue. The protein membrane pattern is very similar to the honeycomb patterns for adipose tissue shown by Katsaras *et al.* (1984), and is in agreement with similar observations by van den Oord and Visser (1971). We did not detect areas like this using light microscopy; instead we observed what appeared to be agglomerated fat globules. It is possible that our sample preparation methods could cause this discrepancy in observations. Figures 13 and 14 were obtained from low viscosity batters (batches 1 and 8 respectively). Perhaps one of the reasons for the observed effect of viscosity upon fat agglomeration is that the adipose tissue is not broken down as much in batters having a lower level of solids.

TEM, like LM, did reveal a greater incidence of larger, irregularly-shaped fat globules in the wieners homogenated at the higher temperature (Figures 15-17). This was especially evident in the wieners not containing starch filler. Figure 15 shows a dispersion of smaller fat globules found in batch 7 (starch and caseinate) which was the most stable of the high temperature homogenates. Figure 16, from batch 5 (no filler, 26°C), shows a large, irregularly-shaped fat agglomerate indicative of instability. In Figure 17, from batch 8 (caseinate, 26°C) the amorphous fat globule contains darkly staining material The source of this material is unknown but it could be broken protein membrane fragments. These materials were more prevalent in the batches homogenized at the higher temperatures. Like SEM, TEM revealed a diverse protein environment for the fat globules. Figures 18 and 19 show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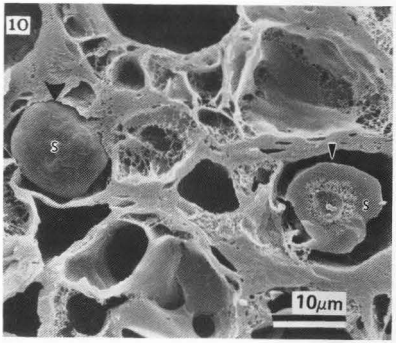
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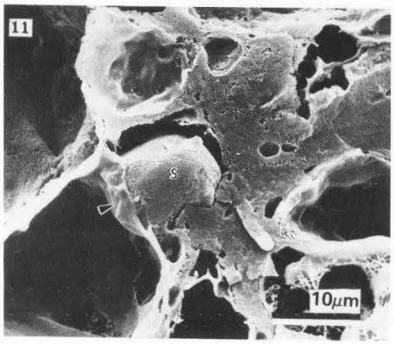
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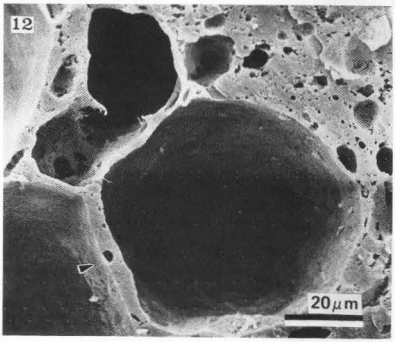
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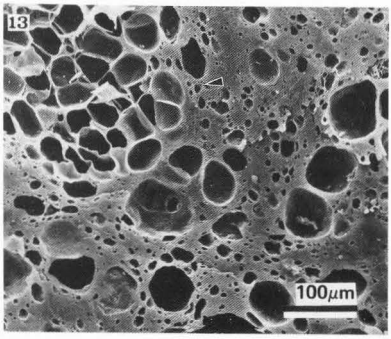
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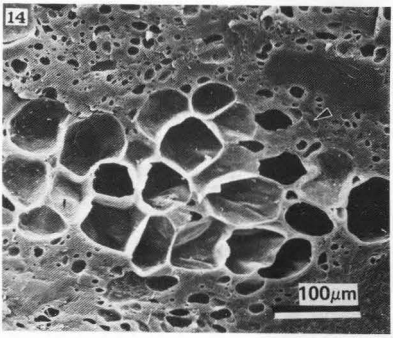
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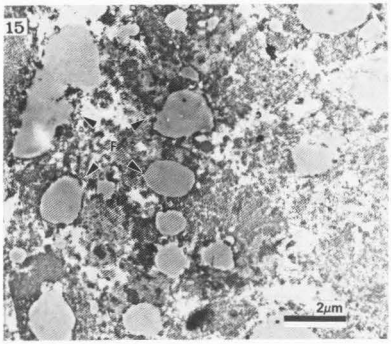
Figure 9. A protein matrix in a starch-containing wiener with fat globules finely dispersed throughout (arrows). Pieces of intact muscle (M) are also seen. (SEM).
Figure 10. Wiener sample containing starch (S). The shrunken granule (small arrow) is broken and sits in a cavity; the intact granule (large arrow) is enclosed in muscle fibre and membrane material. (SEM).
Figure 11. Wiener sample containing starch (S). In this micrograph the membrane (arrow) has taken the shape of the granule. (SEM).
Figure 12. Wiener sample showing a fat globule "void" adjacent to other voids and separated by a membrane (arrow). (SEM).
Figure 13. Sample of a low-viscosity batter wiener showing a cluster of voids (arrows) thought to be undergraded clumps of adipose tissue. (SEM).
Figure 14. Sample of a low-viscosity batter wiener homogenized at 26°C, showing a cluster of voids (arrow). (SEM).

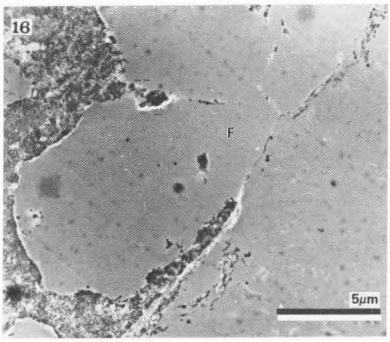
fat globules which are respectively embedded in a complete and partial protein covering. Figure 20 shows a fat globule, almost entirely coated by a membrane of variable thickness positioned in a region of low protein density.

Starch granules were also found in different environments. A characteristic feature of the starch granules is that their shape is distorted as shown in Figure 21. It is likely that the granules are misshapen during the dehydration step of the sample preparation procedure for TEM (Comer *et al.*, 1986). In Figure 21, a small fat droplet is shown adjacent to the starch granule. This illustrates how the starch granules contribute to wiener stability by physically constraining the movement of the fat droplets.

Figure 22 brings us, so to speak, full circle. At first glance, Figure 22 appears to have the same structure as undegraded adipose tissue; however, the fat droplets in Figure 22 are at least two orders of magnitude smaller than are the fat cells of adipose tissue. Figure 22 shows a region of a true emulsion with a fine dispersion of fat globules surrounded by meat protein membranes, as first described by Hansen (1960), using photomicrographs of model meat systems. However, in our observations it was a rare occurrence to find regions of emulsion as shown in Figure 22.

As mentioned previously, we can only speculate on the origins of the protein membranes surrounding the fat globules since the protein staining is





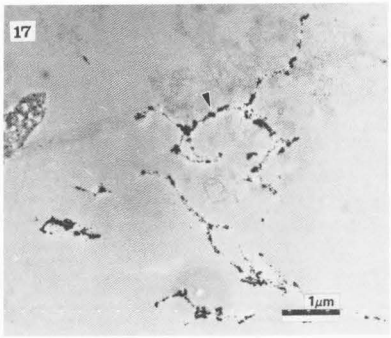


Figure 15. Wiener sample containing both starch and caseinate, which has been homogenized at 26°C shows a dispersion of small fat globules (F). (TEM).
Figures 16. Wiener containing no fillers, homogenized at 26°C. A large, irregularly shaped fat agglomeration (F) is seen, which is indicative of instability. (TEM).
Figure 17. Wiener made with caseinate and homogenized at 26°C. A region of amorphous fat which contains darkly-staining material (arrow) is shown. (TEM).

nonspecific. Both SEM and TEM evidence suggests, however, that the role of membranes in homogenate stability is secondary to that of the continuous matrix. A strong matrix is required for high stability.

In our experiments to evaluate the effects of fillers, we have shown that both starch and protein fillers do strengthen the matrix leading to increased stability and firmness. Homogenizing the batters at higher temperatures (26°C) does not produce a drastic change in stability or texture when fillers are present. From our examination of the microstructure, we conclude that these effects of the fillers are the result of reduced fat agglomeration and a more dense matrix.

Concluding Remarks

As meat prices continue to rise, the economic incentive to use fillers in comminuted meat products increases. Canadian regulations (Agriculture Canada, 1985) offer no restrictions on the use of ingredients as fillers in comminuted meat products, provided minimum meat protein (9.5%) and total protein (11%) requirements are met. Regulatory and economic factors encourage the development of cost effective "binders", i.e., blends of fillers. Experiments to evaluate the functional effects of "binders" are carried out in production size batches by manufacturers of comminuted meat products. Ultimately, this is the only way that the effectiveness of a binder can be determined.

Research experiments, particularly those involving the preparation of small scale batches of wieners, have put a great deal more science into the "art of sausage making" than existed a few decades ago. The model of comminuted meat products as mechanical mixture of ingredients held in a gel matrix, which has resulted from experiments on the functional effects of meat and filler ingredients is proving to be more useful than the simpler emulsion model. Research emphasis is now shifting to the interactions of ingredients, particularly as they relate to gelation properties. The simplicity of model systems and functional property tests is often irresistible, but the data obtained may be of limited value. The functional effects of fillers in comminuted meat products relate to stability and textural improvements. Because these effects are dependent upon the composition of the meat system, it is necessary to design experiments which involve evaluation of ingredients in the actual meat products.

The principal functional fillers are polysaccharides, especially starches, and proteins, i.e. ingredients which have the ability to hold moisture and/or fat in a gel structure after the heat treatment used in processing. The tolerance of a meat system for a filler ingredient is limited by either competition for moisture or a disruption of the meat gel structure. Provided the tolerance limit is not exceeded, then fillers added to a comminuted meat system will generally increase both stability and textural firmness.

Microscopy has had a relatively small influence upon the development of "binders" for comminuted meat products. The contribution of microscopy can best be described as substantiative, since it has often been used in a secondary role to support one theory or another. The data, when taken collectively, however, are unambiguous. Comminuted meat products are mechanical mixtures of fat globules and filler components held in a heat-set gel composed principally of hydrated meat proteins.

The value of microscopy in detecting filler ingredients and in determining their effects in comminuted meat systems will be increased by developing methods which eliminate artefacts and which reveal the fine structure of component interfaces more clearly. The protein and fat staining procedures used in light microscopy obscure fine structural features such as protein membranes. Nonstaining procedures, such as using polarized light, which has been used to reveal collagen membranes in adipose tissue (Lewis, 1979), and selective staining procedures may reveal more detail than previously reported. Comminuted meat products are complex heterogeneous products. The amount of structural detail which has been obtained from SEM and TEM work done to date is overwhelming and may be confused by artefacts.

Time should be spent in documenting these artefacts. Another approach, which may prove to be worthwhile, is to "map" microscopic regions of about 2 mm^2 rather than to randomly scan a particle to obtain "characteristic features". The collection and interpretation of electron microscopy data is a time-consuming process; the practical benefits are not always apparent. However, only through the scrutiny of electron microscopic data can we understand the functional effects of filers in comminuted meat products.

Acknowledgments

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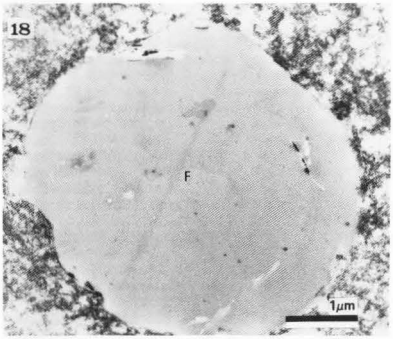

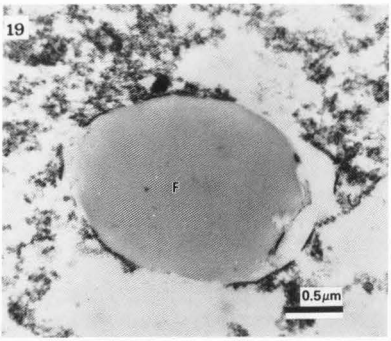

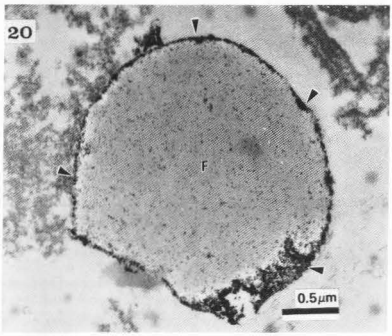

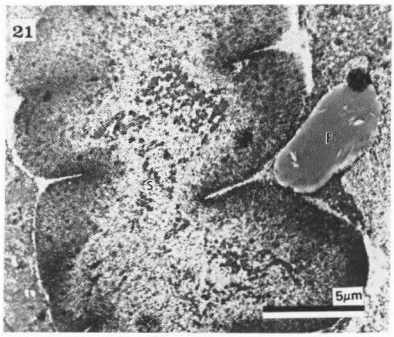

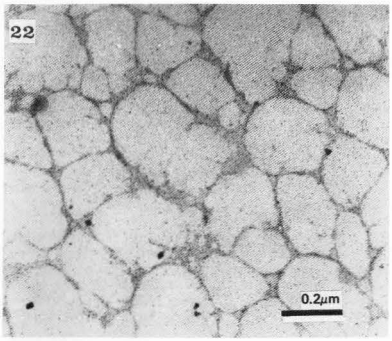

Figure 18. A fat globule (F) is shown, which is completely surrounded by protein. (TEM)
Figure 19. A fat globule (F) which has only a partial protein covering is shown. (TEM).
Figure 20. A membrane (arrows) of variable thickness almost completely coats a fat globule (F) in a region of low protein density. The dark "spots" on the globule result from difficulties encountered at the staining step in this particular sample. (TEM).
Figure 21. A small fat globule (F) is shown adjacent to a starch granule (S), illustrating how starch granules may physically restrain the fat. (TEM).
Figure 22. A rare "true emulsion" area, which has a fine dispersion of fat globules surrounded by protein membranes. (TEM).

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Discussion with Reviewers

G. R. Schmidt: Would it have been useful to measure the pH's of the raw batter and finished products in treatments 1 through 8 in the experiments reported in tables 3 through 5? The various fillers may have modified the pH or, as the authors suggest, the storage of the meat block may have modified the pH. Do the authors feel that this could have happened?
Authors: pH values were not determined. However, we do not believe that the fillers would have changed the pH significantly. The modified starch used has pH 6.0 and the caseinate has a pH of 6.5. The pH of the batters should be in the range of 6.0 to 6.4.

G. R. Schmidt: Do the authors feel that the reason that they did not get as great an effect due to increasing chopping temperature was due to the fact that they are at a much lower fat percentage, *i.e.*, 20% rather than the 30% often used by other researchers? Or perhaps, did the authors use a smokehouse schedule that is much less conducive to cook loss than the model system?
C. Lee: Could the differences in cook stabilities between the wieners and cooked homogenates be due to differences in heating rate?
Authors: We selected a heating cycle for our "model system" which approximates the heating rate achieved in our smokehouse schedule for cooking wieners. The results convince us that our smokehouse schedule is indeed much less conducive to cook loss than "model systems". Since fat agglomeration is a major cause of instability, it is also likely that higher fat percentages would magnify the differences.

C. Lee: How do you rationalize using a water temperature of 40°C for preparation of the meat emulsion? Would this temperature be too high to keep the functionality of myosin?
Authors: The thermal transitions for myosin are above 40°C. Immediately after the water was added the mixture was comminuted to the temperatures of 16°C or 26°C. Warm water is often used in industry to raise the temperature of cold meat blends. Based on the results, we feel there is a negligible effect upon myosin functionality from using 40°C water.

A. M. Hermansson: It is not generally agreed upon that gelation of myosin alone determines the textural properties of comminuted meat products. The solubility of myosin is less than one percent by weight in comminuted meat systems before heating. Why do you stress the importance of gelation?
C. Lee: Have you developed quantitative data on gelation properties?
Authors: We have not tried to develop functional property tests based on gelation phenomena. Gelation mechanisms involve not only the heat setting of soluble proteins and gums, but also the heat-induced swelling of proteins and starch granules. The available moisture in the system has a tremendous effect on the firmness of gels and, indeed, whether a gel will be formed. We believe gelation tests involving excess moisture may have limited value in predicting functional effects.

A. M. Hermansson: You state that the muscle fibers are not destroyed. How can this be if protein gelation is responsible for the network structure and the texture as you claim? The statement is also contradictory to the micrographs shown.
Authors: During the comminution process, the muscle fibers are broken down but not completely destroyed. Figure 9 shows muscle fibers. We were also able to detect heart muscle. Comments on these observations were made to support the description of wieners as mechanical mixtures rather than as emulsions. Complete destruction of the muscle fibers is obviously not required for the formation of a gel. However, a high degree of comminution is required for the solubilization and swelling mechanisms mentioned in our previous comments on gelation.

C. Lee: How do you justify the statements "...good dispersion may lead to interactions"; "...interaction with soluble proteins may result in soft texture"; and "...the incorporation of non-meat proteins may result in a soft texture because their gels are softer than myosin gel"?
Authors: Protein-protein interactions in food systems need to be studied further. However, the fact that soluble proteins interact has been well-established in model systems. We have not carried out model system experiments, but since we did observe a change in the micelle structure of milk proteins in a meat system, we have further speculated that caseinate may interact with soluble meat proteins in the system. We are not aware of gel systems involving non-meat proteins, or blends of non-meat proteins and myosin, which produce the springy gel structure of myosin. Our comments perhaps form the basis of an hypothesis which should stimulate further experimentation.

D. F. Lewis: Soy protein isolates often do not disperse very well when present at the high concentrations and salt contents found in meat products. How do you think that this affects their performance?
Authors: One of the authors (Comer, unpublished) has shown that wieners containing native, dispersible pea protein are much softer than wieners containing heat-denatured pea proteins. Low dispersibility is probably advantageous to the performance of soy protein isolates in wieners, since the dispersible soy proteins are not completely gelled at the cooking temperatures used.

D. F. Lewis: Have you considered the possibility of more than one continuous matrix within a product?
Authors: At high levels of filler, there may be regions where the filler forms a continuous matrix, *e.g.*, Figure 3 shows a "starch matrix". However, once the matrix of meat proteins is severely disrupted by fat, carbohydrates, or non-meat proteins, then textural firmness decreases rapidly. Wieners have essentially one major continuous matrix, but for extended meat pastes it is possible that non-meat matrices may have a larger presence.

C. Lee: You state that starch granules were discrete and several possessed birefringence because of the limited moisture environment in the comminuted meat products. I agree with the effect upon birefringence but how do you justify the effect upon the discrete nature of the granules? Starch granules can merge in water but I believe in the meat system the protein gel would restrict their movement and prevent merging.
Authors: The protein matrix does not prevent the merging of granules as shown in our previous publication (Comer *et al.*, 1986). If there is sufficient moisture available the granules will rupture and the starch will flow. At a 7% starch level, there are many starch granules within close proximity as shown in Figures 3 and 4. The merging of starch granules is analogous to the process of fat agglomeration; but as we have shown, it does not generally occur in meat products because of limited moisture availability.

C. Lee: The authors state that "Figures 10 and 11 show clearly starch granules." How can you prove that they are indeed starch granules? Provided that this sample also contains fat, how can one differentiate starch from fat?
Authors: The starch granules are identified mainly by their shape and size (Swinkles, 1985). They are differentiated from fat through the selective extraction of fat from the samples (using chloroform) that is part of our preparatory process. Simply put, the fat is extracted; the starch is not.

A. M. Hermansson: Figure 22 is said to show the characteristic structure of a starch granule. Are there any other characteristic features of the structure apart from the shape?
Authors: Shape is the first quality that is readily apparent in micrographs. The starch has a characteristic appearance after it has been prepared for TEM (Gallant and Guilbot, 1971). In addition, size is also taken into consideration (Swinkles, 1985). When these factors are considered together, we feel that starch can be identified realiably.

C. Lee: What are the white voids in Figure 2? They appear to be places where fat globules have been removed as a result of preparation error.
Authors: The large voids are believed to be air pockets. We have observed that our laboratory-prepared wieners do have some large air pockets which are clearly visible in the sections.

D. F. Lewis: There is some scope for fat mobility during staining with solubility-based oil dyes. Staining with osmium tetroxide vapour or solution is, in my view, a better fat stain. Please comment.
Authors: Ray *et al.* (1981) have successfully used osmium tetroxide to stain fat in LM sections. It is a method which gives informative results and should be included as a standard LM staining technique for the study of comminuted meat products.

C. Lee: In the photomicrographs, I find it difficult to evaluate the fat dispersion. This is, in part, a resolution problem, but also I believe the field of magnification was not adequate. Please comment on your choice of conditions.
A. M. Hermansson: I believe the quality of the photomicrographs could have been improved by using thinner sections to see finer details. Please comment.
Authors: Our conditions for light microscopy were determined by reviewing the literature and selecting staining techniques which gave good contrast. Sections from 15 µm to 30 µm thickness were evaluated and we did not observe a large enough improvement in detail to cope with the greater difficulties in obtaining and handling the thinner sections. Selecting a magnification level involves compromises. Lower magnification levels provide a more representative view of the microstructure but the fat and starch are more difficult to see. Higher magnification levels provide more detail but the representative view is lost.

A. M. Hermansson: The most serious criticism to this paper is with respect to the SEM micrographs where the fat has been extracted. The discussion of fat globules should be omitted and discussion restricted to features seen in the micrographs. Voids can be due to entrapped air and created during processing, or preparation of the samples for microscopy. Please comment on your approach to the problem.
Authors: The fat has been intentionally removed for the purpose of illustrating specific points about the protein matrix of the samples. Other authors have used a defatting step such as the one we have used to clarify certain structures in their samples (Ray *et al.*, 1979; Ray *et al.*, 1981; Katsaras and Stenzel, 1983; Katsaras *et al.*, 1984). Ray *et al.* (1979, 1981) have discussed the limited use of SEM in the study of comminuted meat products, mainly because of the absence of easily recognizable structures in the micrographs. They have suggested a method where useful information can be obtained from SEM micrographs by doing histology (specific staining and examination in the LM) on the same samples. They were also questioned about identity of the voids shown in their micrographs; they suggested examining meat emulsions of the same composition but mixed using different methods. Katsaras *et al.* (1984) used a defatting step to expose the honeycomb structure of collagenous materials in adipose tissue. Katsaras and Stenzel, (1983) used defatting to illustrate the protein structure of their wiener samples. They also showed techniques to retain fat, as well as cryo-SEM to retain all the components. They discussed how the use of SEM is very limited in the study of comminuted meat products. Sample preparation is not easy, and interpretation of the resulting micrographs is difficult since the structure of muscle and fat tissue is lost. Because of the problems inherent in using SEM as a method of study for meat emulsions, our discussion on fat globules has been kept brief.

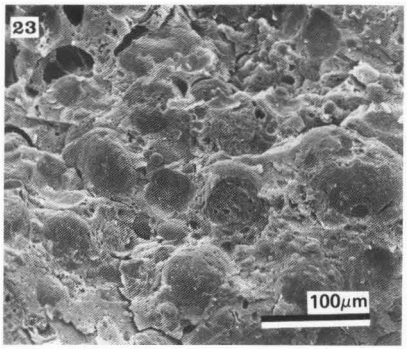
A. M. Hermansson: Was there any shrinkage or deformation of the samples after the preparation for SEM?
Authors: The samples prepared for SEM were dehydrated and critical-point dried according to standard procedures. It is well known that there is a certain amount of shrinkage and deformation which occurs in the sample as a result of these preparatory steps (Cohen, 1979; Boyde, 1978; Boyde and

Maconnachie, 1981). Boyde and Maconnachie (1981) have discussed this problem at length, and have concluded that critical-point drying (and freeze drying) of fixed samples give perfectly acceptable results for SEM. The shrinkage/deformation of our samples was not quantified.

D. F. Lewis: Regarding Figures 18 and 19, is the membrane changed after processing?
Authors: Most likely, processing would cause some changes in the membrane. However, a separate study would be necessary to investigate this phenomenon.

A. M. Hermansson: Is there any evidence that the darkly stained material surrounding fat globules is pure protein?
Authors: The stains that have been used to impart contrast to the sections destined for TEM are the standard stains, uranyl acetate and lead citrate. These are not specific for protein, but are general stains. Carroll and Lee (1981) and Borchert *et al.* (1967) have also observed the electron-dense material surrounding fat globules under conditions of preparation similar to ours. Stanley *et al.* (1977) have also observed material in their bacon samples with apparently the same staining characteristics; they speculate that the material is phospholipid in nature. Dutson (1974) has suggested that specific staining can be accomplished (in muscle tissue) by using specific electron-dense enzyme substrates for enzyme histochemistry, and for specific tissue components such as mucopolysaccharides. This is an interesting approach and should be explored in the area of comminuted meat products.

A. M. Hermansson: Comminuted meat systems are difficult to handle and prepare for microscopy. Conventional techniques for EM may produce artefacts, especially with regard to the structure of fat and starch distributed in the meat structure. What are the authors' opinions about the preparation techniques used in this study?
Authors: Comminuted meat systems have as their main components protein (in the form of muscle), fat, starch, protein additives (such as casein), water and air. Protein can be fixed using aldehydes (glutaraldehyde and/or formaldehyde). Only unsaturated fat can be fixed with osmium tetroxide. Starch cannot be properly fixed with either of the standard methods (Kaláb, 1984) and water is lost through the standard dehydration techniques required to ready the sample for the in vacuo conditions found in the SEM. The work discussed in this paper concentrates on observations of the protein matrix, and so the fat was extracted using chloroform. Information is gained through the use of the preferential extraction step; it was possible to differentiate starch (which was not extracted) from fat (which was extracted). Also, areas of undegraded adipose tissue (see Figures 13 and 14) became obvious after the extraction. Ideally, especially because of the known artefacts which can be associated with different methods of preparation, a number of different types of fixation/preparation conditions should be tried on the samples, since each technique will provide its own type of information. As mentioned in the review, we had done, at the outset, a fixation which allowed fat to be preserved *in situ* (see Figure 23). From analyses of micrographs obtained from samples such as these, very little can be said about the protein matrix, since it is obscured by the retained fat, which cannot be differentiated from starch. Nothing definite can be said about the origin of the voids, which may be due to either water or air in samples prepared in this manner. In cold-stage SEM, where fixation is a physical rather than a chemical phenomenon, and no components of the samples are lost, even less can be said about the microstructure of the sample (see Figure 24). The protein matrix is rather featureless and globules of unknown identity (fat, water, starch) can be seen.



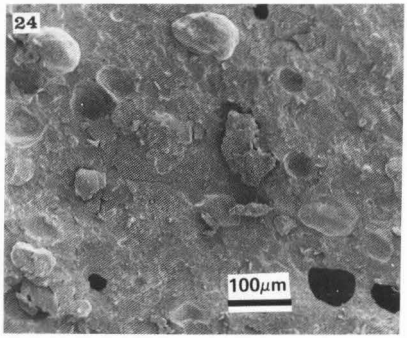


Figure 23. Conventional SEM of a wiener which has been prepared to retain fat in situ. A low-contrast, topographically flat image results in which no positive identification of components can be made. The voids are due to air or removed water.
Figure 24. Cryo-SEM of a wiener in which all components have been retained. The protein matrix appears featureless and globules of unknown identity can be seen. The voids are due to air.

The only information which can be gleaned from a sample prepared in this manner is the identification of the voids due to air. We feel, therefore, that the methods that we have chosen to prepare the samples for conventional SEM are reasonable and provide the illustration of aspects which we have discussed. LM and TEM, which have been done in conjunction with SEM, provide information, respectively, on voids due to air, and the appearance of fat *in situ* and the interface between fat and protein components.

A. M. Hermansson: References are given to a lot of published work without many comments on their quality or expertise with regard to microscopy. Artefact always needs to be taken into account and some analysis of advantages and disadvantages of preparation techniques would improve the paper. Please comment.

Authors: Lee (1985), in his review, has already dealt with this matter, comparing the techniques which have been used for microscopy to study meat emulsions (LM, TEM, SEM). At that time and since then, workers in Germany have carried out microscopy using different preparatory methods for SEM and TEM (Katsaras and Stenzel, 1983; Oelker, 1988, 1987, 1986). There are a number of methods available now which can be selected for different tasks (such as showing different relationships). All, however, have artefacts associated with them. These artefacts arise from the different fixation and fracturing methods which are used, as well as methods which only partially fix or retain fat. These artefacts lead to confusion when one wants to differentiate water from air voids, and other such problems. Because so many techniques are now available for studying comminuted meat products, a thorough review of them is beyond the scope of this paper. Dealing with this problem could provide the substance for a review paper which would discuss only the development and effective use of such techniques in this field.

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THE RETENTION OF CONTRACTILITY OF RABBIT MYOFIBRILS DURING STORAGE AT 25°C

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Abstract

During postmortem storage of muscle at 25°C, myofibrils retained the ability to contract for more than 400 hours under experimental conditions used to prevent bacterial contamination of the meat surface. During this time, rapid breakdown of troponin T was observed. Storage beyond 500 hours resulted in rapid loss of contractility correlating with proteolysis of the heavy chain of myosin, and in this stage some granules were observed along the whole A-band within the sarcomere.

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Introduction

It has not been clearly defined how long the biological activity of muscle can be retained during postmortem storage. Sung et al. (1976) reported that the contractility of porcine skeletal myofibrils measured at 24 h postmortem depends on the pH of muscle and indicated that the myofibrils from muscles whose pH was 5.45 or above exhibited 100% contractility. Addition of Mg^{2+}-ATP to intact myofibrils brings about their contraction, resulting in the formation of the so-called contraction bands (Hanson, 1952, Muguruma et al., 1980).

The present study was conducted to clarify the changes in contractile function of myofibrils during prolonged postmortem storage at room temperature under experimental conditions designed to prevent degradation by bacterial proteases.

Materials and Methods

Materials

Rabbits were euthanized with sodium pentobarbital (90 mg) prior to exsanguination, and the carcasses were soaked in 50 mM NaN_3 solution for a few seconds to prevent bacterial growth on the surface. Three replications with a total of 15 rabbits were used for the present experiment. Fresh longissimus thoracis muscles were cut approximately 2.5 cm thick and 5 cm in length, soaked in 50 mM NaN_3, then individual pieces were wrapped separately in a double layer of polyethylene bags, with toluene between the layers to prevent penetration of the inner polyethylene layer surrounding the meat by exogenous bacteria, and stored at 25°C.

Contractility of myofibrils

The longissimus thoracis muscles were minced with scissors. Approximately 0.2 g minced muscle was homogenized in 20 ml of 0.1 M KCl, 0.039 M borate buffer solution (pH 7.0) with a Waring blender for 30 sec. A drop of the muscle suspension was placed on a glass slide and then a

cover glass placed on it. Thereafter, a drop of Mg^{2+}-ATP solution (1 mM $MgCl_2$, 1 mM ATP, 10 mM Tris-maleate, pH 7.0) was placed at one edge of a cover glass. The appearance of myofibrils in the suspension before and after the addition of the Mg^{2+}-ATP solution was observed at about 20°C with a phase-contrast microscope (Olympus microscope FHT, Olympus Ltd). The number of contracted and uncontracted myofibrils in the suspension after the addition of Mg^{2+}-ATP solution were counted. Contractility of myofibrils in the muscle suspension was expressed as percentage of the contracted myofibrils to the total myofibrils counted in six different experiments. In each case, more than 200 myofibrils were counted.

pH determinations

Minced muscle (3 g) was homogenized in 10 ml of distilled water with a Waring blender for 3 min. The pH of the muscle homogenate was determined with a Hitachi-Horiba pH meter at 20°C.

Protein concentrations

Protein concentrations were determined by the biuret method (Gornall et al., 1949).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on gradient slab gel (7.5-20% acrylamide) at 30 mA employing the discontinuous buffer system of Laemmli (1970).

Electron microscopy

Double fixation in 3% glutaraldehyde for 1 hr and 1.3% osmium tetroxide for 2 hr was followed by dehydration through graded ethanols (50%, 75%, then 100% 3 times), alcohol was replaced with propylene oxide, and the samples were embedded in an epon mixture. Thin sections, approximately 60 nm thick, were stained with uranyl acetate and lead citrate according to the procedure of Reynolds (1963). Specimens were examined with a Hitachi H-300S electron microscope operated at 75 kV.

Results and Discussion

The pH of muscle, 6.8-7.0 antemortem, remained at the minimum value of 5.6 for a relatively long period during postmortem storage, approached the neutral pH region at 400 h storage, and finally neared pH 8.0 at 600 h storage. These pH changes follow the usual pattern observed in muscle that shows normal rigor mortis (Bendall, 1973).

The contractility of myofibrils was almost constant from the time of death up to around 400 h postmortem, but was rapidly lost after 584 h (see Fig. 1).

Fig. 2 shows the effect of storage time on the electrophoretic pattern of myofibrillar proteins. A 30,000-dalton component, presumably due to the degradation of troponin T (Olson et al., 1977) was observed after 30 h storage.

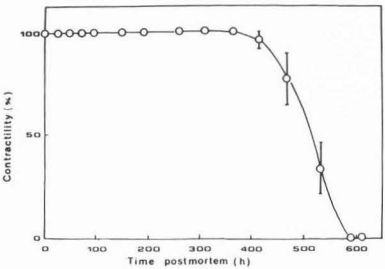

Fig. 1. Contractility of myofibrils prepared from fresh muscle and from muscle stored at 25°C. The contractility was expressed as percentage of myofibrils forming contraction bands, including single sarcomeres lacking H-zone, relative to the total myofibrils observed after addition of Mg^{2+}-ATP solution (1 mM $MgCl_2$, 1 mM ATP and 10 mM Tris-maleate, pH 7.0) under the phase contrast microscope. In each case, about 500 sarcomeres representing 200 myofibrils were evaluated. Each value is the mean SD of six preparations from individual rabbits.

This component was often degradated further, and therefore the gel band was not observed in muscle after 150 hrs storage. Degradation products having molecular weights of 130,000-150,000 daltons appeared after 408 h storage concurrent with the decrease of myosin heavy chain (Fig. 3). This degradation can be attributed to endogenous proteases, because bacterial contamination was prevented. After 584 h of postmortem storage, the myosin heavy chain could not be observed on the gel. A loss of contractility accompanied the disappearance of myosin heavy chain. The other major component of myofibrils, actin, was degraded only after almost 584 h of postmortem storage. Tropomyosin appeared more stable, than α-actinin. M-line protein and C-protein could not be determined, because the molecular weights of the myosin heavy chain breakdown products were very close to the molecular weights of the former proteins.

Under the electron microscope, myofibrils prepared from fresh muscle showed the typical sarcomere pattern of interdigitating thick and thin myofilaments (Yamaguchi et al., 1985), whereas myofibrils prepared from muscle stored for 584 h at 25°C seemed to have lost the regular arrangement of filaments. Fig. 4 shows a single sarcomere obtained from muscle stored for 584 h at 25°C. The thick filaments were not distinct but appeared to be associated with the thin filaments. The

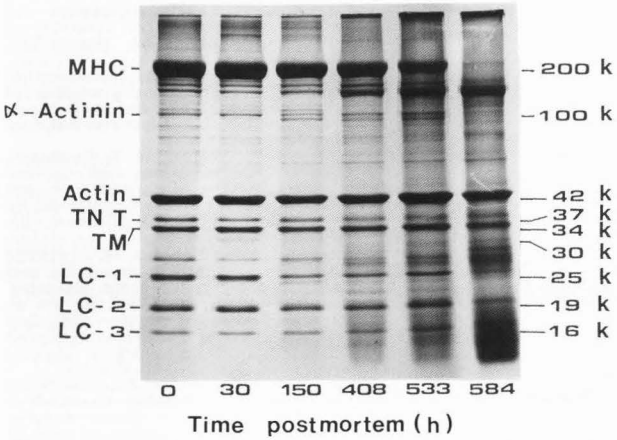

Fig. 2. Effect of storage on the electrophoretic pattern of myofibrils. Myofibrillar suspensions (5 mg/ml protein) were diluted with equal volumes of 0.5 M Tris-HCl, pH 6.8, 1% SDS, 30% glycerol, 1% β-mercaptoethanol, 0.01% bromophenol blue and boiled for 3 min. Aliquots (70 ug protein) were subjected to SDS-gradient PAGE (7.5-20% linear acrylamide gradient). The number under each gel indicates postmortem storage time. MHC, myosin heavy chain; Tn-T, troponin-T; TM, tropomyosin; LC, myosin light chain.

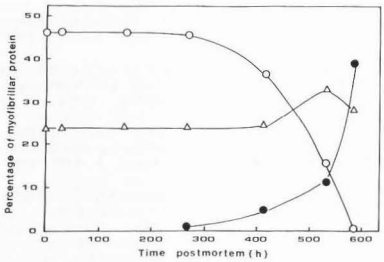

Fig. 3. Amount of myosin heavy chain, actin and 130,000-150,000 dalton component during postmortem storage. Coomassie blue-stained gels were scanned using an LKB 2202 Ultroscan Laser Densitometer. The amounts of the proteins were estimated from the areas of peaks in the densitograms. Myosin heavy chain (O), actin (Δ), 130,000-150,000 dalton component (●).

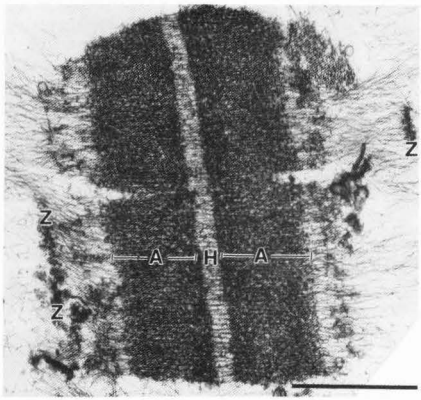

Fig. 4. Electron micrograph of a single sarcomere obtained from muscle stored at 25°C for 584 h. The scale indicates 1 um. A; A-band, H; H-zone, Z; Z-line.

myosin molecules may have been degraded already at this stage, as shown in Fig. 2, but perhaps some heavy meromyosin degradation products remained within the sarcomeres. Some granules were observed throughout the A-band after 584 h of storage.

Recently, Bechtel and Parrish (1983) reported that the myosin heavy chain was degraded to a series of polypeptides having molecular weights between 145,000 and 125,000 when the muscles were stored at 37°C. for 7 days. Ikeuchi et al., (1980) also demonstrated some degradation of rabbit skeletal muscle myosin during storage for up to 12 h at 37°C. A number of studies of the structural changes in myofibrillar

protein during postmortem storage have been performed (Arakawa et al., 1976; Olson et al., 1977; Ikeuchi et al., 1980; Bechtel & Parrish, 1983). The structural changes observed in various studies may be due to endogenous proteinases, including the calcium-activated proteinase (Reddy et al., 1975; Dayton et al., 1976; Ishiura et al., 1979), various catheptic proteinases (Schwartz & Bird, 1977; Matsukura et al., 1981; Zeece et al., 1986) and serine proteinases (Yasogawa et al., 1978; Murakami & Uchida, 1979).

Our results show that myofibrils retain the contractile function for a long period at 25°C in spite of partial degradation of myosin heavy chains. Further studies on chemical changes in myosin molecules of myofibrils during long-term storage are in progress.

Acknowledgements

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Discussion with Reviewers

C. A. Voyle: Does loss of contractility precede the loss of myosin heavy chains?
Authors: Several of our results indicate that degradation of myosin heavy chain is often preceded by the loss of myofibril contractility.

P. J. Bechtel: How does change of pH correlate with protein breakdown?
Authors: The pH of muscle was measured at pH 6.9-7.0 at death and then the pH dropped to 5.5-5.6 after 24 hours. The muscle maintained a pH of 5.5-5.6 for a prolonged period of about 3 weeks until myosin heavy chain started degradation. Then the pH of muscle gradually increased up to pH 7.0, although there are some variations among individual rabbits. (See Winger et al., 1979)

P. J. Bechtel: On average, how long after death were the muscles excised?
Authors: Immediately after death the whole carcass was cooled by ice and after a 15-20 min period from the time of death, the muscles were excised.

P. J. Bechtel: What do the electron micrographs from the other storage days reveal?
Authors: Unfortunately we have not yet examined any of the electron micrographs from other storage days, however we predict that there was some occurrence of structural changes prior to the electron micrograph shown here.

P. J. Bechtel: Would you speculate on why muscle proteins are not rapidly degraded at temperature below 37°C?
Authors: We have performed some experiments at 15 and 20°C in which the muscle retained contractility for more than two months. However, we have not conducted the experiment at temperatures between 25 and 37°C. It may be possible then, that the observed phenomenon is related to enzyme reactivity, which often shows a rapid increase in activity above certain temperatures.

P. J. Bechtel: How stable are the different muscle proteolytic enzymes under the conditions used in these experiments?
Authors: We have no idea of the stability of other enzymes under these conditions, however, we feel that we will obtain similar results using mammalian skeletal muscle.

S. H. Cohen: Results shown in Fig. 2 do not appear to support breakdown of TN-T to a 30K dalton fragment which increases in context after 30 hours of storage. Although the 30K dalton fragment is seen after 30 hours, I would have expected to see it at least as intense after 150 hrs if this fragment is from TN-T as reported by Olson et al. (1977) Will the authors please comment?
Authors: We assume that a 25K component, after 150 hrs of storage, may be the result of the breakdown of the components of the 30K dalton fragment from TN-T. We understand the comments noted by Dr. Bechtel and Dr. Cohen, however, TN-T seems to be the initial protein targeted by endogenous protease after death. Although the specific sample shown in Fig. 2 did not indicate a clear breakdown, we often observe a complete disappearance of TN-T. A complete disappearance may often be indistinguishable from other breakdown products of higher molecular weight proteins.

S. H. Cohen: In a paper by Robbins et al. (1979) the authors described SDS gel electrophoresis of bovine myofibrils and show the breakdown of the major myofibrillar components. How do these results compare to those in your results?
Authors: Any enzymes obtained from spleen are often contaminated with a countless number of proteases. In our experience, even purified DN-ase I from spleen contains several kinds of protease. Therefore, it is often difficult to verify the activity of only a specific type of enzyme as in the case of Robbins et al. Some serine proteases are active at a neutral pH and digest myosin heavy chains and also possibly the light chains. Myosin heavy chain seems to be sensitive to a serine type of enzyme, whether the optimum pH is acidic (as in Robbin's experiments) or neutral (as in our experiments) with the presence of multiple proteases during a period of long storage. We feel that in the Robbins experiments the possible linker protein between α-actinin and actin of the Z-line was probably digested by cathepsin D and other proteases. Thus Z-line was removed while α-actinin and actin were not affected. We cannot give a conclusive explanation for our results, however, the partial degradation of α-actinin was most likely due to the combined effect of several proteases existing in muscle because the myofibril was not isolated from the muscle during treatment.

D. E. Goll: The authors are somewhat vague in the last paragraph about what causes the chemical changes that occur during long-term postmortem storage in the myosin molecules. The SDS-polyacrylamide gels clearly show that the polypeptide chains of the myosin molecule (the heavy chains) are disappearing. Do the authors think that something besides or in addition to proteolysis is causing this change?
Authors: We do not have a definite answer for this question, however, it is possible that some organic elements may become radical due to protein breakdown. The increase in radicals may accelerate the

degradation of proteins.

D. E. Goll: There is a progressive increase in the protein band at the very top of the SDS-polyacrylamide gels with increasing time of postmortem storage. Titin and nebulin, two proteins that might be expected to be located at the top of these polyacrylamide gels, are very labile to proteolysis and have been reported to be degraded during postmortem storage. What explanation do the authors have for the increase in this protein band at the top of the SDS-polyacrylamide gels?
Authors: During the SDS gel preparation, we used a 4.5% stacking gel, which is not shown in figure 2. In the stacking gel, we noticed that there was some trapping of proteins in the stacking gel of the samples with a storage time of up to 150 hrs. The increase of the protein at the top of the gel is probably an increase in fragmented components of the high molecular weight proteins, titin and nebulin, which as you mentioned, are reportedly degraded during postmortem storage. In addition, it is possible that the high molecular weight of membrane associated and/or structural proteins may be degraded during postmortem storage and are difficult to remove completely when the myofibril is prepared. While in fresh samples, these membrane proteins can be more easily removed.

D. E. Goll: The authors indicate that the pH of postmortem muscle after 400 hrs of postmortem storage is near neutrality, a pH at which most catheptic proteases are not active. Although the Ca^{2+}-dependent proteinase is active at pH 7, it has been reported many times that this proteinase does not degrade myosin heavy chains. If the loss of the myosin heavy chain beginning at 408 hrs of postmortem storage is due to proteolysis, what proteases do the authors believe are responsible for this proteolysis?
Authors: This is an excellent question and is related to the response given for the question asked by Dr. Cohen relating to the Robbins et al. paper. Although this cannot be proven, we feel that a plausible possibility is a serine type proteinase (i.e. cathepsin C), which is active at a neutral pH, in combination with other muscle proteases. This could be investigated by injecting chemicals which partially inhibit serine types of proteases, such as PMSF, directly into the muscle (or by making a muscle homogenate with PMSF) after 150 hrs at which time the myosin heavy chain is still intact.

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The Use of Transmission Electron Microscopy to Study the Composition of Pseudomonas fragi Attachment Material

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Abstract

Electron microscopy techniques were used to study the attachment matrix of Pseudomonas fragi ATCC 4973. Scanning electron microscopy (SEM) was used to demonstrate the presence of attachment fibrils of P. fragi adhered to stainless steel. Transmission electron microscopy (TEM) was used to examine thin sectioned cells stained with ruthenium red or alcian blue. Extracellular substances surrounding cells stained with ruthenium red or alcian blue had a mat- or spike-like morphology. This indicated that these substances were acidic mucopolysaccharides that may be involved in the attachment of P. fragi to food contact surfaces.

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Direct inquiries to E. Zottola
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Key words: Pseudomonas fragi, transmission electron microscopy, scanning electron microscopy, ruthenium red, alcian blue, exopolymer, attachment, fibril formation, food contact surfaces, polysaccharide.

Introduction

Bacterial attachment to food and food contact surfaces is of concern as it can result in the transmission of disease and in economic losses due to food spoilage. Pseudomonas fragi, a psychrotrophic food spoilage organism, has been shown to attach to stainless steel, rubber, and glass surfaces, the materials used most often in food and milk processing equipment, as well as to beef surfaces (Speers, et al., 1984; Zoltai, et al., 1981; Schwach and Zottola, 1982). Stone and Zottola (1985a) demonstrated with scanning electron microscopy (SEM) that P. fragi cells remained attached to stainless steel surfaces after suboptimum cleaning-in-place (CIP). This attachment phenomenon has been observed in both stationary and dynamic environmental growth conditions at 4° and 25°C with no major differences between the two conditions (Stone and Zottola, 1985b).

Numerous investigators have studied bacterial attachment of marine pseudomonads to solid surfaces, such as glass and plastic, utilizing transmission electron microscopy (TEM) and histochemical staining methodology. Luft (1964) was among the first to utilize ruthenium red to stain extracellular material around cell walls, and Pate and Ordal (1967) used this cationic dye to demonstrate surface filaments on myxobacteria. The capsular structure of Diplococcus pneumoniae and Klebsiella pneumoniae was investigated using ruthenium red by Springer and Roth (1973). Fletcher and Floodgate (1973) used ruthenium red and alcian blue to demonstrate the acidic polysaccharide exocellular exudate of a marine pseudomonad. Costerton, et al. (1978) reported that the glycocalyx of many adhering microorganisms was polysaccharide in nature. Hayat (1975) states that both alcian blue and ruthenium red are specific stains for acidic polysaccharide and acidic mucopolysaccharides. He stated that alcian blue and ruthenium red contain cationic groups which bind to polyanions.

The objectives of this work were two-fold. First, to demonstrate the attachment of P. fragi ATCC 4973 to stainless steel with SEM, and secondly, to utilize the histochemical stains, ruthenium red and alcian blue, and TEM techniques to characterize the attachment matrix of P. fragi.

Materials and Methods

SEM sample preparation

Pseudomonas fragi ATCC 4973 was cultured through one or two 24 h successive transfers and

used to inoculate trypticase soy broth (TSB) (Difco) at a 1% inoculum level. Two mL of this culture were placed in a vial containing one 6 mm X 6 mm stainless steel chip of the type most commonly used in the food industry (type 304 stainless steel with finish No. 4), and the culture was allowed to grow for 18-24 h at 21°C. The stainless steel chips were then removed and rinsed three times in a vial with 2 mL of 0.1 M cacodylate buffer (pH 7.0) for 1 min to remove organisms not firmly attached. The cells attached to the chips were fixed for SEM according to Hood and Zottola (1987) in a solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7:0) and 500 ppm ruthenium red (Electron Microscopy Sciences, Fort Washington, PA) for 4-18 h at 4°C. A second fixation was done in 2 mL of 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.0) and 500 ppm ruthenium red in water for 30-60 min at 21°C. The cells were dehydrated at room temperature (21°C) in an acetone series of 25, 50, 75, and 99% for 10 min each and 3 changes in 100% for 10 min each. The cells were critical point dried in a Bomar SPC/EX with CO_2 as the transition fluid. The stainless steel chips were then mounted on aluminum stubs with carbon paint and coated with gold-palladium in a Kinney vacuum evaporator, Model KSE-2AM. Observations of the cells attached to the chips were made using a Philips 500X scanning electron microscope at an accelerating voltage of 12 kV.

Preparation of thin sections of TEM

A P. fragi culture at a 10% inoculum level was grown in 1 L of TSB for 24 h in a reciprocal shaker bath at 21°C. Alternately, cells were grown on TSB containing 1.5% agar (TSA) for 24 h, removed with a sterile spatula, and suspended in cacodylate buffer (pH 7.0). Cells were harvested with centrifugation at 12,000 X g for 10 min at 4°C in a Sorvall RC2-B Superspeed centrifuge. The pellets were rinsed with 0.1 M cacodylate buffer, centrifuged, and then fixed in 4 mL of a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer and 500 ppm ruthenium red in water for 1-4 h at 4°C. The cells were then pelleted with centrifugation and resuspended in 2% osmium tetroxide in 0.1 M cacodylate buffer and 500 ppm ruthenium red for 30-60 min at 21° C. Identical fixations were done with 1% alcian blue (Electron Microscopy Sciences, Fort Washington, PA) in glutaraldehyde/cacodylate buffer and osmium tetroxide/cacodylate buffer at pH 6.5 (Behnke and Zelander, 1970). Control cells without stain were prepared by substituting 2 mL cacodylate buffer at pH 7.0 for the stains. The cells were pelleted and then dehydrated in an acetone series as described in the SEM procedure, and embedded in Spurr's epoxy (Spurr, 1969), see Table 1. The embedded cells were thin sectioned (gold) on an LKB Ultratome, placed on Formvar coated copper grids, post stained with uranyl acetate (3 min) and lead citrate (20 sec), and viewed using a Philips 300 TEM at 60 kV.

Negative stain preparations

Negative stains of P. fragi cells in late log phase (12-16 h) were made by placing a drop of culture in TSB on a Formvar coated copper grid and removing surplus fluid with filter paper, leaving a thin film to air dry within 5-10 min. A drop of a 1% (w/v) potassium phosphotungstic acid (PTA) at pH 7.0 was added to the grids and immediately removed with filter paper. The grids were then examined in the TEM as described above.

Table 1. Spurr's epoxy embedding resin formula

Component	g
Vinylcyclohexanedioxide	10
Digycidyl ether of polypropyleneglycol	6
Nonenylsuccinic anhydride	26
Dimethylaminoethanol	0.4
Cure schedule at 70°C (h)	8

Results and Discussion

SEM examination of P. fragi ATCC 4973 attached to stainless chips revealed fibrous fibrils of varied lengths and thicknesses extending from the cells to the corrugated surface of the chips and also to neighboring cell surfaces (Fig. 1A). These attachment matrices had the appearance of peritrichous fibrils around single cells while groups of cells had a matted network of fibrils between them. Cells were also observed adhered to the stainless steel chips without the aid of visible fibrils. Fraser and Gilmour (1986) have suggested that fibril formation results from the dehydration procedures during fixation causing a collapse of any extracellular material surrounding the cells. They proposed the use of cryo-SEM to prevent this shrinkage and to aid in determining whether fibrils are distinct attachment appendages or result from collapse of exopolymer material. Cells fixed with solutions containing ruthenium red had their ultrastructure preserved with greater detail of the cell surface (Fig. 1A) than cells prepared without ruthenium red (Fig. 1B), as observed in preliminary work (Schwach, 1982). This finding agrees with work by Garland et al. (1979) who reported that ruthenium red preserved mucous in a strand form on epithelial cells and resulted in attached microorganisms being preserved in higher numbers.

TEM examination of thin sectioned cells stained with ruthenium red or alcian blue showed regions of amorphous, electron dense material exterior to the cell wall. The ruthenium red stained cells exhibited a characteristic border "fringe" or reticulum (Fig. 2A) while the alcian blue stained cells had their matrix preserved in a "spike-like" pattern around the cells (Fig. 2B). However, some alcian blue stained cells had smooth electron dense borders around cells cut longitudinally whereas the spiked appearance was observed in transversely cut cells (Fig. 2B). Figure 2C shows an alcian blue stained cell exhibiting fibril-like appendages. The multilayered structure of the cell wall was evident in ruthenium red cells but, due to poorer contrast in alcian blue stained cells, the cell wall structure had to be enhanced with post staining. The unstained control cells had very low contrast in TEM (Fig. 2D), and only when post stained were faint mat-like substances observed around a few cells. The use of ruthenium red or alcian blue greatly enhanced these substances. The observations in this study are similar to those reported by Springer and Roth (1973) who studied the ultrastructure of bacterial capsules with ruthenium red. They attributed the spike-like and mat-like

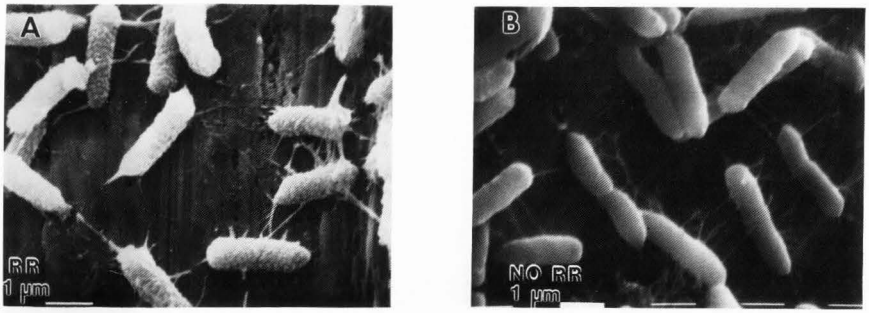

Fig. 1 (A) SEM micrograph of P. fragi ATCC 4973 attached to a stainless steel chip fixed with ruthenium red (RR) in fixatives. (B) cells fixed without RR.

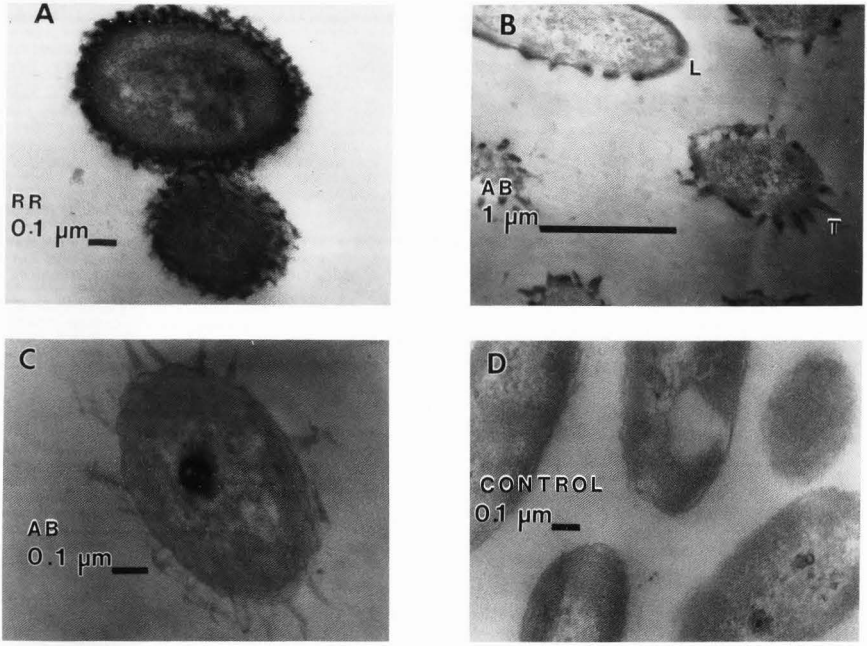

Fig. 2 TEM micrographs of P. fragi ATCC 4973. (A) Cells stained with ruthenium red (RR) showing mat-like border. (B) Cells stained with alcian blue (AB) demonstrating smooth electron dense borders in cells cut longitudinally (L) and the spiked appearance of transversely cut cells (T). (C) Cell stained with AB exhibiting thinner, fibril-like appendages. (D) Unstained control cells.

appearances of the stained material to dehydration and embedding procedures. They found that alcohol dehydration causes some crosslinking of fibrils whereas dehydration with epoxies kept fibrils separated. The spike-like appearance of tangently-cut sections were attributed to the "tips" of the fibrils being sectioned. Because little or no extracellular material was observed in the unstained controls, the ruthenium red and alcian blue may have aided the fixation of the polymers surrounding the cells (Garland et al., 1979).

Because ruthenium red and alcian blue are reported to preferentially stain acidic mucopolysaccharides in cells and tissues (Hayat, 1975), the attachment of P. fragi may be attributed to a matrix of acidic mucopolysaccharide surrounding the cells.

P. fragi ATCC 4973 has been reported to possess peritichous fimbriae (Fuerst and Hayward, 1969). In order to demonstrate the presence or absence of fimbriae on our culture, negative staining with PTA was employed. Electron dense borders were seen around cells stained with PTA while the flagella were outlined in a thin layer of PTA (Fig. 3A). One would expect that if fimbriae were present, they would also be outlined in a thin layer of PTA. Groups of cells exhibited spike-like appendages stretching between cells similar to those observed in the alcian blue stained cells (Fig. 3B). It is possible that any fimbriae present were obscured by the polysaccharide material surrounding the cells as demonstrated with histochemical staining. Fimbriae also could have been coated with the polysaccharide and formed the spiked-like appendages seen in the thin sections. Because negative staining of P. fragi ATCC 4973 failed to demonstrate the presence of fimbriae, it is possible that, under the conditions of this study, our culture did not produce fimbriae. Duguid et al. (1966) found that fimbriae production in Enterobacteriaceae can be influenced by culture conditions such as substrate, incubation temperature, growth stage, and number of serial transfers.

Conclusions

This study suggested that the attachment matrix of P. fragi is composed of an acidic polysaccharide or mucopolysaccharide material. Although fimbriae have been reported on this species, none were observed in our culture under the culture conditions used indicating that their role, if any, in attachment to stainless steel was overshadowed by the polysaccharide material surrounding the cells.

Future work is needed to remove the matrix from cells and further identify its composition. This could possibly aid in the formulation of detergents or sanitizers to remove cells attached to food contact surfaces, and the development of an alternative methodology for the determination of attachment.

Acknowledgements

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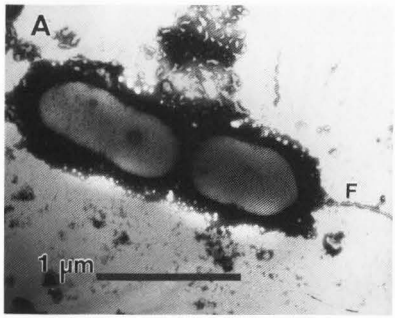

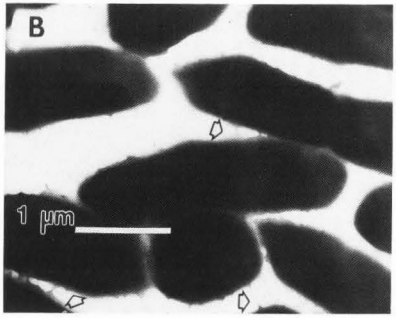

Fig. 3. TEM micrographs of P. fragi negatively stained cells. (A) PTA stained cells with heavy border and flagella (F) outlined in a thin layer of PTA. (B) PTA stained cells with material stretching between cells.

Discussion with Reviewers

P.Stanley: During preparation for TEM, the cells were rinsed in cacodylate buffer before fixation. Was any significant portion of the outer cell wall removed during this rinse? Since the outermost wall layers are responsible for attachment, one would want to be sure none were inadvertently removed in a study of this type.
Authors: Cacodylate buffer (0.1 M, pH 7.0) was used in the SEM rinsing procedures without any obvious removal of attachment fibrils from the cell surface and, although no investigation was performed to determine its effect upon the cell wall, it appeared to have little or no effect upon the removal of the outer cell wall, as the ruthenium red and alcian blue stains illustrated. Glauert (1975, in "Fixation, Dehydration and Embedding of Biological Specimens," North Holland Publishing Company, Amsterdam, 16-17) reports that cacodylate performs with similar results as phosphate buffers, except that it does not support cell growth and the arsenic may also act as a fixative.

Reviewer 1: How many cells were observed without fibrils? Does this relate to your observation concerning longisections?
Authors: As noted in the text, some cells were observed attached to stainless steel without the presence of fibrils. Cells without fibrils usually occurred as single cells rather than in large groups of cells. Although fibrils were absent, they still had the characteristic rough cell surface as depicted in Figure 1. No effort was made to enumerate the proportion of cells without fibril formation, but a majority of the cells observed had fibrils. It is possible that these cell differences may aid in explaining the observation of smooth borders around longitudinally cut cells in Figure 2B rather then the fact that they are longisections, but this was not investigated.

P. Stanley: Two lines of evidence suggest that proteins could be involved in attachment. First, the exopolymer of a sediment bacterium (Mittelman and Gessey, Astr. Ann. Mtg. ASM. 1983. Q5) and outer layers of a marine pseudomonad (Forsberg et al., J. Bacteriol. 104:1338. 1970) were shown to contain approximately 15% protein. Second, proteases improved removal of biofilms from R-O membranes (Whittaker, et al., App. Environ. Microbiol. 48:395. 1984). Did the authors examine the outer surface of P. fragi for proteins which might have been involved in attachment?
Authors: The ruthenium red stain is specific for acidic mucopolysaccharides which Hayat (1985) stated are "invariably associated with protein." Also, the mucopolysaccharides themselves may have amino groups present. In the investigation presented above, no attempt was made to determine if proteins were involved in the attachment process; however, studies are currently being performed to characterize the material responsible for attachment.

P. Stanley: Cells used for TEM analysis were grown in broth or on colonies on agar, whereas cells for SEM analysis grew while attached. Is there evidence that the outer layers of bacteria remain constant under these various growth conditions making the SEM-TEM comparisons valid?
Authors: Cells were grown on the agar surface and then removed in order to simulate growth during attachment to solid surfaces and broth cultures were used to examine the cell surface in a liquid medium. In general, no differences were observed in the thin sections from agar grown and broth cultures. Previous work in our laboratory (Stone and Zottola, 1985b) has shown that attachment to stainless steel with fibril formation occurred within 30 minutes of contact time in broth culture. This would suggest that the cells possess an attachment matrix while in broth culture. However, fibril formation increased with increasing contact time.

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EFFECTS OF LIGNIFICATION, CELLULOSE CRYSTALLINITY AND ENZYME ACCESSIBLE SPACE ON THE DIGESTIBILITY OF PLANT CELL WALL CARBOHYDRATES BY THE RUMINANT

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Abstract

Intrinsic characteristics of plant cell walls limiting susceptibility of structural carbohydrates to microbial attack in the ruminant's gastrointestinal tract are lignification of the cell wall, covalent bonding of phenolic acids to cell wall polysaccharides, the crystalline structure of cellulose and limited fibrolytic enzyme accessible space. The exact mechanism by which or degree to which each of these characteristics affect rate and/or extent of cell wall polysaccharide hydrolysis by gastrointestinal tract microbes is not well understood. Lignification and limited enzyme accessible space probably affect the extent of cell wall degradation by preventing contact between microbial enzymes and cell wall polysaccharides. Phenolic acids may limit cell wall carbohydrate degradation by steric hindrance of the fibrolytic enzyme, which could affect both rate and extent of degradation, and by their potentially toxic effects on microbes. Crystalline cellulose, occurring in secondary cell walls, may be degraded at a slower rate than amorphous cellulose. Further research is needed to gain a better understanding of the mechanisms by which these characteristics limit structural polysaccharide degradation by gastrointestinal tract microbes and to determine to what degree each contributes to limiting digestibility of cell wall carbohydrates by ruminants.

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Key Words: Ruminant, plant cell wall, hemicelluloses, cellulose, lignin, phenolic acids, cellulose crystallinity, enzyme accessible space, gastrointestinal tract microbes, lignocellulose degradation.

Introduction

Pond et al. (1980) identified the poor digestibility of lignocellulose as a major obstacle constraining animal protein production in the face of an expanding world population. Digestion of plant material by ruminants is dictated, in part, by the rate and extent with which gastrointestinal tract microorganisms can degrade cell wall polysaccharides. Increasing digestibility of lignocellulosic fiber by ruminants, therefore, is dependent upon a better understanding of the reasons that cell wall carbohydrates are limited in their susceptibility to microbial degradation.

Based on the model of the cell wall proposed by Albersheim (1978), there are three possible factors primarily responsible for limiting susceptibility of cell wall polysaccharides to microbial degradation in the ruminant's gastrointestinal tract: (1) the close physical and chemical association among cellulose, hemicelluloses and lignin; (2) the presence of crystalline regions within cellulose; and (3) a limited enzyme accessible space (Stone et al., 1969; Northcote, 1972; Bailey, 1973; Van Soest, 1973; Cowling, 1975; Rowland, 1975; Gordon et al., 1977; Fan et al., 1980 Chambat et al., 1981; Jung and Fahey, 1983). Before these limitations to cell wall degradation can be overcome by plant breeding or chemical treatments, one must understand how these factors exert their negative influence on microbial degradation of cell wall carbohydrates.

Factors limiting microbial degradation of cell wall polysaccharides

Phenolic acids / lignin

A comprehensive review of the nutritional implications of phenolic monomers and lignin was presented by Jung and Fahey (1983). Microbial degradation of lignocellulosics is negatively correlated with total phenolic acid content (Burns and Cope, 1974; Jung and Fahey, 1981) and cell wall lignification (Patton and Gieseker, 1942). While phenolic acids and polyphenolic polymers appear to be primary factors limiting susceptibility of cell wall polysaccharides to microbial digestion, their mechanism of protection is unclear (Chesson et al., 1983).

Cell wall polymeric lignin is covalently bound to hemicelluloses in the plant cell wall (Van Soest, 1981; Brice and Morrison, 1982). Smith and Hartley (1983) noted that ferulic and para-coumaric acids were esterified to cell wall polysaccharides, and appear to be the primary means of lignin attachment to

cell wall polysaccharides. The mechanism by which ester linkages between cell wall polysaccharides and ferulic and (or) para-coumaric acids limit enzymatic hydrolysis of cell wall polysaccharides is not well understood. These phenolic acids probably limit structural carbohydrate degradation by inhibiting microbial growth and (or) enzyme activity, by inactivating the enzyme or by sterically hindering its attachment to the structural carbohydrate.

Chesson et al. (1982) showed that phenolic acids inhibited growth and cellulolytic activity of ruminal bacteria. Akin (1982) found that para-coumaric, ferulic and sinapic acids depressed in vitro cellulose digestion by ruminal bacteria. Akin (1982) found that para-coumaric acid was more inhibitory to cellulose degradation than ferulic acid. Jung (1985), however, found ferulic acid more inhibitory to cellulose degradation than para-coumaric acid. As shown by Chesson et al. (1982; Table 1), different bacterial species respond differently to the various phenolic acids present in plant cell walls. The contrasting results of the previously mentioned experiments could be explained by differences in the primary species of bacteria degrading cellulose in these experiments. Whether phenolic acids actually affected total microbial numbers, lowering the total amount of bacterial cellulase present, or decreased the cellulase enzyme activity, is unclear. In either case, in vitro cellulose degradation would be reduced. Further research is needed to identify the mechanisms by which free phenolic acids and (or) complexes of phenolic acids covalently bound to cell wall monosaccharides depress microbial degradation of plant cell wall polysaccharides.

Jung and Sahlu (1986) found that filter paper cellulose degradation by ruminal bacteria was depressed when phenolic acids were esterified to cellulose fibers (Table 2). The negative effect of phenolic acids on structural carbohydrate degradation was apparently greater when phenolic acids were esterified to the cellulose than when free in solution. Jung and Sahlu (1986) also found that different sources of cellulose, presumed to vary in their structure, differed in terms of which phenolic acids were most inhibitory to microbial degradation of the cellulose. If the different celluloses used in these experiments were degraded by different species of cellulolytic bacteria, variation in the negative effects of the various phenolic acids tested on cellulose degradation could have been due to differences in the predominant cellulolytic organism present (as previously discussed). Further research needs to be conducted to determine (1) the primary bacterial species degrading various hemicelluloses and cellulose in the plant cell wall and (2) the effects of various phenolic acids esterified to cell wall structural carbohydrates on rate and extent of microbial degradation.

Smith and Hartley (1983) isolated a lignin-carbohydrate complex from wheat bran cell wall after fungal cellulase treatment. The complex was composed primarily of xylose, arabinose and ferulic acid. They identified the complex as 2-0-[5-0(feruloyl)-β-L- arabino-furanosyl]-D xylopyranose. Because this compound could be isolated, it appears that steric hindrance inhibited hydrolyses of monosaccharides bound to phenolic acids. Chesson et al. (1983) noted that xylans substituted with arabinose residues were preserved during ruminal digestion, and the extent of substitution at the 0-5 position of arabinose was closely related to the amount to phenolic material present further indicating that ruminal microbes are limited in their ability to degrade cell wall polysaccharides bound to phenolic acids.

Core lignin (Gordon and Neudoerffer, 1973) is a complex three-dimensional structure formed by free radical-induced polymerization of phenolic monomers synthesized by the shikimic acid pathway (Harkin, 1973). The mechanism by which core lignin limits cell wall polysaccharide digestion is also unknown, but it is possible that this limitation is due to lignin's physical protection of cell wall carbohydrates and its hydrophobic character (Van Soest, 1982). The physical protection and hydrophobic nature of core lignin would exclude microbes from reaching and attaching to the polysaccharides, thus reducing their ability to hydrolyze the cell wall carbohydrates. Disrupting the structure of core lignin which encrusts the cell wall polysaccharides should result in increased attachment and penetration by microbes and, subsequently, in an increased digestibility of the cell wall polysaccharides. Completely removing core lignin from the cell wall with permanganate oxidation was shown to increase microbial degradation of cell wall polysaccharides (Barton and Akin, 1977; Table 3). Kerley et al. (1985) demonstrated that partial (approximately 50%) delignification of plant cell walls by alkaline hydrogen peroxide treatment (Gould, 1984) allowed extensive attachment of ruminal microbes, accompanied by rapid degradation of cell wall carbohydrates (Figure 1).

It is not known to what extent core lignin's negative effect on digestion is dependent on its binding with cell wall polysaccharides. It is known that non-core (alkali-labile) phenolic acids form diaryl (Hartley and Jones, 1976) and alkyl-alkyl (Stafford and Brown, 1976) bonds with proteins. Scalbert et al. (1985) provided evidence of ferulic acid (non-core lignin) attachment to core lignin by a similar bonding mechanism, indicating that core lignin may be bound to cell wall polysaccharides via non-core lignin. This could limit digestion, in that core lignin could physically exclude and non-core lignin could sterically hinder enzymatic attachment to and hydrolysis of cell wall carbohydrates.

Cellulose crystallinity.

Crystalline cellulose, in contrast to amorphous cellulose, refers to aggregates of cellulose polymers held tightly together by extensive hydrogen bonding. Cellulose is a polymer of β-1,4-linked D-glucose units (Frey-Wyssling, 1969). This type of linkage results in the relative inversion of alternate glucose units. This places the C-3 hydroxyl of one glucose unit in close proximity to the ring oxygen of the next glucose unit in the chain. Hydrogen bonding between the hydroxyl and ring oxygens stabilize the cellulose polymer, giving it a straight, flat structure (Frey-Wyssling and Muhlethaler, 1963). This linear structure of cellulose allows adjacent polymers to fit closely together, favoring hydrogen bond development between the C-6 hydroxyl glucose in one chain with C-2 or C-6 hydroxyls of glucose in an adjacent chain. Since cellulose chains consist of 8,000 to 15,000 glucose residues, extensive hydrogen bonding can occur, conferring considerable strength to the microfibrils (Frey-Wyssling and Muhlethaler, 1963).

Cellulose polymers are held so tightly together in the microfibril structure by hydrogen bonding that water molecules may be excluded from the crystal

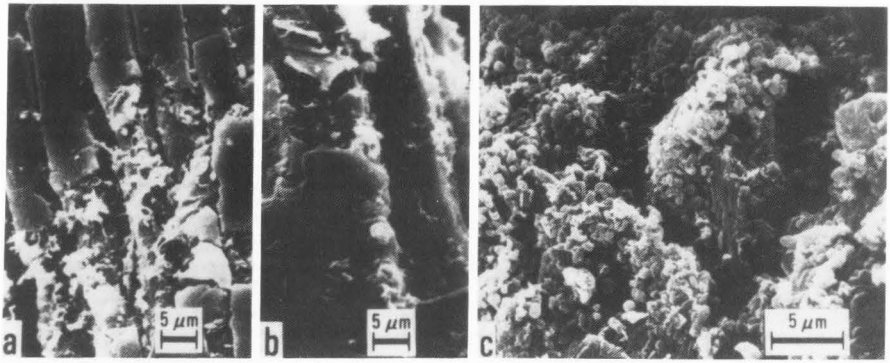

Figure 1. Scanning electron micrographs of straw particles isolated from the rumen of fistulated mature sheep fed diets containing 72 percent untreated wheat straw (a and b) or 72 percent wheat straw treated with alkaline hydrogen peroxide (c; Gould, 1984).

Table 1. Effect of phenolic acids on the cellulolytic activity of ruminal bacteria[a]

Phenolic acid	Concentration (mM)	% of cellulolytic activity retained[b]		
		Bacteroides succinogenes BL2	Ruminococcus flavefaciens 007	Ruminococcus albus SY3
p-Coumaric acid	1	89	96	100
	5	87	71	83
	10	37	39	55
Ferulic acid	1	93	96	97
	5	58	47	84
	10	29	27	81
Vanillic acid	1	100	100	100
	5	90	100	100
	10	89	100	100

[a](Chesson et al., 1982).
[b]The amount of cellulose digested after 7 to 10 days of incubation at 39°C is expressed as a percent of that digested by control cultures without added acid under the same conditions.

Table 2. Effects of ester-linked cinnamic acids on in vitro filter paper cellulose digestion by ruminal microorganisms[a]

Compound	Cellulose digestion (%)
Control	23.9
Cinnamic acid	24.0
p-Coumaric acid	22.7
Caffeic acid	11.7[b]
Ferulic acid	19.1[b]
Sinapic acid	22.4[b]

a(Jung and Sahlu, 1986).
[b]Significantly different from the control (P § 0.05)

Table 3. Percent in vitro dry matter disappearance of untreated and permanganate-treated neutral detergent fiber fraction of Tall Fescue and Coastal Bermudagrass[a]

Grass	Neutral detergent fiber residue	Permanganate-treated neutral detergent fiber residue
Tall Fescue	63.1	77.0
Coastal Bermudagrass	63.5	79.8

[a](Barton and Akin, 1977).

inner structure. The inability of water to penetrate the microfibril prevents hydration of the internal cellulose polymers of the microfibril, which in turn prevents cellulose hydrolysis by cellulolytic enzymes or microorganisms. As a result, microbes are limited to attacking cellulose polymers on the outer surface of the unhydrated microfibril unit. Haworth et al. (1969) noted that 44% of the cellulose polymers were on the surface of the microfibril unit, which is rectangular in cross-section with eight and ten cellulose polymers along each of two sides. Assuming that this is the correct structure of the cellulose microfibril, 56% of the cellulose polymers in the microfibril would be protected from microbial hydrolysis until the outer layer of cellulose polymers was removed. This could greatly affect rate of cellulose degradation by ruminal microbes. Fan et al. (1980) demonstrated that the degree of crystallinity affected the rate of cotton cellulose hydrolysis by Trichoderma reesei cellulase.

Enzymatic hydrolysis of glucosidic bonds in crystalline cellulose may also be hindered by the restricting influence of hydrogen bonding (Rowland, 1975). Hydrolysis of the glucosidic bonds in solution is reversible. For hydrolysis to occur, the glucosidic linkage must be available for protonation and the chain ends must move apart to implement hydrolysis. Separation of chain ends may be prevented or delayed by the restricting influence of interchain hydrogen bonding. Therefore, the microbes must first disrupt the hydrogen bonds. Decreased hydrogen bonding could be controlling the rate at which fragmentation, swelling, loss in tensile strength, transverse cracking and lowering of the degree of polymerization occurring in cellulose prior to release of glucose and cellobiose by cellulase enzymes (Lee and Fan, 1980). All of these occurrences would be expected if the extent of hydrogen bonding was reduced.

The process of bacterial attachment and hydrolysis of cellulose is further complicated by the manner in which crystalline microfibrils are interconnected with one another. In the past, microfibrils were thought to be interconnected by cell wall matrix components (hemicelluloses and lignin). Therefore, it was hypothesized that the major factor limiting the cellulose microfibril from bacterial attack was encrustation and attachment of lignin and hemicelluloses to the cellulose polymers. While this undoubtedly occurs, Colvin and Sowden (1985) reported that microfibril units in cotton cellulose were interconnected with one another by cellulose polymers, which themselves were arranged in a crystalline structure. If the crystalline arrangement prevents microbial access to cellulose, separation of the microfibrils, which is necessary for extensive microbial attachment, would be limited, slowing the rate of microfibril hydrolysis.

Limited data exist regarding the degree of cellulose crystallinity of lignocellulosics commonly fed to ruminants or the effect of their crystalline arrangement on cellulose degradation by ruminal microbes. Since microfibrils in plant cell walls are known to become more tightly packed and lie more parallel to one another upon maturation and secondary cell wall formation (Northcote, 1972), it would be expected that most crop residues, which are harvested at advanced stages of maturity, are comprised primarily of crystalline cellulose. Therefore, determining the extent of cellulose crystallinity in lignocellulosics and understanding the effect of cellulose crystallinity on bacterial hydrolysis of cellulose might be important in predicting the degree of susceptibility of lignocellulosic materials to microbial attack, providing crystallinity is an important component affecting cell wall breakdown.

Gould (1984) reported that treating wheat straw with dilute, alkaline solutions of hydrogen peroxide greatly increased its water absorption capability. Alkaline hydrogen peroxide treatment also increased susceptibility of wheat straw structural carbohydrates to ruminal microbial degradation (Kerley et al., 1985). These findings were attributed to a decrease in the crystallinity of the cellulose in wheat straw. However, based upon X-ray and neutron diffraction studies, it was concluded that no change in the degree of cellulose crystallinity occurred due to alkaline hydrogen peroxide treatment (Martel and Gould, 1987), indicating that other factors are involved. Therefore, cellulose crystallinity does not appear to greatly deter microbial hydrolysis of cellulose in forages.

Cellulase Enzyme Accessible Space

The surface area of cell wall carbohydrates accessible to ruminal cellulase enzymes could also limit their degradation. The accessible surface area is defined by size, shape and surface properties of microscopic and sub-microscopic capillaries within the fiber in relation to size, shape and diffusibility of microbial cellulase enzyme molecules themselves. Microscopic capillaries include the cell lumina, pit apertures and pit-membrane pores that are visible under the light microscope and range between 20 nm and 10 or more microns in diameter (Cowling, 1975). Sub-microscopic capillaries include spaces between microfibrils and cellulose polymers in the amorphous regions of cellulose. Some sub-microscopic capillaries expand to 20 nm in diameter when fully hydrated, but most are considerably smaller. Total surface area exposed in microscopic capillaries is approximately 2×10^3 cm^2 per g of wood or cotton, whereas total surface ares exposed in sub-microscopic capillaries is approximately 3×10^6 cm^2 per g of wood or cotton (Cowling, 1975). If cellulolytic bacteria could penetrate into the sub-microscopic capillary area, substantially greater rates of cellulose degradation would be expected than if they were prevented from entering this area of the cell wall.

The maximum dimensions of various cellulolytic enzymes studied (Ishikawa et al., 1963) appear to be smaller than microscopic capillaries of both wood and cotton. Only a small fraction of the sub-microscopic capillaries in hydrated wood or cotton, however, are sufficiently large enough to allow penetration of the microbial cellulase enzymes. Stone et al. (1969) showed that the initial rate of cellulose hydrolysis by Trichoderma cellulase was proportional to the surface area accessible to a solute molecule of 4 nm. Ruminal bacteria, ranging from 0.3 - 2.0 m in diameter and 1.0 - 6.0 m in length (Church, 1976), would be greatly limited in their ability to enter the sub-microscopic capillary space in the plant cell wall. Since the cellulase enzyme complex is probably bound to the bacterial cell wall or subcellular membrane vesicles of ruminal microorganisms (Groleau and Forsberg, 1981; Forsberg et al., 1981), the surface area of the sub-microscopic capillaries would be

inaccessible to the cellulase enzyme complex.

Dehority (1961) and Dehority and Johnson (1961) found that physical reduction of forage particle size by ball milling increased the amount of cellulose degraded by ruminal microbes (Table 4). The increase in cellulose digestion may have been due to an increase in the cellulase accessible surface area of the forage due to ball milling. Lin et al. (1985) found that increasing the surface area of cornstalk residue was necessary for effective increases in cellulose digestion. Further research is needed to determine the effects of cellulase accessible surface area on plant cell wall degradation by ruminal microbes.

Table 4. Effect of surface area on in vitro cellulose digestibility by ruminal microorganisms[a]

	Cellulose digested (%)	
Grass	Ground (40 mesh screen)	Ball milled (72 h)
Bromegrass	42.7	75.8
Orchardgrass	53.7	82.3

[a](Dehority and Johnson,1961).

Conclusion

The plant cell wall is a complete entity rather than merely a complex of isolated fractions. Use of techniques such as those involved in the determination of cellulase enzyme accessible space allows the cell wall to be treated as a holistic unit. Chesson (1982) noted that the rate of plant cell wall degradation by microbes was determined more by the nature of the cell walls themselves than by the physicochemical properties of their individual component polymers. Therefore, to identify factors constraining degradation of cell wall structural polysaccharides by ruminal microorganisms, researchers must view the cell wall as a single entity and not as a complex of individual fractions which are studied independently of each other. This is exemplified by the findings of Chesson et al. (1982) which showed that residual fractions of barley straw cell wall remaining after extensive ruminal degradation had a similar cell wall composition as the undigested, original cell wall material. Even though cell wall composition was similar between undigested and digested residues, the residual material could not be further degraded by ruminal microorganisms, indicating that analyses of individual components of the cell wall, aimed at identifying factors limiting structural carbohydrate degradation by ruminal microbes, may not totally encompass the major factors constraining cell wall degradability.

As indicated by Harbers (1985), further research is needed to separate components or fraction of the plant cell wall based on their susceptibility to microbial degradation in the ruminant's gastrointestinal tract, without destroying the infrastructure of the cell wall. These fractions need to be characterized according to their structure and composition, using microscopic and chemical techniques, so that differences in structure and composition among the various fractions might be used to explain differences in their susceptibility to microbial attack in the ruminant's gastrointestinal tract. Once the limitations to microbial degradation of cell wall carbohydrates has been elucidated, plant breeding methods and chemical treatments can be developed to increase utilization of plant carbohydrates by the ruminant.

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Discussion with Reviewers

H.G. Jung: Published data suggest virtually no free phenolic acids exist in plant tissue. How can the study of inhibition of microbial fermentation by free phenolic acids aid our understanding of plant cell wall biodegradability?

Authors: Because of syntrophic relationships in the rumen, it is not illogical to think that free phenolic compounds do exist in the rumen liquor. Also, it is possible that microenvironments around the microbial cells do contain phenolic monomers generated upon plant cell wall hydrolysis. Effects of phenolic monomers in solution around the microorganism on the cells microenvironment is unknown. Another important area of consideration is the effect of phenolic-carbohydrate complexes on microbial activity, as both a toxin and a sterical hindrance to structural polysaccharide hydrolysis.

H.G. Jung: Could the differential results seen for phenolics esterified to different cellulose preparations be an example of crystallinity effects on microbial fermentability?

Authors: It is possible that crystallinity has some affect on selection of bacterial species by a particular microorganism having a competitive advantage in hydrolyzing crystalline cellulose. Our research, as

does others, indicates that cellulose crystallinity of forages is not sufficient to limit cellulose hydrolysis. Therefore, in forage research, while crystallinity may play a minor role in limiting cellulose digestion, the limited susceptibility of structural carbohydrates appears to be a composite of several factors negatively affecting digestion.

D. E. Akin: Is there any direct evidence that cellulose crystallinity varies in forages or that crystallinity affects forage breakdown?
Authors: The hypothesis that crystallinity affected mature forage cellulose hydrolysis was derived from research examining the degradation of wood cellulose, which does appear to be affected by crystallinity. It is our opinion, based on X-ray crystallinity research, that cellulose in forages examined to date does not appear to affect digestion.

D. E. Akin: Do plants vary in size of submicroscopic capillaries?
Authors: It is expected that plants, like wood, have a wide range of submicroscopic capillary pore sizes. Extensive research into the various range of sizes which exist and the effect of pore size on microbial degradation has not been done to our knowledge.

D. E. Akin: Are a, b and c of the same plant region? Figure a and b seem to show cuticle (usually with little attachment) while c is of internal plant tissue. This should be addressed as it will influence attachment.
Authors: Panel a and b of Figure 1 show a broken portion of the external face of a wheat straw particle. The upper-left half of panel a probably is the actual outer surface of the straw, while the lower-right half reveals the inner surfaces of the underlying tissue. Attached cells are apparent in the lower-right area, but are sparsely distributed. Panel b shows the inner surface of an epithelial cell, again with relatively sparse cell attachment. As Dr. Akin has so elegantly shown in many publications, these attachment patterns are typical for relatively indigestible lignocellulosic materials such as straw. Panels a and b merely reiterate the many pictures documenting this fact that are already in the literature. Panel c, on the other hand, shows a typical view of rumen-incubated alkaline peroxide-treated wheat straw. Because the treatment process so completely disrupts the organization of the straw tissue, it is impossible to tell what portion of the original tissue is present in this view (for additional SEM data detailing the effects of alkaline peroxide treatment on wheat straw tissue morphology, see Gould, J. M., Biotechnol. Bioeng. 27, 225-231 (1985). In any event, the panel shown is representative of all samples of rumen-incubated treated straw samples examined. In other words, untreated straw samples were characterized by having regions where the density of attached cells was very low as well as by regions where the density was moderate. In contrast, treated straw was characterized by a uniform, dense coat of attached cells on all surfaces.

L. H. Harbers: How well do the currently accepted methods of fiber analyses (crude fiber, neutral detergent fiber, acid detergent fiber, etc.) aid in explaining the limitations of cell wall degradation?
Authors: It is our belief that neutral detergent fiber and acid detergent fiber offer superior alternatives to fiber analyses of foods and feeds compared to crude fiber. Neutral detergent fiber and acid detergent fiber have obvious merit in non-specific fractionation of food and feed, and for use as adequate predictors of digestibility and intake. However, detailed analyses of components in food and feeds requires more extensive study of the composition and structure of the cell wall than can be achieved by detergent analyses.

L. H. Harbers: Assuming all three factors discussed do limit cell wall degradation, what types of tests should we concern ourselves with in reference to feed formulation in the future?
Authors: Cellulose crystallinity will probably have little influence on ration formulation. Enzyme accessible space could be important in determining digestibility of forage material. The most potential in predicting digestibility and use of forages in ruminant diets lies in understanding intrinsic factors in the cell wall structure which limit structural polysaccharide hydrolysis.

L. H. Harbers: Do each of the limitations to digestion affect monocotyleden (C3 versus C4) and dicotyledon species equally?
Authors: There are obvious differences between species of C3, C4 and legume forages. The differences within a class (i.e. legumes), or even within a variety, can be as great as are expected to occur among classes. It may well be that factors(s) limiting hydrolysis is similar among all forages (particularily C3 and C4), but the repitition or number of these negative factors varies from one class to another. It is also possible that each class and possibly species within a class differ in factor(s) which control digestion, which would make the search for a specific factor controlling digestion of forages by the ruminant virtually impossible.

S. H. Cohen: Figure 1 is an electron micrograph of wheat straw, untreated or treated with alkaline hydrogen peroxide, fed to sheep, and then isolated from the rumens. According to the text the alkaline hydrogen peroxide removed lignin and allowed extensive attachment of rumen organisms and rapid digestion of cell wall carbohydrate. When I examined the figure these effects were not apparent to me. The figures need some indicators pointing them out.
Authors: The effects of alkaline peroxide treatment on the disruption of tissue integrity as a result of cell wall delignification are pretty well obscured in these pictures by the attached bacteria. A better view of the effects of treatment on tissue morphology is given in Gould, J. M., biotechnol. Bioeng. 27, 225-231 (1985).

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CHANGES IN THE ULTRASTRUCTURE OF EMULSIONS AS A RESULT OF ELECTRON MICROSCOPY PREPARATION PROCEDURES

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Abstract

Various methods of preparing emulsions for electron microscopy were examined with peanut oil/protein and ice cream mix emulsions. For transmission electron microscopy (TEM), fresh peanut oil/bovine serum albumin emulsions were mixed with 2% agar, fixed in phosphate-buffered (pH 7.0) 4% glutaraldehyde solution and postfixed in phosphate-buffered (pH 7.0) 1% osmium tetroxide; alternatively, the glutaraldehyde-fixed samples were briefly rinsed in acetone prior to postfixation. Both preparations yielded satisfactory fat globule preservation. Similar emulsions were prepared on loops and suspended over vapors of 25% glutaraldehyde and 1% osmium tetroxide. This preparation resulted in angular fat globules surrounded by a heavy protein precipitate.

Ice cream mix emulsions were prepared for TEM study by mixing with 4% agar, mixing with 2% agarose or using agar tubes. After fixation in phosphate-buffered 4% glutaraldehyde (pH 7.0) solution, the samples were postfixed in either phosphate/imidazole buffered (pH 7.0) or phosphate-buffered (pH 7.0) 1% osmium tetroxide. Mixing with 2% agarose and postfixing in imidazole/phosphate buffered osmium tetroxide yielded the best results. A clearly visible fat membrane and well-delineated fat crystals were observed.

Scanning electron microscope (SEM) studies of peanut oil/casein emulsions mixed with 4% agar yielded good results whereas in ice cream mix emulsions, the results were inconclusive.

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Key Words: agarose, agar, emulsion, fat globule, gels, ice cream, membrane, peanut oil, protein, scanning electron microscope, transmission electron microscope, ultrastructure.

Introduction

There has been a great deal of interest in the microstructure of food emulsions in recent years but because of variations in emulsion properties, standard preparation methods for electron microscopy study may have to be modified. It is well known that changes in preparation procedures for electron microscopy study can alter the specimen (Hayat, 1970; Ericsson et al. 1965; Sjostrand, 1967). Chabot et al. (1979) discussed these effects on the ultrastructure of white bread; Khoo et al. (1975) experimented with vapor fixation of dough in order to eliminate artifacts caused by dehydration but discarded this method as unsatisfactory and adopted a more standard fixation method. Carroll et al. (1968) found that glutaraldehyde preserved micelle structure better than osmium tetroxide or formaldehyde. Parnell-Clunies et al. (1986) noted that electron dense particles were not observed when yoghurt was fixed in glutaraldehyde alone whereas the addition of osmium tetroxide as a postfixative in conjunction with glutaraldehyde as a primary fixative resulted in the appearance of dense particles. In a study of muscle tissue, Colquhoun and Rieder (1980) found that the relatively minor change of initiating the dehydration step at 75% ethanol rather than 30% ethanol caused an increase in contrast of EM images in phosphate buffered specimens whereas cacodylate buffered specimens were unaffected.

Fluid multiphase samples, such as peanut oil/protein or ice cream mix emulsions present additional difficulties because they cannot be prepared as simply as semi-solid or solid samples. The use of freeze-etch (Buchheim and Precht, 1979; Buchheim, 1974) and cold stage SEM (Kalab, 1985) to study liquid samples may not provide the type of information needed or simply may not be available. In addition, some specimens are better preserved using the traditional critical point drying method rather than the cold stage for SEM (Kalab et al., 1988). The purpose of this research was to evaluate different electron microscopy preparation procedures for studying the ultrastructure of emulsions.

Specimen changes that occurred using peanut oil/protein and ice cream mix emulsions were examined.

Materials and Methods

TEM - Peanut Oil/Bovine Serum Albumin (BSA)

Peanut oil/BSA emulsions were prepared as described by Haque and Kinsella (1988). The fresh emulsions were mixed with warm 2% agar (3 parts emulsion:1 part agar), mixed gently with a wooden applicator stick and allowed to set. The resultant gel was cut into 1mm^3 pieces and fixed in 4% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) overnight. After several brief rinses in phosphate buffer, the samples were post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.0) for 2h or rinsed briefly in acetone between the glutaraldehyde and osmium step, dehydrated in a graded series of acetone, embedded in Spurr resin and polymerized in a vacuum oven overnight at 70°C.

Loops were prepared as follows: Wire loops approximately 2 mm in diameter were fashioned from nichrome wire. The loops were dipped into the peanut oil/BSA emulsions causing the formation of an emulsion droplet within the loop by virtue of surface tension. The emulsions were then suspended over vapors of 25% glutaraldehyde for 1/2 h followed by suspension over 1% osmium tetroxide vapors overnight. After this process, the samples were brittle enough to be removed from the loop, dehydrated and embedded as described above. In some cases, the droplet did not retain its integrity within the loop and there was no material left to embed.

TEM - Ice Cream Mix

Ice cream mixes were prepared as follows: 10% milkfat, 11% milk solids not fat, 10% sucrose, and 5% corn syrup solids. Fresh cream, skimmilk, and nonfat dry milk were used as the sources of milk solids. The 8kg mixes were blended, pasteurized at 74°C for 30 minutes, homogenized at 17.2 MPa (2500 psig), 3.4 MPa (500 psig) second stage, cooled to 5°C, and aged 24 h. Following aging, ice cream mix emulsions were warmed to 15°C and combined with either 2% agarose (22°C), an ultra low gelling temperature agarose derived from agar (Sea Prep Agarose, FMC Marine Colloids Div., Rockland, ME) which remains liquid at room temperature if stirred constantly or with warm 4% agar in the proportion of 3 parts sample:1 part agar or agarose. The 2% agarose was prepared as follows: Deionized, distilled water was heated to 95°C. The agarose was added slowly until it dissolved while the water was constantly stirred. The agarose solution was allowed to cool to 22°C with constant stirring. If stirring was stopped, the solution became quite viscous. The samples were gently mixed with a wooden applicator stick and allowed to gel overnight at 4°C.

Agar microtubes were prepared as follows: the flame sealed thin portion of a Pasteur pipette was repeatedly dipped into warm 4% agar and allowed to harden until a thick film had adsorbed, forming an agar tube. After cutting the distal end of the tube with a razor blade, the emulsion was drawn into the tube by dipping the pipette into the emulsion and partially withdrawing the pipette. The agar tube containing some of the emulsion was placed on a glass slide and the pipette was removed. After trimming and sealing the ends of the agar tube with warm agar, the tube was momentarily dipped in warm 4% agar to complete sealing and prevent leakage. The tube was then allowed to gel. This method is a variation of the one described by Allan-Wojtas and Kalab (1984b). The three sample types were cut into approximately 1 mm^3 pieces and fixed in 4% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) overnight.

In addition, the 2% agarose mixtures were fixed in 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.0). The samples were briefly rinsed in several changes of 0.1M phosphate buffer (pH 7.0) and postfixed in 1% osmium tetroxide in 0.1M phosphate/imidazole buffer (pH 7.0) (1:1, v/v) 12 h. The agar mixtures were also postfixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.0) alone. After dehydration in a graded series of ethanol, the samples were embedded in Spurr resin and polymerized in a vacuum oven at 70°C.

Sectioning was done on a Sorvall Porter-Blum ultramicrotome. The sections were picked up on carbon-coated Formvar grids, stained with uranyl acetate followed by Reynold's lead citrate and examined with a Philips 300 TEM at 80 kV.

SEM-Peanut Oil/Casein

Peanut oil/casein emulsions were prepared as described by Haque and Kinsella (1988). They were mixed with 4% warm agar. A spatula was used to lift the gelled emulsion, causing several free fractures and exposing fresh surfaces for examination. The samples were treated as described in the TEM section (peanut oil/BSA) until the dehydration step. The SEM samples were dehydrated in a graded series of ethanol and critical point dried in a Tousimis Auto Dri critical point drier. The fresh surface of the samples were oriented for viewing, mounted on aluminum stubs covered with double sticky tape, and coated with gold/palladium in a Balzers Union Sputter Coater.

SEM - Ice Cream Mix

Ice cream mix emulsions mixed with 4% agar, 2% agarose or encapsulated in agar tubes were prepared as described in the TEM section (ice cream mix) up to and including the ethanol dehydration step. Fresh surfaces of the 4% agar and 2% agarose mixtures were obtained as described in SEM section (peanut oil/casein). The agar tube samples were sliced lengthwise with a razor blade after critical point drying. Specimen samples were taken from areas untouched by the razor blade. Samples near the center of the tube rather the ends were selected in order to avoid the areas most exposed to heat. The samples were critical point dried in a Tousimis Auto Dri critical point drier, mounted on aluminum stubs covered with double-sticky tape

and coated with gold/palladium in a Balzer's Union sputter coater.

All SEM samples were examined with an AMRay 1000 SEM at 10 kV.

Results and Discussion

The first emulsions studied, peanut oil/BSA, were mixed with warm 2% agar, fixed in 4% glutaraldehyde and postfixed in 1% osmium tetroxide. This preparation resulted in fat globules that were well-preserved, regular in shape, and surrounded by a fine, evenly distributed protein precipitate (Figure 1).

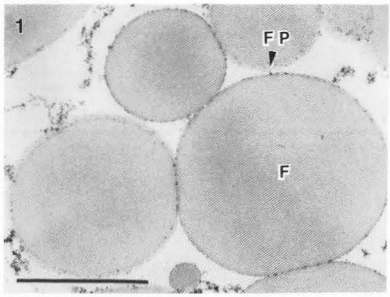


Figure 1. TEM micrograph of peanut oil/BSA emulsion mixed with agar. No acetone treatment. F = well-preserved fat globule. FP = fine protein precipitate. Bar = 1 μm.

The same emulsions were prepared as described above except for a brief acetone rinse between the glutaraldehyde and osmium step. This step was included as a result of the observation by Henstra and Schmidt (1970) that in milk, the saturated fat which is not fixed with osmium tetroxide was extracted during the dehydration, leaving an empty space surrounded by a well-stained fat globule membrane. Since peanut oil is composed of unsaturated fat which is well fixed with osmium tetroxide, a brief acetone rinse before the osmium step was tested in order to extract the unsaturated fat, possibly enhancing TEM visualization of the protein film at the lipid interface. The acetone treated emulsion also yielded well-preserved fat globules which had a regular shape, but the protein precipitate surrounding the globules was denser and less homogeneous in size. The distribution of the precipitate around the globules was uneven (Figure 2). The fat globules in the acetone treated samples did not appear to be less densely stained than those in the non-acetone treated samples, suggesting that the acetone rinse may have been too brief to extract the fat.

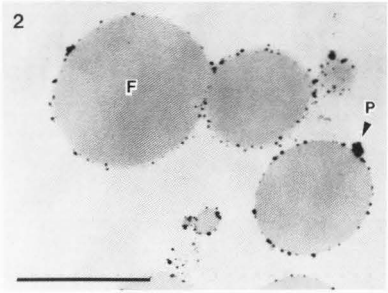


Figure 2. TEM micrograph of peanut oil/BSA emulsion mixed with agar. Acetone treatment. F = globule. P = protein precipitate. Bar = 1 μm.

Loops were tested in order to avoid the sample dilution and agar network visualization associated with the above methods as noted by Kalab (1981). Loop preparation yielded fat globules with an angular shape surrounded by very dense protein precipitates (Figure 3). It was possible that surface tension changes within the emulsion suspended in the loop caused angular distortion of the fat globules. Brooker (1985) and Brooker et al. (1986), working on milk foams and whipped cream suspended in loops over fixative vapors, obtained excellent results. However in this study, the loop method was time consuming, tedious and did not always yield usable material since the emulsion droplet sometimes fell out of the loop after a period of time.

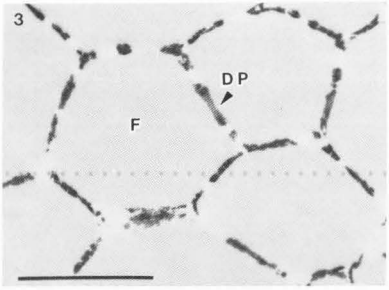
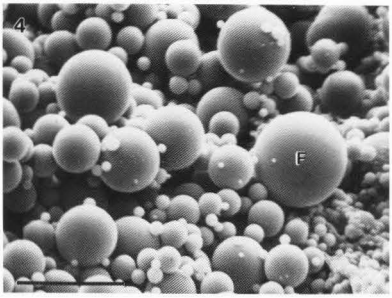


Figure 3. TEM micrograph of peanut oil/BSA emulsion mixed with agar. Loop method. F = well-preserved fat globule with angular shape. DP = dense protein precipitate. Bar = 1 μm.

It was concluded that the simple procedure of mixing the emulsions with agar (or agarose discussed below) without acetone treatment gave the most satisfactory results.

SEM samples of peanut oil/casein emulsions mixed with warm 4% agar, fixed in 4% glutaraldehyde and postfixed in 1% osmium tetroxide yielded well-preserved fat globules with round, regular shapes. Size distribution was well-illustrated (Figure 4). Occasionally an agar film covered the emulsion, but with careful scanning of the field it was possible to find an unobstructed area (Figure 5).



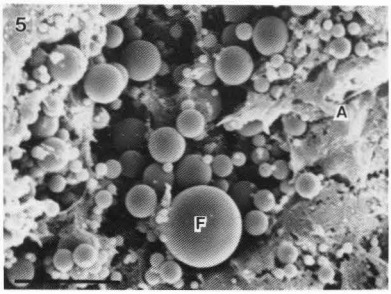
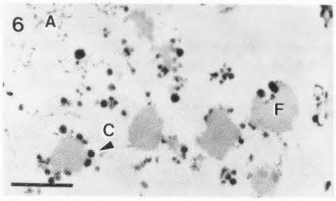


Figure 4. SEM micrograph of peanut oil/casein emulsion mixed with agar. F = well-preserved fat globule. Bar = 10μm.

Figure 5. SEM micrograph of peanut oil/casein emulsion mixed with agar. F = well-preserved fat globule. A = agar. Bar = 10μm.

Since the micrographs of peanut oil/protein emulsions mixed with agar were satisfactory, the first samples of ice cream mix emulsions were prepared using the same method (agar mix, 4% glutaraldehyde fixation, 1% osmium tetroxide post-fixation). However, since saturated fatty acids were a major component in the ice cream mix samples, osmium tetroxide was buffered in phosphate/imidazole in addition to phosphate buffer alone. Angermüller and Fahimi (1982) found that imidazole-buffered osmium tetroxide stained lipid droplets more intensely than aqueous or cacodylate buffered osmium tetroxide. They suggested that unsaturated fatty acids, particularly linolenic, oleic and linoleic acid reacted intensely with imidazole-buffered osmium tetroxide. Allan-Wojtas and Kalab (1984a) and Kalab (1985) in a study of yoghurt, found that the liquid matrix of the fat globule assumed to be composed of unsaturated fatty acids such as oleic and linoleic acids were deeply stained when postfixed with osmium tetroxide in veronal-acetate/imidazole buffer. The lightly stained fat crystals composed of saturated fatty acids contrasted sharply with the well-stained unsaturated fatty acids.



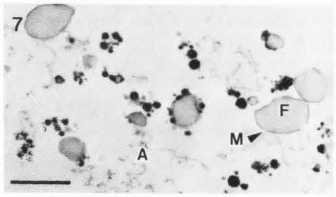


Figure 6. TEM of ice cream mix emulsion mixed with agar and postfixed in phosphate-buffered osmium tetroxide illustrating fat globule distortion and lack of fat globule membrane. F = fat globule. A = agar fibrils. C = casein micelle. Bar = 1μm.

Figure 7. TEM micrograph of ice cream mix emulsion mixed with agar, postfixed in imidazole/phosphate buffered osmium tetroxide illustrating fat globules that are somewhat distorted. The fat globule membrane is clearly evident. F= fat globule. A = agar fibrils. M = fat globule membrane. Bar = 1μm.

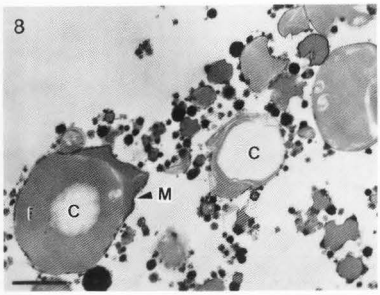


Figure 8. TEM micrograph of ice cream mix emulsion prepared in agar microtubes illustrating fat globule distortion, clearly evident fat globule membrane and melted fat crystals. F = fat globule. M = fat globule membrane. C = melted fat crystals. Bar = 1 μm.

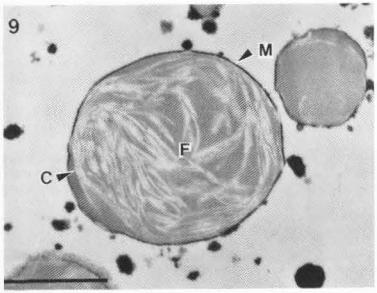


Figure 9. TEM micrograph of ice cream mix emulsion mixed with agarose fixed in 4% glutaraldehyde illustrating well-preserved fat globule, clearly evident fat globule membrane and well-delineated fat crystals. F = fat globule. M = fat globule membrane. C = fat crystals. Bar = 1 μm.

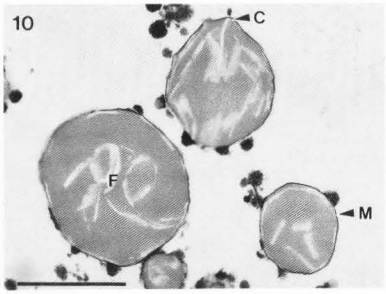


Figure 10. TEM micrograph of ice cream mix emulsion mixed with agarose, fixed in 2% glutaraldehyde illustrating well-preserved fat globule, clearly evident fat globule membrane and well-delineated fat crystals. C = fat crystal adjacent to distorted membrane. F = fat globule. M = fat globule membrane. Bar = 1 μm.

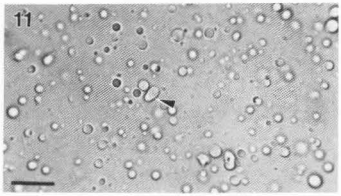


Figure 11. Light micrograph under oil immersion of diluted ice cream mix emulsion in depression slide. Arrow indicates a distorted area in the fat globule. Bar = 10 μm.

Ice cream mix emulsions postfixed in phosphate-buffered osmium tetroxide contained fat globules that appeared distorted and disrupted. There were no visible fat globule membranes and no fat crystals were evident (Figure 6). The emulsions postfixed in phosphate/imidazole buffered osmium tetroxide contained fat globules whose shape appeared somewhat less disrupted but the fat globule membranes were clearly visible (Figure 7).

It was concluded that the presence of imidazole in phosphate-buffered osmium tetroxide improved the image of the fat globule membrane (Figure 6 vs. Figure 7). It appeared that unsaturated fatty acids present in the fat globule membrane reacted with the imidazole, producing a well-stained membrane. A similar reaction was noted between the fat globule matrix of yoghurt and imidazole (Allan-Wojtas and Kalab, 1984a; Kalab, 1985) discussed earlier in this section. In addition, this reaction may have resulted in a less delicate membrane. This could have contributed to fat globule stability causing less disruption to globular shape (Figure 6 vs. Figure 7).

In order to improve on these results, a number of different procedures were studied. The ice cream mix emulsions were prepared in agar tubes. This procedure drastically reduced the amount of time the samples were in contact with heat, which could cause distortion of fat

globule shape. Henstra and Schmidt (1970) and Jewell (1981) used variations of this technique introduced by Salyaev (1968) for the preparation of milk and orange juice for TEM. Allan-Wojtas and Kalab (1984b) used this method for preparation of yoghurt for SEM. After preparation of samples in the agar tubes, they were fixed in 4% glutaraldehyde and postfixed in 1% osmium in phosphate/imidazole buffer. The membranes of the fat globules were well-defined with this preparation (Figure 8). However, gross distortion of many fat globules was evident and the fat crystals seemed to have melted, losing their definition (Figure 8). Therefore, it appeared that ice cream mix emulsions were very sensitive to temperature changes. The brief exposure to heat when sealing the agar tubes appeared to have caused melting of some of the fat crystals and fat globule distortion.

In order to circumvent heat induced alterations, the ice cream mix emulsions were mixed with 2% agarose (Sea-Prep) which is liquid at room temperature. To process single cells for TEM, Yuan and Gulyas (1981) used an agarose preparation (Sea Plaque Agarose, Marine Colloids Inc., Biomedical System, Rockland, ME) that gels at 30°C. However, Strausbauch et al. (1985) used the newly introduced Sea Prep agarose for TEM preparation of cell suspensions. They found that 2% agarose solutions were best suited for this purpose. Concentrations of less than 1% did not give consistent gelling while higher concentrations were difficult to prepare.

The samples were fixed in 2% or 4% glutaraldehyde and postfixed in 1% osmium tetroxide in phosphate/imidazole buffer. Ice cream mix emulsions fixed in 4% glutaraldehyde showed well-preserved fat globules which had minimal irregularities in shape compared to the fat globules in Figures 6, 7 and 8. The membrane was clearly evident and the fat crystals were well-delineated (Figure 9). Fixation in 2% glutaraldehyde was compared to 4% glutaraldehyde and seemed to have had no noticeable effect. The membrane was clearly evident and the fat crystals were well delineated (Figure 10). There were some fat globules that were more regular in their shape than others. Some of the fat crystals appeared to cause distortion of the fat globule membrane (Figure 10). In order to determine whether fat globules were distorted before preparation for TEM study, light micrographs were taken of diluted ice cream mix emulsion (1 drop emulsion: 10 drops distilled, deionized H_2O) in a depression slide under oil immersion. Light microscopy illustrated that some fat globules were distorted before preparation for TEM study (Figure 11). Van Boekel and Walstra (1981) hypothesized that crystals in an oil-in-water emulsion may protrude through the membrane into the aqueous phase. Darling (1982) suggested that fat crystals pierce the film between two approaching interfaces in dairy emulsions. The TEM and light microscopic data indicated that in some cases this appeared to be the mechanism that caused fat globule distortion.

SEM of ice cream mix emulsions proved unsatisfactory. The fat globules were obscured by either agar or an undefined precipitate probably composed of proteins. This occurred regardless of whether the emulsions were mixed with agar, agarose or in agar microtubes.

It was concluded that for TEM study of liquid emulsions, especially those sensitive to heat such as ice cream mix emulsions, mixing with agarose was preferable to mixing with agar or preparation in agar tubes. This technique minimized fat globule distortion and prevented melting of the fat crystals. Postfixation in phosphate/imidazole buffered osmium tetroxide resulted in well-preserved fat globules, clearly defined membranes and well-delineated fat crystals. These results correlate well with those reported by Allan-Wojtas and Kalab (1984a) and Kalab (1985). Fixation in 2% or 4% glutaraldehyde produced similar results.

Further study on ice cream mix emulsions using cold stage SEM on frozen, hydrated specimens may produce useful results.

Acknowledgements

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Discussion with Reviewers

M. Kalab: Do the authors have any explanation why the "loop method" was unreliable? I used Brooker's loop procedure too and obtained excellent results with various foams.

Authors: It appeared that the surface tension between the loop and some of the emulsions was not strong enough to allow the emulsions to remain intact until the completion of EM preparation procedures. Many times the emulsions destabilized and "fell out" of the loop several hours after preparation resulting in a complete loss of sample.

R. Martin: How do you know the absence of fat crystals is due to heat and not a fixation artefact?

Authors: Samples mixed with 4% agar (Figure 7), encapsulated in agar tubes (Figure 8) and mixed with 2% agarose (Figure 9) were fixed (4% glutaraldehyde) and postfixed (1% osmium tetroxide in phosphate/imidazole buffer) in a similar manner. Since there was no exposure to heat while preparing samples with 2% agarose, we believe that this was the variable that affected preservation of fat crystals. In addition, there appeared to be a temperature dependent progression of fat crystal preservation. Samples mixed with agar were subject to the most heat and had few, if any, fat crystals. Samples encapsulated in agar tubes thereby subject to less heat than the agar mix exhibited fat crystals but in many cases they appeared to have melted. The samples mixed with agarose exhibited the most satisfactory fat crystal preservation.

R. Martin: Are these fat crystals or merely the location where fat crystals were once present?

Authors: We believe that these are fat crystals because they are stained, albeit very slightly, compared to the background. However, without conducting chemical analyses of the residues after fixation, postfixation and dehydration, it cannot be stated with certainty what proportion, if any, of the saturated or unsaturated fatty acids were washed out. Allan-Wojtas and Kalab (1984a) and Kalab (1985) observed fat crystals in yoghurt very similar in appearance to those we observed.

B.E. Brooker: Perhaps some explanation might be given to account for the great difference in appearance of the fat globule membranes in Figures 1 and 2.

Authors: It is possible that protein membrane surrounding the fat globule is very fragile, even after fixation with glutaraldehyde. The acetone appears to have caused the protein to coagulate but additional work on this aspect of sample preparation is needed.

B.E. Brooker: Are the authors implying a connection between crystal melting and globule distortion? If not, will they please comment on the possible cause of fat globule distortion.

Authors: We believe that the primary cause of

fat globule distortion is due to heat, although fat globule melting could be a contributing factor. Preliminary experiments with emulsions composed solely of unsaturated fatty acids and protein (devoid of fat crystals) also exhibited globule distortion. The minimal fat globule distortion noted in the 2% agarose/emulsion might be explained by the hypotheses of Darling (1982) and Van Boekel and Walstra (1981) stated briefly in the text.

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LACTOSE CRYSTALLIZATION IN COMMERCIAL WHEY POWDERS AND IN SPRAY-DRIED LACTOSE

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Abstract

Two kinds of whey powder, one containing only amorphous lactose, and the other containing precrystallized lactose, and spray-dried amorphous lactose were examined for particle structure by scanning electron microscopy. Lactose crystallization under 75% relative humidity was studied by X-ray diffraction analysis. Scanning electron microscopy appeared to detect changes due to lactose crystallization on the smooth surface of the particles examined. Lactose crystallization started within 30 minutes after exposure to the humid atmosphere. Both α-lactose hydrate and β-lactose crystals were detected in the spray-dried amorphous lactose by X-ray diffraction within 40 minutes under the humid conditions. Only crystals of α-lactose hydrate developed in the whey powders. Additional crystallization of lactose in the amorphous portion of precrystallized whey powder was seen by scanning electron microscopy but increased crystallinity was not clearly detected by X-ray diffraction analysis. Several physical treatments involving storage at -20 to 55°C along with a desiccating agent, failed to crystallize β-lactose in the whey powders. It is hypothesized that the presence of milk serum solids inhibited the crystallization of β-lactose.

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Key Words: Whey powder, amorphous lactose, spray-dried lactose, lactose hydrate, beta-lactose, X-ray diffraction, crystallization, scanning electron microscopy.

Introduction

Utilization of whey is attracting the attention of researchers and manufacturers in the dairy industry because of a) effective utilization of whey proteins and lactose (Zall, 1984), and b) whey disposal problems. Lactose is being used for various purposes, including as a constituent in dairy foods (Hobman, 1983; Zadow, 1983). Whey powder is the main product produced from whey. The whey drying process has been summarized by many researchers (Webb and Whittier, 1948; Hall and Hedrick, 1971; Morrissey, 1985). Some reports showing the particle structure of whey powder have been published (Buma and Henstra, 1971a; Roetman, 1979; Linko et al., 1981, Saltmarch and Labuza, 1980a,b; Saito and Taguchi, 1980), although they are not as numerous as those concerning the structure of skim milk powder particles (Verhey, 1972; Buma, 1978; Roetman, 1979; Saito, 1985). The composition of whey varies slightly depending on the type of cheese that is made due to differences in processing. Demineralization processes using either ion exchange resin or electric dialysis may be applied to whey depending on the requirements of the users of the whey powder. This is another reason for variation in the composition of the whey powder. Lactose, however, is always the major constituent of whey powder, being present at 65 to 88% (Hall and Hedrick, 1971) by mass. Properties of lactose are reflected in the behavior of the whey powder particles. There are two types of whey powder in respect to the physical state of lactose. One type contains the amorphous form of lactose, and the other type contains both the crystalline and the amorphous forms. In either type, whey proteins are dispersed in a continuous phase of amorphous lactose having the β/α ratio of 1.25 (Roetman and Schaik, 1975). Since lactose in the amorphous state is highly hygroscopic, the whey powder which is spray-dried without the crystallization of lactose is also highly hygroscopic. On the other hand, a whey powder in which crystallization of lactose is allowed to take place before and/or after drying is considerably less hygroscopic and flows easily. The properties of whey powder in relation to the characteristics of lactose, particularly its physical state, have been discussed in other publications (Nickerson, 1974; Hynd, 1980; Parkinson, 1980).

It generally has been accepted that in spray-dried milk powder lactose occurs in the amorphous state and crystallizes as α-lactose hydrate in skim milk powder as reviewed by King (1965), in whole-

milk powder (Troy and Sharp, 1930; Saito, 1985), and in buttermilk powder (Kalab, 1980) by moisture uptake from the atmosphere. Lactose crystallization is also important in whey powder and must be controlled for desired flow and solubility characteristics. Amorphous lactose in whey powder behaves like that in skim milk powder with respect to the transition into its crystalline form (Roetman, 1979; Saltmarch and Labuza, 1980a,b).

The processes usually applied to produce dried milk and whey powder cannot lead to the crystallization of β-lactose (Webb and Whittier, 1948). In a few cases, however, crystalline β-lactose was detected in dried milk (Knoop and Samhammer, 1962), and in drum-dried whey (Sharp, 1938; Sharp and Doob, 1941). Crystals of β-lactose also developed in whole milk powder stored in a sealed metal can at 60°C (Würsch et al., 1984), and that stored at low humidity at 37°C for 5 months (Saito, 1985). Saito (1986) also treated instant skim milk powder in the same manner as the whole milk powder, and found crystals of β-lactose and α-lactose hydrate, even though the X-ray diffraction peaks showing crystallization were lower in the skim milk powder than in the whole milk powder. Subsequent storage under 75% relative humidity at 37°C for 2 days resulted in the disappearance of β-lactose crystals and an increase of α-lactose hydrate crystals in the instant skim milk powder, whereas no change (and thus no formation of α-lactose hydrate) was observed in the whole milk powder. In other literature (Knoop and Samhammer, 1962), formation and detection of β-lactose crystals have been described only in a limited number of whole milk powders, but never in other spray-dried products such as skim milk powder and whey powder.

Some patents describe a process where seeding a partially crystallized whey with β-lactose at a temperature above 93.3°C was applied to prepare a dried whey in which lactose crystallized in the β-form (Webb and Whittier, 1948). Many procedures to crystallize β-lactose from aqueous solutions have been published (Olano et al., 1983). Crystalline β-lactose also was detected in amorphous lactose after moisture uptake (Herrington, 1934) and on the surface of crystals of α-lactose hydrate (Sharp, 1938).

In a pervious paper (Saito, 1985), lactose crystallization in whole milk powders and skim milk powders as well as the structure of the powder particles were studied. The present paper extends the previous work, and includes a study of the particle structures of whey powder and spray-dried lactose, and the effect of milk constituents on the crystallization of lactose.

Materials and Methods

Whey powders: Three commercial spray-dried powders were used. Lactose was present only in the amorphous form in two of them (ordinary whey powder and partially demineralized whey powder). In the third powder (precrystallized whey powder), lactose was partially crystallized prior to drying.

Spray-dried lactose: A 33% lactose solution was kept at 70°C for 1 h and then was spray-dried (drying air: 160°C at inlet, 90°C at outlet).

The materials described above were packed in a polyethylene bag, then placed in another polyethylene bag along with a drying agent (silica gel), and kept at room temperature until used.

Scanning electron microscopic examination was carried out without any fixation procedure. The samples were sprinkled on a piece of a double adhesive tape attached to a specimen holder, coated with gold by an ion-sputtering method, and observed in a JEOL 25 SII scanning electron microscope (SEM) operating at an accelerating voltage of 15 kV.

A computerized X-ray diffractometer (Rigaku Geigerflex RADIIA, Cu Target) was used to obtain X-ray diffraction patterns. The forms of crystalline lactose were determined by comparing X-ray diffraction patterns with published patterns (Knoop and Samhammer, 1962; Buma, 1967; Saito, 1985).

Relative humidity (RH) of 75% was provided by placing a saturated sodium chloride solution in a closed container (Rockland 1960) at room temperature.

To learn how rapidly the lactose crystallized, the following procedures were attempted. Small amounts of the samples were attached to the adhesive tape on a specimen holder for SEM and kept under humid conditions (75% RH) for 30 min and then coated with gold. Samples were also packed in a shallow dent on a glass plate, which served as a sample holder for X-ray analysis, and kept for 40 min at 75% RH prior to analysis by X-ray diffraction.

Results

Structure of whey powder particles

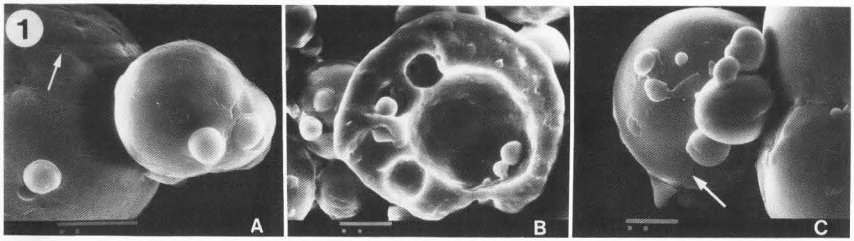
Whey powder particles seemed to be more fragile than skim milk powder particles and were easily damaged by the electron beam in a way similar to that demonstrated by Kalab and Emmons (1974).

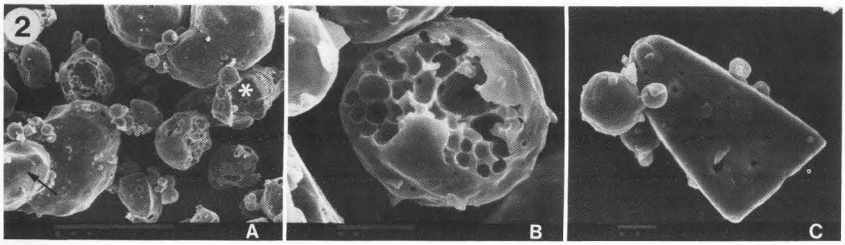
SEM demonstrated that particles of ordinary and demineralized whey powders were spherical (Fig. 1). Demineralization apparently had no effect on particles structure. Surface folds and dents such as those observed in skim milk powder (Roetman, 1979; Saito, 1985) were not present in whey powder particles. Their absence had already been mentioned by Buma and Henstra (1971a). Broken particles showed vacuoles of various sizes (Fig. 1B). Thin streaks, which could be a type of crack, were observed on the surface of particles (Figs. 1A,C). It is unknown how these streaks formed. The occasional presence of thin streaks was also in agreement with the observations of Buma and Henstra (1971a).

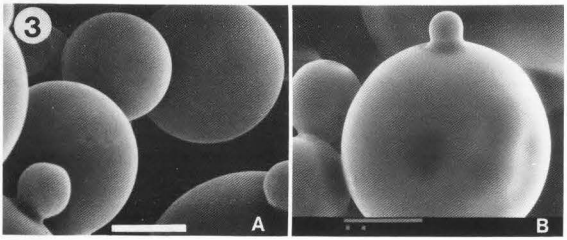
The precrystallized whey powder contained spherical particles and lactose crystals covered with amorphous lactose and other constituents such as whey proteins (Fig. 2). A somewhat squared structure was observed in some of the spherical particles suggesting inclusion of lactose crystals even in the particles (Fig. 2A). Small pieces of dust-like material, probably parts of crushed particles, were adhering to the surface of the powder particles (Figs. 2A,B). Some crystals were covered only slightly with other constituents (Fig. 2C), but contained small holes in the surface which were probably caused by evaporation of water from the covering material, i.e., amorphous lactose involving whey proteins and salts.

Structure of spray-dried lactose particles

Particles of spray-dried lactose were intact spheres of various sizes having smooth surfaces (Fig. 3) as was shown earlier by Roetman (1979) and Buma and Henstra (1971b). Deformed particles were rarely observed, even though some of the large particles carried small particles on them (Fig. 3B).







Figs. 1 - 3. Scanning electron micrographs. Bars = 10 μm, except in 2A = 100 μm.
Fig. 1. A,B = ordinary whey powder, B = broken particle showing vacuoles, C = demineralized whey powder. Arrows show streak.
Fig. 2. Precrystallized whey powder. A,B = spherical particles and lactose crystal (*) formed before spray drying and covered with whey solids. C = lactose crystal slightly covered with whey solids. Arrow shows square structure in a particle.
Fig. 3. Spray-dried lactose.

Crystallization of lactose under humid conditions

Amorphous lactose in the ordinary as well as in the demineralized whey powder and in spray-dried lactose crystallized easily by uptake of moisture under a 75% RH.

SEM observations of the particles of whey powder (Fig. 4A) and spray-dried lactose (Fig. 4B) attached on specimen holders and exposed to 75% RH for 30 min revealed that lactose crystallization was initiated on the surface of the particles within such a short time. However a sample, of about 5 g, placed in a small beaker (4.2 x 6.0 cm, height of powder: 0.6 cm) and kept under 75% RH for 30 min at room temperature did not show the peaks of lactose crystals in X-ray diffraction pattern. Crystallization of lactose during 30 min might be limited to the surface portion of particles in the upper zone and not detected by X-ray diffraction. Thus, scanning electron microscopy was more sensitive than X-ray diffraction analysis for detecting initial crystallization of lactose in whey powder particles.

The X-ray diffraction pattern for spray-dried lactose (packed in a dent of a sample holder for X-ray analysis and exposed to 75% RH for 40 min) showed the formation of crystals of α-lactose hydrate and β-lactose. Such patterns, which would correspond to lactose crystals, could not be shown by X-ray diffraction in the two whey powders under study.

Lactose crystals developed sufficiently within 1 day under 75% RH in both whey powders and in spray-dried lactose. Electron micrographs of the whey powders and the spray-dried lactose kept for 10 h and for 3 - 6 days in the humid atmosphere are shown in Figs. 5 and 6. Additional crystallization of lactose in the precrystallized whey powder seemed to proceed only to a small extent and produced tiny crystals on the surface of the original crystals as well as on the spherical particles (Figs. 5C,D). Spray-dried lactose retained its spherical structure after crystallization (Figs. 6A,C) but the inner

structure consisted just of an aggregate of lactose crystals (Figs. 6A,B).

According to the X-ray diffraction patterns, the crystals formed in spray-dried lactose were a mixture of α-and β-forms (Fig. 7A) similar to some roller-dried milk powder (Knoop and Samhammer, 1962), whereas the crystals in ordinary whey powder (Fig. 7B) were α-lactose hydrate.

In the case of the precrystallized whey powder, in which some of the lactose had crystallized as α-lactose hydrate (Fig. 7D), the proof of the additional development of crystals was supported by SEM observations of coarser particle surfaces following the uptake of moisture. The changes in X-ray diffraction patterns, however, were small and did not clearly demonstrate any additional crystallization of lactose (Fig. 7C).

To find a physical treatment which might promote the development of β-lactose crystals, as in the case of whole milk powder (Saito, 1985), the storage of whey powders and spray-dried lactose for 6 months at 37°C and 55°C together with a desiccating agent (silica gel) was attempted. The samples were also stored at -20°C to 5°C for 6 months. All these treatments failed to cause the crystallization of either α-lactose hydrate or β-lactose. The samples treated as above produced lactose crystals in the same manner as the untreated sample by subsequent exposure to 75% RH for 1 day.

Discussion

Particles of spray-dried lactose were almost ideal spheres. On the other hand, the ordinary whey powder containing only amorphous lactose contained some deformed particles. This deformation was probably related to the particle size and to the presence of whey proteins. The latter might affect the passage of moisture from the interior of the particles to the surface at the time of drying. The particles of skim milk powder, whole milk powder, and whey powder were similar in that they all were spherical, but differed in surface structure. Buma and Henstra (1971b) reported that casein micelles may cause folds and dents on the surface of skim milk powder particles. According to their results, the smooth surface of whey powder particles was due to the absence of casein.

Only α-lactose crystallized as a monohydrate in whey powders after moisture uptake. Impurities, i.e., whey proteins and salts, must influence the initiation and progression of the crystallization process in various ways. Nickerson and Moore (1974) demonstrated that demineralized whey accelerated the crystallization of α-lactose from supersaturated solutions, even though the continued presence of the impurities depressed the rate of crystal growth. It has been reported that β-lactose could retard the growth rate of α-lactose hydrate (Michaels and van Kreveld, 1966).

In the case of precrystallized whey powder, the crystallization of lactose under humid conditions was much less noticeable than in the whey powder which contained only amorphous lactose. Precrystallized whey powder contains not only crystalline lactose but also amorphous lactose, in which whey proteins and salts are concentrated. Crystallization of lactose from the amorphous state may be retarded by whey proteins and β-lactose. Further growth of precrystallized lactose crystals may not be anticipated because of contamination of the crystal surface with impurities. Therefore, the growth of lactose crystal was limited.

Bushill et al. (1965) demonstrated an unusual crystalline form of lactose, an anhydrous molecular compound consisting of α-lactose and β-lactose in a molecular ratio of 5:3, in a spray-dried lactose solution after absorption of moisture and its subsequent loss. In the present study, the X-ray diffraction patterns of spray-dried lactose kept under humid conditions consisted of a combination of typical patterns of α-and β-forms, i.e., there were peaks at $2\theta = 22.0°$, $16.4°$ (α-form); and $2\theta = 20.9°$, $10.5°$ (β-form); whereas $2\theta = 22.0°$, which was a typical peak for the unusual crystalline form reported by Bushill et al. (1965), was missing. The crystals, which appeared in the particles of spray-dried lactose after the uptake of moisture were, therefore, a mixture of α-lactose hydrate and β-lactose, and not the anhydrous molecular compound of Bushill et al. (1965). It is, however, possible that the unusual crystalline form develops after subsequent drying and/or prolonged storage.

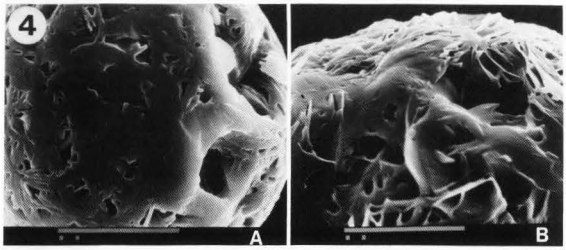
Since amorphous lactose is in a supersaturated state of both α-and β-lactose, both forms may crystallize if right conditions to initiate crystallization exist. No information about the crystallization of β-lactose form an amorphous state is available at present. Storage at relatively high temperatures did not succeed in initiating the crystallization of β-lactose unlike in the case of whole milk powder (Würsch et al., 1984, Saito, 1985). Only the uptake of moisture produced crystals of β-lactose as a mixture of α- and β-forms in spray-dried lactose. The formation of β-lactose crystals on the surface of α-lactose hydrate (Sharp, 1938) suggested a possibility that the crystallization of α-lactose could stimulate the initiation of β-lactose crystallization. In the case of whey powder, however, the surface of the α-lactose hydrate crystals was covered with other constituents which retarded additional lactose crystallization. Therefore, no crystallization of β-lactose was induced by the crystallization of α-lactose hydrate.

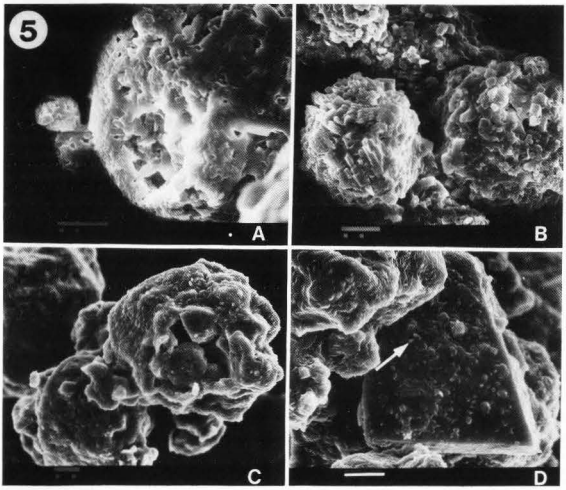
The literature available suggests that the crystallization of β-lactose from amorphous state is favored in whole milk powder (Würsch et al., 1984; Saito, 1985; Saito 1986) but not in skim milk powders and in whey powders. Milk fat might help the crystallization of β-lactose probably because free fat interferes with the movement of moisture and retards the crystallization of α-lactose as monohydrate.

The crystallization of amorphous lactose in whey powder and spray-dried lactose causes caking of them, but makes their physical properties more stable with respect to moisture uptake. Thus the crystallization of amorphous lactose, with subsequent grinding procedure, could provide rather stable types of whey powders and spray-dried lactose which are easy to handle.

Acknowledgements

The author is indebted to Snow Brand Milk Products Co. for supplying the samples of whey powder and for preparing spray-dried lactose. The author is also grateful to Yotsuba Milk Products Co. for providing another sample of whey powder. This research was supported, in part, by a grant from the CS Whey Research Association, Tokyo.





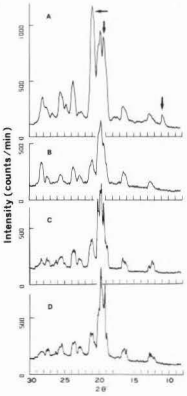


Fig. 7 (above). X-ray diffraction patterns of whey powders and spray-dried lactose. A = Spray-dried lactose, B = ordinary whey powder, C, D = precrystallized whey powder. A, B, C = kept under 75% RH at room temperature for 1 day. D = not exposed to the humid condition. Arrows show characteristic peaks of β-lactose crystal.

Figs. 4 - 6. Scanning electron micrographs. Bars = 10 μm.

Fig. 4. Ordinary whey powder (A) and spray-dried lactose (B) kept under 75% RH at room temperature for 30 min.

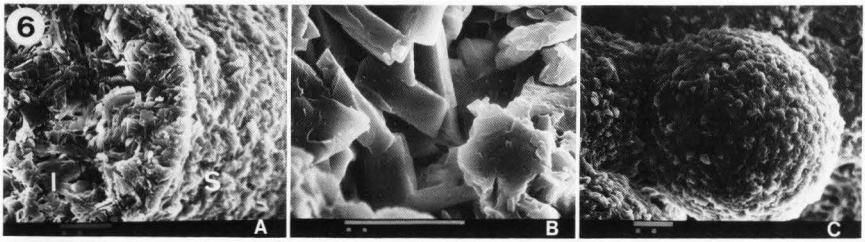


Fig. 5. Whey powders kept under 75% RH at room temperature for 10 h (A), 3 days (B), and 6 days (C, D). A, B = ordinary whey powder. C, D = precrystallized whey powder. Arrow shows tiny crystals produced on the original crystal after moisture uptake.

Fig. 6. Spray-dried lactose kept under 75% RH at room temperature for 10 h (A), 3 days (B), and 6 days (C). I = inner portion, S = particle surface.

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Discussion with Reviewers

M. Kalab: "Somewhat squared structure" inside the particles (Fig. 2A) would mean that crystallization took place before the particles were formed. I doubt it that Fig. 2C shows a lactose crystal because that structure has no attribute of a crystal such as sharp edges, smooth planes etc., but has holes.

Author: Squared structure inside the particles means that crystallization took place before the particles were formed. In the manufacturing of precrystallized whey powder, lactose crystals are dispersed in saturated lactose solution which contains salts and whey proteins, before spray-drying. Therefore, all of the lactose crystals are covered, more or less, with a continuous layer of amorphous lactose which transformed from the saturated lactose solution after drying. Some of the relatively small crystals of

lactose may be included in the particles and give squared appearance at the surface of the particle.

On the other hand, large crystals covered with amorphous lactose show the shape of lactose crystals but not sharp edges and smooth planes, typical for crystals, because amorphous lactose containing whey proteins and salts covers them.

M. Kalab: What are the practical implications of this study?

Author: Crystallization of amorphous lactose stabilizes the physical properties of whey powder and spray-dried lactose. This is convenient for users and may provide a new way in their usage. For instance, caking of spray-dried lactose containing chemicals, such as pesticide, is avoided by crystallization of amorphous lactose followed by subsequent grinding. If we could crystallize amorphous lactose in whey powder as β-lactose, even though we did not succeed, the usage of whey powder may be expanded.

Thus, crystallization of amorphous lactose has some practical implications. So I plan to continue this research to control crystallization behavior of amorphous lactose.

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DEVELOPMENT OF MICROSTRUCTURE IN RAW, FRIED, AND FRIED AND COOKED PANEER MADE FROM BUFFALO, COW, AND MIXED MILKS

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Abstract

Paneer was made from cow, buffalo, and mixed cow and buffalo milk by coagulation with citric acid at pH 5.5. All milk samples were heated to 90°C. Cow milk was coagulated at this temperature but buffalo and mixed milks were cooled to 70°C before coagulation. Differences in the composition and the treatments of the cow and buffalo milks were reflected in the composition and structure of the paneers. Electron microscopy revealed that raw paneer samples had a granular structure consisting of protein particles having a core-and-lining ultrastructure. Deep-frying in vegetable oil at 175°C for 4-5 min led to the compaction of the paneer structure and also the individual protein particles. Cooking of the fried paneers by boiling in salt water (1.5% NaCl) for 5 min resulted in partial restoration of the overall structure of the paneers and the ultrastructure of the protein particles. The restoration was most obvious in the paneer made from cow milk.

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KEY WORDS: Acidulated curd, Buffalo milk, Casein, Cooked curd, Core-and-lining structure, Electron microscopy, Fried curd, Milk, Paneer.

Introduction

Paneer is the curd obtained by acid coagulation of hot milk, subsequent drainage of whey, and washing and pressing of the curd [11]. The milk (cow, buffalo, or mixed milk) is first heated to 90°C, cooled to 70°C, and coagulated with citric acid. Citric acid is the acidulant of choice for large-scale manufacture but tartaric acid is often used by street vendors who prepare fresh paneer in small quantities for their customers. The curd is drained and washed in cold water to reduce the lactose content and then is pressed manually into patties. In India, raw paneer is very popular as an ingredient in vegetarian dishes and is also consumed after frying in peanut oil. Fried paneer may be further cooked by boiling in salt water. Paneer is also available in Canada, where it is made from cow milk.

Coagulation of cow milk at 90°C and pH 5.5 is known to lead to the formation of a core-and-lining ultrastructure of protein particles [6]. This structure is also found in Queso Blanco, the Latin-American White cheese [7], which is made by a procedure [9] similar to that used to produce paneer. One of the objectives of this study was to examine whether a similar structure develops in paneer.

Frying of paneer in oil introduces additional heat treatment to milk curd and dehydrates it to some extent. Additional structural changes may develop while fried paneer is cooked in salt water. The other objective, therefore, was to study changes in the microstructure of the fried and subsequently cooked paneer.

Materials and Methods

Preparation of paneer

Separate batches of pooled buffalo and cow milks were obtained from the herds maintained at the National Dairy Research Institute in Karnal. Cow milk had 4.0% fat and 12.8% total solids. Buffalo milk (7.7% fat and 18.2% total solids) was clarified and standardized to 6.0% fat and 16.5% total solids. Standardized buffalo milk and cow milk were mixed in equal proportions (1:1) to obtain mixed milk, which contained 5.0% fat and 14.5% total solids. Each batch (5 L) of milk was heated to 90°C without holding. It took 5 min for the milk to attain the temperature of 90°C. Cow

milk was coagulated at 90°C by the addition of 1% (w/v) citric acid. Buffalo and mixed milks were first cooled from 90°C to 70°C (it took 2 min to cool the milk) and then coagulated within 1 min. The coagulated curd was placed in cheese cloth and transferred into a wooden hoop (16x7.5x5 cm). The curd was pressed with a pressure of 0.05 kg/cm^2 for 15 min and then chilled for 2-3 h by immersion in tap water at 8° to 10°C. The chilled curd (paneer) was drained for 15 min and analyzed.

The paneer was cut into cubes (3.0x2.0x1.5 cm) and deep-fried in hydrogenated vegetable oil at 175°C for 4-5 min. The fried paneer was boiled in a 1.5% sodium chloride (w/v) solution for 5 min to simulate the usual cooking procedure.

Characterization of paneer

The moisture content of the paneer was determined by drying samples (1 to 2 g) at 100°±2°C to constant weight. The fat content of raw, fried, and fried-and-cooked paneer was determined gravimetrically by taking 1.0 to 1.5 g accurately weighed samples, digesting them in a hydrochloric acid-water (2:1, v/v) solution in a fat-extraction flask and by extracting the fat using diethyl ether and petroleum ether according to the method by Roese and Gottlieb as outlined in the Official Methods of Analysis [2].

Firmness of all paneer samples was measured using an Instron Model 4301 Universal Testing Machine equipped with a 100 N cell. A cylindrical paneer plug, 20 mm in diameter and 20 mm high, was placed on the platform and compressed to 80% of its initial height at crosshead speed of 50 mm/min. The results are expressed in mN/mm^2 [5].

Electron microscopy

Samples (approx. 1x1x10 mm) obtained at the National Dairy Research Institute in Karnal at various stages of production were fixed in a 2.8% glutaraldehyde solution, sealed in vials, and mailed to the Food Research Centre in Ottawa for electron microscopy [1]. For scanning electron microscopy (SEM), the samples were dehydrated in a graded alcohol series, defatted in chloroform, returned to alcohol, rapidly frozen in Freon 12 cooled to its freezing point with liquid nitrogen, and freeze-fractured. The fragments were melted in absolute alcohol, critical-point dried from carbon dioxide, mounted on aluminum SEM stubs, coated with gold in a Technics Hummer II sputter coater, and examined in an AMR-1000A scanning electron microscope operated at 10 kV. Micrographs were taken on 100 ASA 35-mm film [8].

For transmission electron microscopy (TEM), the samples fixed in glutaraldehyde were trimmed into pieces approximately 0.5x0.5x0.5 mm, post-fixed for 2 h in a 2% OsO_4 solution in 0.05 M veronal-acetate buffer, pH 6.8 [8], dehydrated in alcohol, embedded in a Spurr's low-viscosity medium (J. B. EM Service, Inc., Pointe Claire-Dorval, Quebec, Canada), and sectioned. Sections, approximately 90 nm thick, were stained with uranyl acetate and lead citrate solutions and examined in a Philips EM-300 electron microscope operated at 60 kV. Micrographs were taken on 35-mm film [8].

Results and Discussion

Chemical composition

Heating of the milk to 90°C prior to coagulation increases the yield of the curd because whey proteins are coprecipitated with casein in the form of a β-lactoglobulin-κ-casein complex [12]. Cooling of buffalo and mixed milk to 70°C prior to coagulation is necessary as coagulation at 90°C produces excessively firm curd with a low yield.

Table 1.

Characterization of paneer made from cow, buffalo, and mixed milks

CHARACTERISTICS	COW MILK PANEER			MIXED MILK PANEER			BUFFALO MILK PANEER		
	Raw	Fried	Fried + cooked	Raw	Fried	Fried + cooked	Raw	Fried	Fried + cooked
pH of milk	6.75	-	-	6.7	-	-	6.65	-	-
Total solids of milk (%)	12.8	-	-	14.5	-	-	16.5	-	-
Temp. of coagulation	90°C	-	-	70°C	-	-	70°C	-	-
pH of coagulation	5.50	-	-	5.40	-	-	5.4	-	-
Yield (%)	14.50	-	-	18.75	-	-	21.0	-	-
Moisture of paneer (%)	52.4	28.7	64.6	49.0	26.8	68.7	48.3	22.8	70.1
Moisture (%) relative to raw paneer	100.0	54.8	123.3	100.0	54.7	140.2	100.0	47.2	145.1
Total solids of paneer (%)	47.6	71.3	35.4	51.0	73.2	31.3	51.7	77.2	29.9
Fat content:									
(a) in paneer (%)	23.4	36.1	17.6	26.1	39.6	16.0	29.2	43.7	16.6
(b) rel. to total solids (%)	49.2	50.6	49.7	51.2	54.1	51.1	56.5	56.6	55.5
Protein content:									
(a) in paneer (%)	20.3	30.3	15.0	20.8	28.5	12.8	18.5	27.7	10.4
(b) rel. to total solids (%)	42.6	42.5	42.4	40.8	38.9	40.9	35.8	35.9	34.8
Firmness (mN/mm^2)	15.8	43.0	9.2	12.5	36.8	5.7	16.4	29.5	5.5

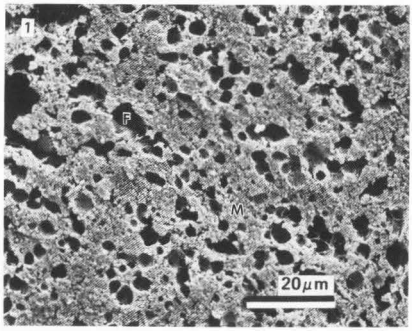
The differences in the composition of cow and buffalo milks and in the temperature of coagulation affected the composition and structure of the paneer (Table 1). Thus, the total solids content of the cow milk was 12.8%, whereas the buffalo milk contained 16.5% total solids, *i.e.*, 29% more. Because of the higher fat content of the buffalo milk, the yield (21.0%) of buffalo milk paneer was considerably higher than the yield (14.5%) of the cow milk paneer. The difference in the yield was almost 45% higher with the buffalo milk paneer, not taking its higher total solids content of 51.7% into consideration (compare with 47.6% total solids in the cow milk paneer). Of the total mass of the paneer, fat represented more than one half of it (56.5%) in the buffalo milk paneer and slightly less than one half of it (49.2%) in the cow milk paneer.

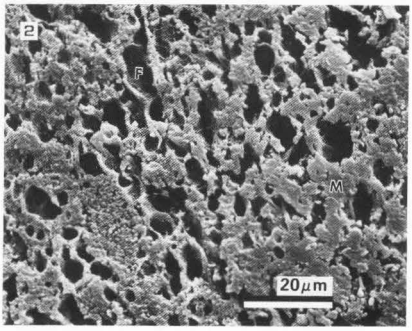
Frying of the paneers in vegetable oil substantially reduced their moisture contents. Relatively more water was lost from the cow milk paneer, where the moisture content decreased to 28.7% (*i.e.*, to 54.8% of the initial moisture present in the raw paneer) than in the buffalo milk paneer, where the moisture content decreased to 22.8% (*i.e.*, to 47% of the initial value). Contrary to what was expected, the fat content remained relatively stable during frying as well as during the subsequent cooking of the paneer; it was approximately 50% of the total solids content in the cow milk paneer and approximately 56% in the buffalo milk paneer.

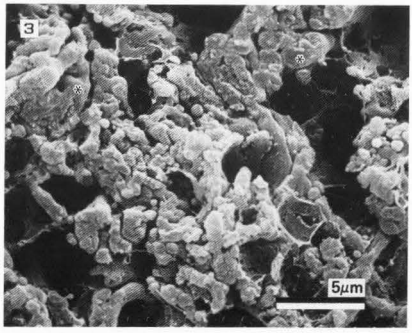
Cooking of the fried paneer increased its moisture content, and consequently, the total solids contents dropped to 29.9% in the buffalo milk paneer and to 35.4% in the cow milk paneer. Frying and cooking had a considerable effect on the firmness of the paneer made from both kinds of milk. The firmness of the cow milk paneer almost tripled by frying but dropped to about 60% of the initial value in cooked paneer. The lower protein content and the higher fat content of the raw, fried, and fried-and-cooked buffalo milk paneer probably caused the products to be softer than the cow milk paneers. The paneer samples made from mixed milk had all their parameters between the paneers made from pure cow and from buffalo milks (Table 1).

Microstructure

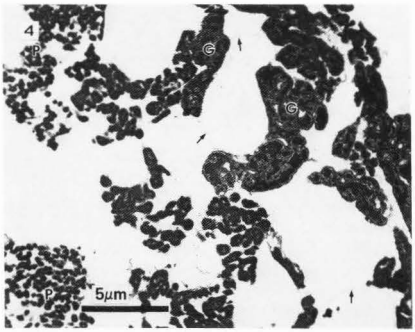
Milk in all three experimental variants was heated to 90°C, but the cow milk was coagulated with citric acid at a higher temperature (90°C) than the buffalo milk (70°C). However, the final pH values were similar in both paneers, *i.e.*, 5.4

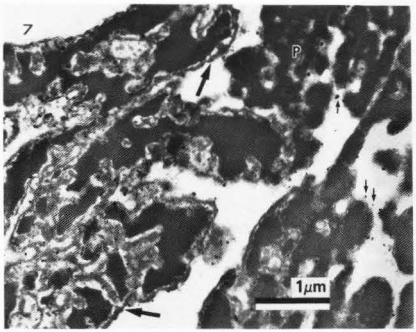


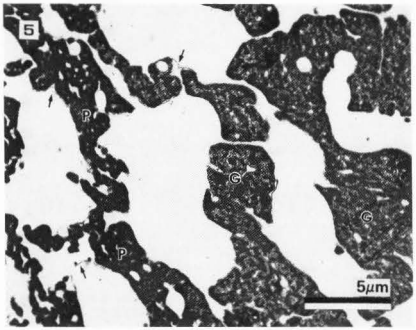


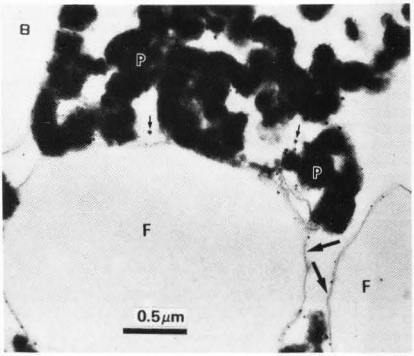


Figs. 1 and 2. Structures of raw cow milk paneer (Fig. 1) and raw buffalo milk paneer (Fig. 2). Void spaces (F) in the protein matrix (M) indicate the location of fat prior to its removal during preparative steps for SEM.
Fig. 3. Detail of the protein particles in raw cow milk paneer. Large particles are marked with asterisks.









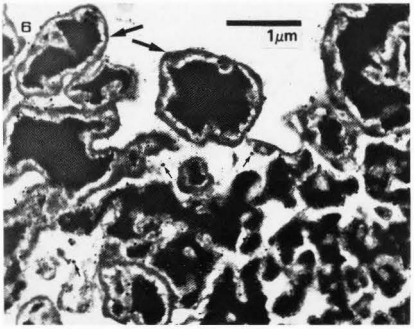


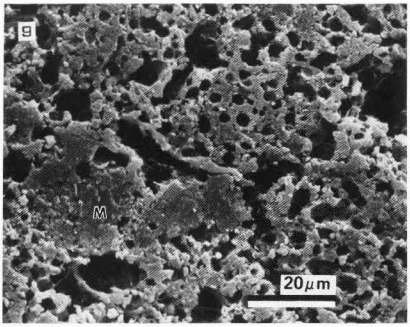
Fig. 4. Thin section of raw cow milk paneer shows large protein particles (G), small protein particles (P), and fat globule membranes (arrows). The core-and-lining structure is noticeable in the large protein particles.

Fig. 5. Thin section of raw buffalo milk paneer shows large protein particles having the core-and-lining structure (G) and clusters of individual compact protein particles (P). Remnants of fat globule membranes (arrows) are also noticeable.

Fig. 6. Detail of the core-and-lining structure (large arrows) in raw cow milk paneer. Minute black dots (small arrows) are contaminants.

Fig. 7. Detail of the core-and-lining structure (large arrows) in buffalo milk paneer. Some compacted protein particles (P) are free of the core-and-lining structure. Minute black dots (small arrows) are contaminants.

Fig. 8. Detail of fat globules (F) with compact protein particles (P) attached to the fat globule membranes (large arrows). Minute black dots (small arrows) are contaminants.



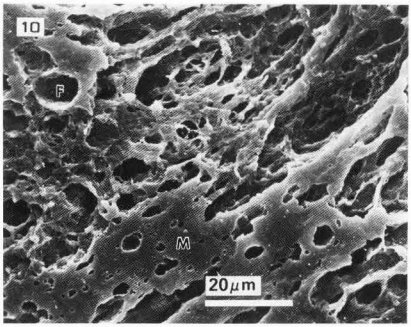
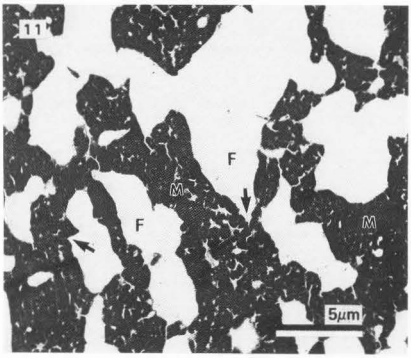


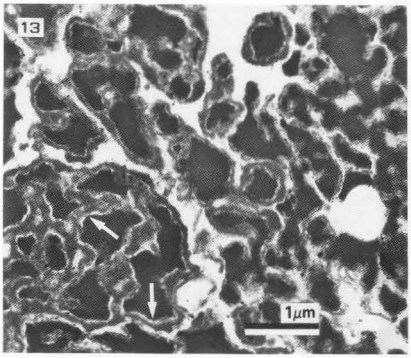
Fig. 9. SEM of cow milk paneer which had been fried in oil shows a compacted protein matrix (M).
Fig. 10. Buffalo milk paneer fried in oil consists of a severely compacted protein matrix (M). Void spaces (F) indicate the presence of fat particles in the paneer prior to the preparation of the samples for SEM.

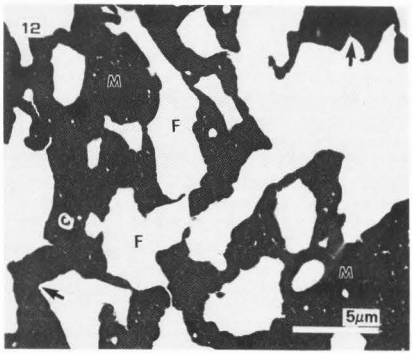
and 5.5, respectively. In the raw state, both paneers consisted of aggregated protein particles. At a low magnification, SEM showed that the structures were apparently uniform and fat globules were evenly distributed in the protein network (Figs. 1 and 2). At a higher magnification, however, protein particles varying in dimensions (Fig. 3) were observed. TEM confirmed the existence of the granular structure in the paneer and also revealed the internal ultrastructure of the protein particles (Figs. 4 to 8). In raw cow milk paneer, the small protein particles were uniform in density (Figs. 4 and 8) and resembled those found in other milk products such as Cottage cheese [4]. In larger particles, the core-and-lining structure (Fig. 6), characteristic of curd obtained by the acidulation of hot milk to pH 5.5, was well developed. A similar core-and-lining structure was found in the buffalo milk paneer (Figs. 5 and 7). Protein particles lacking this structure were also present and, in contrast to the cow milk paneer, were more densely packed and were fused. Micrographs showing areas where different structures were in close proximity to each other have been used for illustration (Figs. 4 to 7). Intact fat globules with casein particles attached to the fat globule membranes (Fig. 8) were frequently seen in the raw paneer. Minute black dots in Figs. 6 to 8 are probably contaminants consisting of a glutaraldehyde-osmium tetroxide complex which developed during the preparation of the samples for electron microscopy [10].

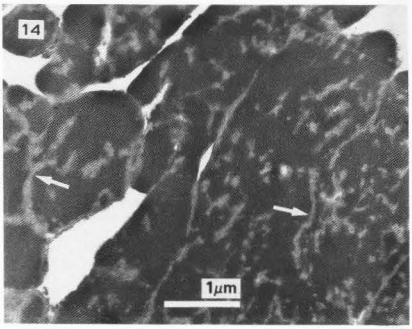
The heterogeneity in the structure of the paneer may be explained by local differences in the pH value during the coagulation of the milk. It was shown earlier [6] that pH of 5.5 is essential for the development of the core-and-lining structure. It is possible that a part of the milk may have been acidified below the critical pH value before uniform acidity was achieved in the curd by stirring. The casein particles in the areas of localized overacidulation thus did not develop the core-and-lining structure. The development of the core-and-lining ultrastructure in bovine protein particles was shown to depend on the temperature at which the milk is coagulated and the final pH value [6]. The ultrastructure develops fully only if the milk had been heated to 90°C, apparently as the result of the formation of a complex between κ-casein and β-lactoglobulin [12]. At a lower temperature, only a part of β-lactoglobulin present in the milk reacts with κ-casein; consequently, the lining around the casein particles does not develop fully. The structure of the mixed milk paneer was more similar to the buffalo milk paneer than to the cow milk paneer probably because the same lower temperature of 70°C was used to coagulate the milk. In this study, the paneer was made in accordance with the commercial operations and all three milks had been heated to 90°C. It is recognized, however, that a separate study is required to establish the relationship between the temperature at which the milk is coagulated and the texture of the resulting paneer and to examine the role of the core-and-lining ultrastructure in this relationship.

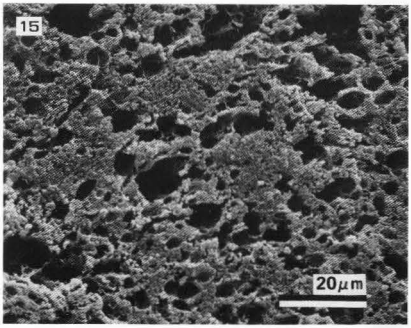
Frying in oil severely altered the structure of the paneer. SEM shows that compaction suppressed the fine granularity of the protein matrix in the cow milk paneer (Fig. 9). The granularity completely vanished in the buffalo milk paneer (Fig. 10). The compaction is even more clearly evident in TEM micrographs (Figs. 11 and 12). The structure of the protein matrices resembles that in young Cheddar cheese [3]. In spite of a severe compaction of the core-and-lining structure observed at a low magnification, the existence of this structure has been confirmed by an examination at a higher magnification (Fig. 13) although it deviates from the original structure in the buffalo paneer (Fig. 14). The compaction of the







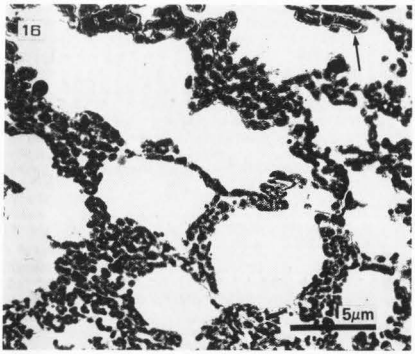


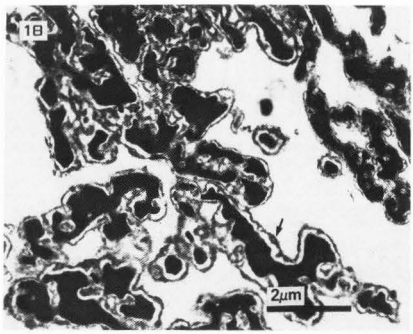


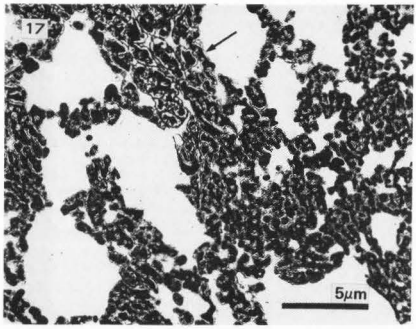
Figs. 11 and 12. *Thin sections of cow milk paneer (Fig. 11) and buffalo milk paneer (Fig. 12) which had been fried in oil show compacted protein matrices (M) and areas with sharp and pointed (arrows) outlines presumed to contain fat (F).*

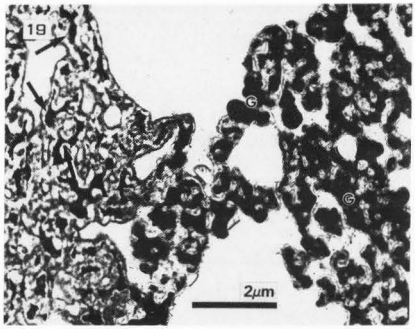
Figs. 13 and 14. *Details of the cow milk paneer (Fig. 13) and buffalo milk paneer (Fig. 14) which had been fried in oil show differences in the core-and-lining structures (arrows) in both paneers.*

Fig. 15. *SEM of cow milk paneer which had been fried and subsequently cooked in salt water. The granular structure appears to be restored (compare with Fig. 1).*









Figs. 16 - 19. Thin sections of cow milk paneer (Figs. 16 and 18) and buffalo milk paneer (Figs. 17 and 19) which had been fried in oil and subsequently cooked in salt water. The restoration of the core-and-lining structure (small arrows) to a varying extent is noticeable in all micrographs. Localized disintegration of the protein particle 'cores' (large arrows) and partial disintegration of the 'lining' (G) are noticeable in the buffalo milk paneer (Fig. 19).

paneer structure caused by frying also altered the shapes of the fat globule clusters. The fat particles acquired sharp and pointed outlines (Figs. 11 and 12) as compared to their near-globular shapes in the raw paneer. The fat globule membranes vanished or were broken and the fragments became convoluted.

Cooking in salt water restored both the granular structure of the fried paneer (Fig. 15) and the core-and-lining structure of the protein particles, particularly in the paneer made from cow milk (Fig. 16). Interestingly, swelling of the protein matrix during cooking considerably loosened even the initially more compact curd of the buffalo milk paneer (Fig. 17). The core-and-lining structure of the protein particles was also restored in both paneer samples (Figs. 18 and 19). Disintegration of the 'cores' and abundant occurrence of the 'lining', as well as the opposite situation characterized by partial disintegration of the 'lining', were localized to small areas; in this respect, the structure of the cooked buffalo milk paneer appeared to be quite different from the structure of the cooked cow milk paneer.

The finding of a stable core-and-lining structure of the milk protein particles resistant to the effects of frying in vegetable oil is in

agreement with an earlier report that this structure, which also develops in Latin-American White cheese made from cow milk, was found almost intact in process cheese in which the White cheese was part of the natural cheese blend [3].

This study showed that the microstructure of paneer, irrespective of the milk used, is similar to other milk products obtained by coagulating hot milk with an acid to a pH in the vicinity of 5.5. Frying in oil expelled water and compacted the protein matrix but did not alter its fat content to any considerable extent. Cooking of the fried paneer in salt water increased the moisture content of the product beyond the initial level and restored the structure of the curd as well as the ultrastructure of the protein particles.

Acknowledgments

Skillful technical assistance provided by Mrs. Paula Allan-Wojtas and Mr. J. Emery is acknowledged. The authors thank Dr. H. W. Modler for useful suggestions. Electron Microscope Unit, Research Branch, Agriculture Canada in Ottawa provided facilities. Contribution 760 from the Food Research Centre.

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Discussion with Reviewers

R. Nath: Please comment on organoleptic preference of variously prepared paneer by the consumer.
Authors: Most paneer in Indian cuisine is fried and is used as an ingredient in curried vegetable dishes such as muttar paneer. Some customers may prefer to buy nonfried paneer and process it at home to their liking. Paneer may also be breaded and then deep-fried.

Reviewer I: Please explain how the buffalo milk was standardized.
Authors: Addition of skimmed buffalo milk was used to reduce the fat content in the whole buffalo milk. Fine adjustment was achieved using separated buffalo cream.

Reviewer I: What is clarification and what effect has it on milk composition or microstructure?
Authors: Clarification means the removal of corpuscular contaminants such as leucocytes from milk by centrifugation. It is not supposed to affect the composition and microstructure of the milk.

Reviewer I: The authors state that "...the fat content remained relatively stable during frying...". Do the authors know whether there was any interchange of the vegetable (frying) oil and the milk fat? This means that although the total content of fat was "relatively stable", its composition could have changed. It might be interesting to know what microstructures would allow such exchange to take place. One might wonder if a "fat exchange" experiment could be done in a way analogous to the common deuterium experiment done with proteins.
Authors: The exchange of vegetable oil and milk fat was not studied. In our opinion, fat globule membranes represent a barrier which would, to some extent, limit such an exchange. The porosity of the paneer protein matrix would certainly play the essential role. Chemical differences in the fatty acid composition between vegetable oil and milk fat as detected by gas chromatography (GC) could be used to study the fat and oil exchange.

R. Cartwright: The observation of the core-and-lining structure is important in understanding the behavior of these products. Do the authors agree that this phenomenon would be a good candidate for application of gold-labeling technology

currently being practised? Also are the authors aware of any attempts to study this phenomenon using gold-labeling technology?
Authors: The core-and-lining structure in milk products has received little attention although its incidence is common to product obtained by the acidulation of hot milk to final pH of around 5.5. The gold-labeling technique will probably help explain the development of the structure. The technique would require the preparation of antibodies against κ-casein, β-lactoglobulin, and their heat-induced complex, and interacting them with gold granules in order to stain thin sections of the core-and-lining structure.

R. Cartwright: In your paper you state that a pH of 5.5 is essential for development of the core-and-lining structure. An article recently published by Haque and Kinsella [13] acknowledges the interaction of heat treated β-lactoglobulin and κ-casein at a pH of 6.8. Is it possible that the core-and-lining structure develops during heat treatment, due to the interaction between β-lactoglobulin and κ-casein? Would it then be possible that the core-and-lining structure is already present before coagulation and is reduced as the pH is lowered to 4.6 indicating a dependence on colloidal calcium or net charge of the micelle for the existence of the lining seen in the micrographs?
Authors: High temperature (>70°C) is necessary for the core-and-lining structure to develop. The presence of β-lactoglobulin is also essential for this development to take place. Recently, the milk salt system has been found to be involved in the formation of the structure [14]. Effects of the fixation of the acidulated milk at varying temperatures and the stability of the core-and-lining structure at varying pH would be interesting to study.

R. Cartwright: In the Harwalkar and Kaláb reference [6], it appears as if the micelles may have an individual lining structure. However, in some of the micrographs you present here, it appears as if a group of micelles may be surrounded by a single lining. How do you interpret the micrographs in this respect?
Authors: The appearance of the lining depends on the acid used. As the development of the core-and-lining structure in general is not yet fully understood, we don't know why in some cases the cores are considerably larger than in others.

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AMINO ACID COMPOSITION AND STRUCTURE OF CHEESE BAKED AS A PIZZA INGREDIENT IN CONVENTIONAL AND MICROWAVE OVENS

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Abstract

Amino acid compositions of stirred-curd Mozzarella, stretched Mozzarella, and process Cheddar cheeses were similar and did not change as the result of baking in a conventional oven. D-glutamic acid (D-Glu) and D-phenylalanine (D-Phe) were present at low concentrations in all cheese samples, the lowest concentrations having been found in unbaked stirred-curd Mozzarella cheese (2.7% D-Glu of total Glu present and <1.0% D-Phe of total Phe present). The highest concentrations were detected in unbaked stretched Mozzarella cheese (5.6% and 1.2%, respectively). The changes were not significant and were not the result of baking, indicating that the heat treatment during baking did not cause racemization of the amino acids.

Each cheese had a characteristic structure before baking. The structures of the Mozzarella cheeses were altered by baking in the conventional oven and also in a microwave oven and their original features such as curd granule junctions and fat globule membranes vanished. Stirred-curd Mozzarella cheese melted most rapidly and partly flowed down from the pizza dough over the edge. Electron microscopy revealed aggregation of the fat globules and a laminar orientation of the protein matrix as the result of the flow. Stretched Mozzarella cheese melted easily but did not flow away. Process Cheddar cheese melted slowly. Fat particles in this cheese aggregated only slightly during baking.

The effects of microwave baking were comparable to those produced in the conventional oven.

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KEY WORDS: Amino acid composition, Baking, Cheese, Conventional oven, Electron microscopy, Enantiomeric analysis of amino acids, Microwave oven, Process cheese, Racemization.

Introduction

Severe heating is known to induce chemical changes in proteins, e.g., losses of lysine (Lys) [12, 31], serine (Ser), and threonine (Thr) [11, 12, A. Paquet: unpublished results] and racemization, i.e., the conversion of amino acids from the L-form into the D-form [9, 11, 18, 19, 21]. Ser, aspartic acid (Asp), glutamic acid (Glu), and phenylalanine (Phe) have been reported [11] to be particularly susceptible to racemization in dietary proteins upon heat and/or alkali treatments.

Cheese is heated during processing and also in culinary practice, where heating by baking is quite common. Pizza, which is topped with shredded cheese [4, 7] is an example. In a conventional oven, the pizza surface is exposed to overheating which results in the formation of a crust on the dough until the interior of the pizza is baked. Browning of the pizza dough has been associated with a loss of Lys present in wheat proteins [31]. Heating causes the cheese to melt and the fat to separate from the protein, thus altering the original structure of the cheese. In a microwave oven, heating is generated throughout the body of the moist material [22] by oscillating water, carbohydrate, and fat molecules. The food is heated from the interior towards the surface which may be the last place to bake [1]. Thus, no crust would be formed on a pizza unless a crust-forming infrared heater is used or the dough is prepared by a newly developed process [23]. Depending on the microwave energy used, high temperature is achieved considerably more rapidly than in the conventional oven.

It has been well established in the literature [21] that protein-bound amino acids are susceptible to racemization when exposed to elevated pH or temperature [10-12, 21]. Natural dietary proteins are composed almost exclusively of L-amino acids. During the treatment of these proteins, e.g., by high temperature or by alkali, some of the L-isomers tend to partially change into the D-isomers, i.e., they racemize. Racemization is a process during which a proton is abstracted from the α-carbon of an amino acid and changes the α-carbon into a negatively charged planar carbanion. As the proton returns, it has an equal chance of rejoining the planar carbanion either from the same side, thus regenerating the original L-form, or from the opposite side of the molecule, thus forming the isomer of an opposite configuration, i.e., the D-amino acid. Racemization of amino acids in proteins has detrimental nutritional consequences because it leads to the formation of nutritionally unavailable D-amino acids as well as unhydrolyzable D-L- and L-D-peptide bonds which are generated

around the racemized amino acid residue [24-26, 29].

Earlier work on the racemization of amino acids in proteins was done using samples treated under severe conditions, some of which never occur in food processing [10, 18, 21]. One of the objectives of this study was to determine whether regular culinary treatment such as baking can cause changes in the amino acid composition and configuration of milk proteins. The effects of baking in a conventional oven on the amino acid composition and amino acid racemization were studied using 2 types of natural cheese and a process cheese. The other objective was to examine the effects of baking in the conventional oven and in a microwave oven on the microstructure of the cheeses.

Materials and Methods

Baking the cheese.

Natural cheeses (Italian-style "stretched" Mozzarella and American-style "stirred-curd" Mozzarella) and process cheese (processed Cheddar cheese) of commercial origin were used. The cheeses were shredded using a shredder with openings 3 mm in diameter. Shredded cheese was spread on a wet cotton cloth circle placed on commercially produced pizza dough discs that were, on an average, 18 cm in diameter and 1.2 cm high. All baking was done in duplicate. The cloth was used to separate the pizza dough from the cheese in order to isolate the cheese during baking for subsequent amino acid analysis and for electron microscopy. In the first run, the cloth was 16 cm in diameter to make observation of the pizza dough possible. Because the cheese flowed during baking, in the second run the cloth size was increased to 20 cm in diameter making it larger than the pizza dough. Each pizza contained 65 g of the shredded cheese in a layer 0.5-1.0 cm thick.

The temperature of the conventional oven was set at 205°C. The temperature of the pizzas was monitored using a contact thermometer inserted into the dough below the cotton cloth. The pizzas were baked for 12 min and the final temperature of the cheese was measured at several points using a contact thermometer immediately after each pizza was taken out of the oven.

Based on a preliminary run in a microwave oven (Panasonic, The Genius II model with a turntable), each pizza was baked for 4 min at the medium energy setting. The temperature of the cheese was measured only in the preliminary test. Experimental baking was carried out without interruption.

Nitrogen determination.

Cheese samples (approximately 5 g each) taken before and after baking were freeze-dried at -20°C for 24 h and were pulverized. The cheese powders were analyzed for the content of nitrogen by the automated Dumas method [2] using a Coleman Model 29 nitrogen analyzer. The values found were used to calculate the protein content by multiplication with the factor of 6.38 in order to express the contents of the individual amino acids (amino acid composition).

Amino acid analysis.

Freeze-dried cheese samples (approximately 80 mg) were each hydrolyzed in 5 mL of 6 N HCl at 110°C for 23 h. The hydrolyzate was cooled and filtered through a sintered glass disc and brought up to 10.0 mL with distilled water. Aliquots (200 uL) were brought up to 1.0 mL with a 2 M sodium citrate buffer, pH 2.2. These solutions were analyzed in a Beckman Model 120 B amino acid analyzer.

Determination of amino acid enantiomers.

The method for the separation of enantiomeric amino acids [3], recently modified and extended for the determination of enantiomers in dietary proteins [A. Paquet: unpublished results] was used as follows:

The cheese protein hydrolyzates obtained for the amino acid analysis were divided into two aliquots and evaporated *in vacuo* at 50°C. One of the two dry residues was reacted with ethoxycarbonylphenylalanine N-hydroxysuccinimide ester (Eoc-Phe-ONSu) and the other residue was reacted with ethoxycarbonylvaline N-hydroxysuccinimide ester (Eoc-Val-ONSu) in the presence of a 10% sodium bicarbonate solution (pH 7.5-8.0) in aqueous acetone (acetone and water, 1:1, v/v). The resulting ethoxycarbonylvaline dipeptides (Eoc-Val-Xx) and ethoxycarbonylphenylalanine dipeptides (Eoc-Phe-Xx) (where Xx is the amino acid under study) were isolated and analyzed by reverse phase high-pressure liquid chromatography (HPLC) (Vista Series 5000, Varian) on a C_{18}-column using aqueous acetonitrile as the solvent. Eoc-Phe-Xx was used to determine the D-isomers of polar amino acids (such as Asp, Glu, Ser, etc.) in the protein hydrolyzates as Eoc-Phe-L-Xx and Eoc-Phe-D-Xx. The enantiomers of Phe in the hydrolyzates were separated in the form of Eoc-Val-L-Phe and Eoc-Val-D-Phe. The Eoc-Phe-ONSu and Eoc-Val-ONSu reagents used in this analysis were synthesized from Eoc-Phe and Eoc-Val, respectively, that had been prepared by a standard condensation of ethoxycarbonyl chloride with Phe or with Val [8]. Esterification with N-hydroxysuccinimide was carried out as shown previously [25]. Synthetic HPLC standards were prepared using the same reactions of Eoc-Val-ONSu and Eoc-Phe-ONSu with the corresponding amino acids purchased from the Sigma Co., St. Louis, MO.

Electron microscopy.

Cheese samples taken before and after baking were examined by scanning electron microscopy (SEM) and by transmission electron microscopy (TEM). For SEM, samples 1 x 1 x 15 mm were fixed in a 2.8% aqueous glutaraldehyde solution for 48 h at 6°C, dehydrated in a graded ethanol series, defatted in chloroform, impregnated with absolute ethanol, and freeze-fractured under liquid nitrogen [16]. The fragments were critical point-dried from carbon dioxide, mounted on SEM stubs using a silver-based cement, coated with gold, and examined in an ISI DS-130 electron microscope operated at 20 kV. Micrographs were taken on 35-mm film. For TEM, 1 mm^3 samples were fixed in a 2.8% aqueous glutaraldehyde solution for 48 h at 6°C, washed with water, postfixed with a buffered (0.05 M veronal-acetate buffer, pH 6.75) 2% osmium tetroxide solution for 6 h, dehydrated in a graded ethanol series, and embedded in a low-viscosity Spurr's resin (J. B. EM Service, Inc., Pointe Claire-Dorval, Quebec, Canada). Sections (approximately 90 nm thick) were stained with uranyl acetate and lead citrate solutions and were examined in a Philips EM-300 electron microscope operated at 60 kV [14].

Results and Discussion

Amino acid composition and enantiomeric analysis of unbaked and baked cheeses.

All the three cheeses under study had similar amino acid compositions before baking that were in agreement with the data published in the literature [18, 31]. Baking increased the temperature of the cheeses as shown in Fig. 1. In the conventional oven, the increase was more gradual than in the microwave oven. Heating above

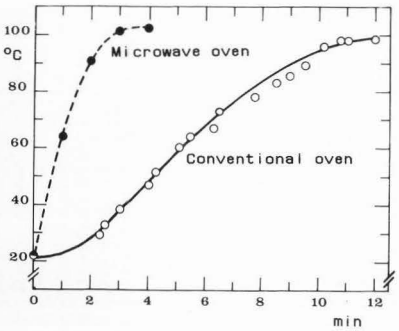


Fig. 1. Temperature of the pizzas during baking in microwave and conventional ovens. Ordinate: time in minutes. Abscissa: temperature in degrees Celsius.

80°C, that has been shown to induce changes in the protein structure of process cheese [16], lasted for 4 min in the conventional oven and for only 2.5 min in the microwave oven. However, baking in the conventional oven did not alter the amino acid composition of any of the cheeses under study and no decrease in the Ser or Thr concentrations was observed. This is interesting in view of our earlier analyses of evaporated cow and goat milks (A. Paquet: unpublished observation), where slightly decreased levels of both hydroxyamino acids were found. The loss is caused by the elimination of the hydroxy groups from the two amino acid residues in proteins treated with alkali and heated at high temperature, which leads to the formation of dehydroamino acids. The dehydroamino acids may then react (cross-link) with the terminal amino group of Lys in the protein molecule and form lysinoalanine [12]. Apparently, the effects of the heat treatment, to which the cheeses were exposed during baking in the conventional oven, were not severe enough to cause dehydration of Ser and Thr.

As baking in the conventional oven produced no significant changes in the amino acid composition of the cheese proteins, it was of interest to examine whether some of the amino acids underwent racemization. Masters and Friedman [21] determined that the rate of racemization of protein-bound amino acids decreases in the order of Asp>Phe>Glu>Ala>Pro>Val>Leu, where Asp, Phe, and Glu are racemized at a rate approximately one order higher than the other amino acids. However, Kemp [17] found that Ser was racemized even more rapidly than Asp. Recently, Liardon and Ledermann [19] also showed that Ser was most sensitive to racemization under moderate alkaline treatment of proteins. Thus it may be considered that Ser, Asp, Phe, and Glu are the most sensitive amino acids in dietary proteins toward racemization occurring at elevated pH or temperature. The side chains of these amino acids have electron-withdrawing capacity which greatly facilitates the proton abstraction from the α-carbon causing easy racemization [17, 21]. Although racemization occurs most readily as the result of the alkali treatment, it may also proceed to a lesser extent during the cooking of proteins at high temperature, particularly in the presence of lipids and/or reducing sugars [13]. Because the proteins in the cheese during baking are exposed to high temperature as well as to other effects such as the presence of salts and carbohydrates in the cheese, it was of interest to determine whether such conditions may cause racemization of the amino acids. The four amino acids mentioned above were used as indicators of the overall racemization.

Two types of diastereomeric dipeptides (Eoc-Phe-Xx and Eoc-Val-Xx) served for the detection of D-amino acids in baked cheeses. Fig. 2 shows the separation of L- and D-Phe standards as Eoc-Val-L-Phe and Eoc-Val-D-Phe. The separation of the corresponding amino acid enantiomers in baked cheeses is shown using process cheese as an example (Fig. 3). The relative concentration of D-Phe in this sample was less than 2% of the total concentration of L- and D-Phe. The stirred-curd Mozzarella and the stretched Mozzarella cheeses contained comparable concentrations of D-Phe both before and after baking (Table 1). Polar amino acids in the cheese samples were separated in the form of Eoc-Phe dipeptides, exemplified in Fig. 4. D-Asp and D-Ser were not found in any of the samples, but D-Glu was found in small quantities in all samples. The concentrations of D-Glu ranged from 2.4% of the total L- and D-Glu concentration in fresh stirred-curd Mozzarella to 6.2% in baked stretched Mozzarella. The differences between the concentrations of the D-amino acids in unbaked and baked cheeses are statistically not significant as the standard deviations in Table 1 indicate. These D-amino acids were already present in the unbaked cheeses. D-enantiomers of unpolar amino acids (Val, Leu, and isoleucine) were not detected. Potential racemization during acid hydrolysis was not considered in this study. It was shown by Manning [20] that methionine (Met) is the amino acid most susceptible

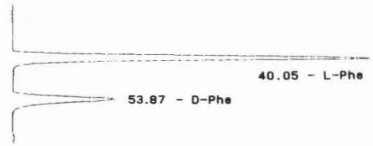


Fig. 2. High-pressure liquid chromatography separation of a standard mixture of L-Phe (retention time: 40.05 min) and D-Phe (retention time: 53.87 min) as Eoc-Val-L-Phe and Eoc-Val-D-Phe. Solvent: 83% water (containing 0.1% H_3PO_4) and 17% acetonitrile. Flow rate: 2.0 mL/min. UV detector (208 nm).

Fig. 3. High-pressure liquid chromatography separation of L-Phe (retention time: 39.87 min) and D-Phe (retention time: 53.91 min) as Eoc-Val-Xx dipeptides in baked process cheese. Solvent: 83% water (containing 0.1% H_3PO_4) and 17% acetonitrile. Flow rate: 2.0 mL/min. UV detector (208 nm).

Table 1.
Contents of D-glutamic acid and D-phenylalanine in the proteins of unbaked cheeses and cheeses baked in a conventional oven

Amino acid[a]	Stirred-curd Mozzarella		Stretched Mozzarella		Process Cheddar cheese	
	fresh	baked	fresh	baked	fresh	baked
D-Glu	2.7 ± 0.4	3.6 ± 0.5	5.6 ± 0.4	5.9 ± 0.5	<1	<1
D-Phe	<1	<1	1.2 ± 0.6	2.2 ± 0.2	<1	1.6 ± 0.2

[a]The values listed [100D/(D+L)%] represent the means from two HPLC determinations.

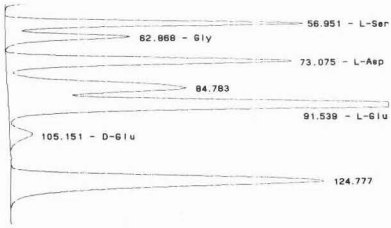


Fig. 4. High-pressure liquid chromatography separation of polar amino acids in unbaked stretched Mozzarella cheese as Eoc-Phe-Xx dipeptides. D-Glu (retention time: 105.15 min) was detected as the only D-enantiomer. Solvent: 94% water (containing 0.1% H_3PO_4) and 6% acetonitrile. Flow rate: 3.0 mL/min. UV detector (208 nm).

to racemization under acid hydrolysis conditions. Since only traces of D-Met (<1%) were detected in the cheeses under study, it is assumed that no D-epimers were formed during acid hydrolysis.

It may thus be concluded that baking of cheese in the conventional oven did not alter the nutritional quality of the cheese proteins that could be attributed to changes in the amino acid composition and configuration. The temperature and the duration of baking were apparently insufficient to induce such changes. As the temperature treatment in the microwave oven was even more moderate than that in the conventional oven (Fig. 1), it is highly improbable that measurable racemization would have occurred in the cheeses baked in the microwave oven.

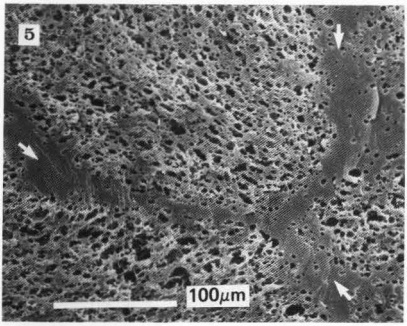
Microstructure.

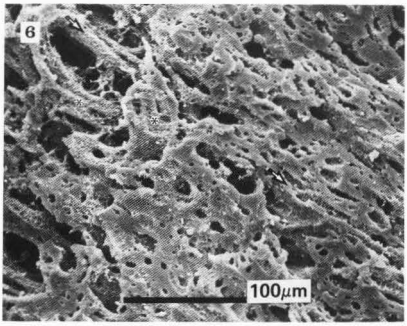
Control cheese samples. SEM revealed considerable differences in the original microstructure of the three cheeses used in this study. Before baking, the stirred-curd Mozzarella cheese (28% fat, 42% moisture) had the structure similar to other stirred-curd cheeses with the curd granule junctions [15, 28, 30] clearly visible as areas depleted of fat (Fig. 5). Lactic acid bacteria were associated with the curd granule junctions more frequently than with the interior areas of the granules. Fat in the form of fat globule clusters was distributed relatively evenly throughout the granules. The stretched Mozzarella cheese (15% fat, 52% moisture) revealed an oriented structure under the SEM provided that the samples were fractured along the protein fibres (Fig. 6). This orientation was not noticeable in cross fractures (Fig. 7). Fat particles consisted of clustered fat globules. Fat globule membrane residues and lactic acid bacteria were clearly noticeable (Fig. 8).

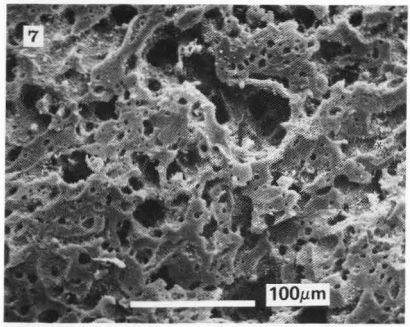
The process Cheddar cheese (28% fat, 44% moisture) contained uniformly emulsified fine fat particles in a protein matrix free of melting salt residues (Fig. 9). Although the process Cheddar cheese had approximately the same fat content as stirred-curd Mozzarella and almost twice as much fat as the stretched Mozzarella, the small dimensions of the fat particles made the process cheese appear under the microscope as having a considerably lower fat content. The fat particles in process Cheddar cheese contained no membranes as the latter had been disrupted during cheese processing. In contrast to the uniform structure of the Mozzarella cheeses, the process Cheddar cheese used in this study was found to contain osmiophilic areas (Figs. 10 and 11) already in the original state before baking. This is an interesting finding because such areas have not been reported [5, 6, 27] to exist in laboratory-made or commercially produced process cheese but were found in process cheese that had been heated excessively for 5 h at 82°C or in process cheese, in which such excessively heated cheese was used as so-called "rework" [16]. It may thus be anticipated (as direct information on the manufacture of the cheese was not available) that the process Cheddar cheese used in this study contained an excessively heated cheese as one of the ingredients.

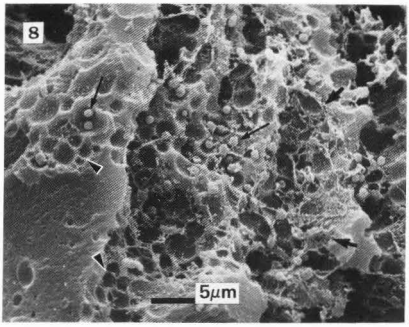
Cheeses baked in the conventional oven. The rise of temperature in the pizza dough during baking in the conventional oven is shown in Fig. 1. After 12 min, the pizza dough acquired a baked appearance and the cheeses melted to a varying extent. Although the process cheese and the stirred Mozzarella cheese had similar fat and moisture contents, the latter cheese melted rapidly and flowed down over the edge of the pizza dough whereas the process Cheddar cheese melted without flowing. The behaviour of stretched Mozzarella was closer to that of the process cheese. However, brown blisters up to 10 mm in diameter developed on the cheese surface, probably as the result of the low fat content.

The microstructure of the natural cheeses was affected by baking to a considerably greater extent than that of the process cheeses. Agglomeration of the fat particles in the natural cheeses was noticeable under a low-magnification dissecting microscope. To visualize these changes by SEM, magnifications lower than those used with the unbaked cheeses had to be used. The









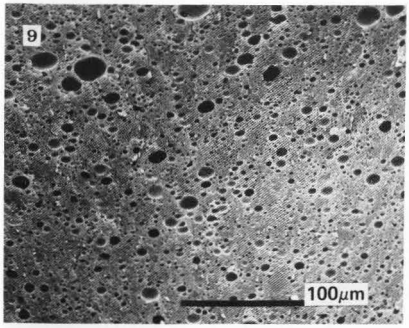


Fig. 5. Curd granule junctions (arrows) in stirred-curd Mozzarella cheese.

Fig. 6. Parallel orientation of protein fibres (arrows) in stretched Mozzarella cheese is evident from a longitudinal freeze-fracture. Fat globules have been removed during the preparation of the samples for SEM from places marked with asterisks.

Fig. 7. Cross freeze-fracturing of stretched Mozzarella cheese reveals no orientation of the protein matrix. Fat globules were aggregated in clusters (asterisk).

Fig. 8. Fat globules (arrowheads) clustered in stretched Mozzarella cheese. Large arrows point to fat globule membrane fragments. Lactic acid bacteria (small arrows) are also noticeable.

Fig. 9. In the process Cheddar cheese, fine fat globules are relatively uniformly distributed in the protein matrix. There is no evidence of melting salt crystals in this sample.

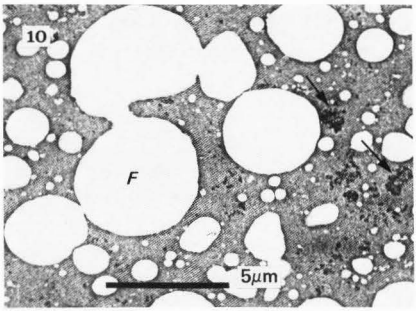


Fig. 10. The distribution of fat (light circles - F) and dark osmiophilic areas (arrows) in a thin section of process Cheddar cheese.

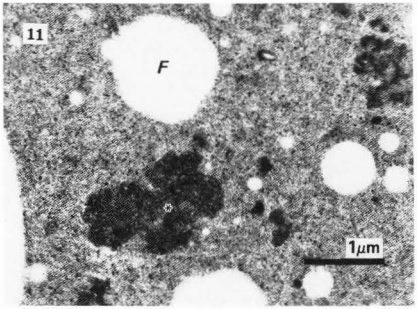
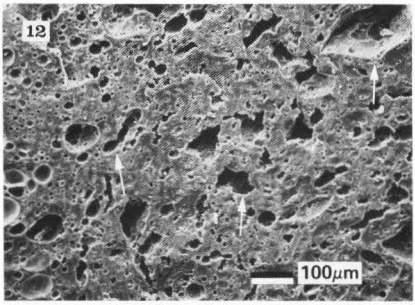
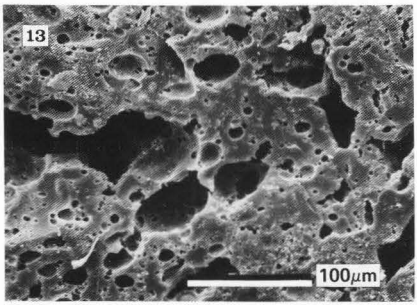
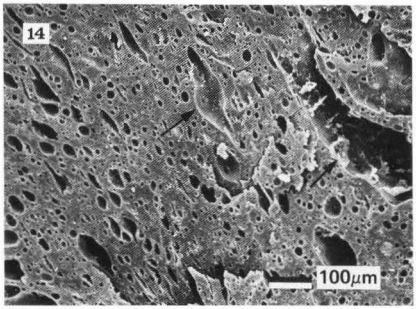


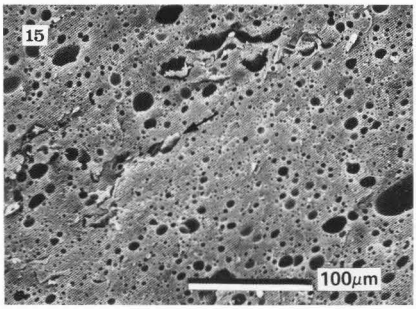
Fig. 11. Detail of an osmiophilic area (asterisk) in process Cheddar cheese. (F = fat).

Figs. 12 to 15: See next page for legends.









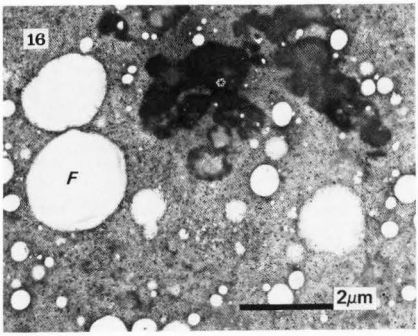


Fig. 16. TEM of a thin section of process Cheddar cheese that had been baked in a conventional oven reveals the presence of dark osmiophilic areas (asterisk) in the protein matrix.

general view of the coarse laminar structure of the stirred-curd Mozzarella cheese shows that fat globules agglomerated into larger particles. Distortion of the fat particle shapes shown in this figure was apparently the result of the collapse of the original structure which led to the flow of the fat out from the cheese (Fig. 12). Interestingly, salt crystals similar to those of calcium phosphate reported elsewhere [5, 14, 27] developed in this cheese as the result of baking. The same magnification in Figs. 5 and 13 makes it possible to compare the structures of unbaked and baked cheese. The agglomeration of the fat particles also took place in the stretched Mozzarella cheese (Fig. 14) with occasional occurrence of large fat particles. Fat globule membrane fragments were displaced by this process in both Mozzarella-type cheeses. This development is particularly noticeable in the stretched Mozzarella cheese by comparing Fig. 14 with Figs. 7 and 8 obtained at the same magnification. The agglomeration of the fat particles was least advanced in the process Cheddar cheese (Fig. 15).

Fig. 12. Microstructure of stirred-curd Mozzarella cheese that had been baked in a conventional oven. Fat globules are aggregated in large fat particles which acquired irregular shapes (arrows).

Fig. 13. Detail of the microstructure of stirred-curd Mozzarella cheese that had been baked in a conventional oven.

Fig. 14. Microstructure of stretched Mozzarella cheese that had been baked in a conventional oven. The orientation of the protein matrix is apparently due to the flow in the oven rather than due to the original stretching. Fat globules are aggregated in large fat particles which acquired irregular shapes (arrows).

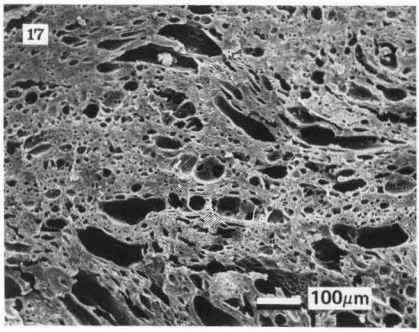
Fig. 15. Microstructure of process Cheddar cheese was only slightly altered by baking in a conventional oven. Arrow points to a disturbance in the structure.

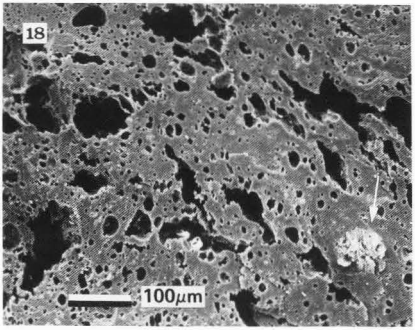
The changes, which took place in the cheeses during baking including the loss of oriented structure in stretched Mozzarella cheese, made them more difficult to prepare for TEM than the control cheeses. The aggregation of fat produced relatively larger fat particles. The protein matrix depleted of the fat became considerably more compact. This compaction, particularly severe in the low-fat stretched Mozzarella cheese and the blisters, led to problems when impregnating the samples with the resin and resulted in poor micrographs suffering from several artefacts. Visual examination of the cheese protein matrices failed to reveal changes other than those associated with the increased structural heterogeneity of the baked cheeses. In process Cheddar cheese, the dark osmiophilic areas found before baking were noticeable in the protein matrix after baking (Fig. 16).

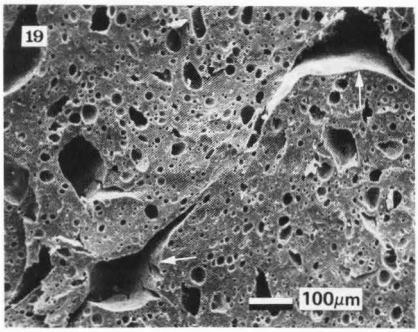
Cheeses baked in the microwave oven. Although the duration of heating was considerably shorter in the microwave oven than in the conventional oven, the cheeses were heated at or above 90°C for similar periods (2 to 2.5 min) as is evident from Fig. 1. The effects of microwave baking on the microstructure of the cheeses were similar to the effects caused by baking in the conventional oven but were not as severe. However, the characteristic features found in the natural Mozzarella cheeses vanished (Figs. 17 to 19). Fat globules and their clusters aggregated into larger particles and became distorted by the flattening of the cheese or its flow. Compared to the baking in the conventional oven (Figs. 12 to 14), where the flow of the cheeses was almost completed, the cheeses baked in the microwave oven were sampled while they were still flowing. This is particularly clearly seen in Figs. 17 and 19. Although heating of the cheese in the microwave oven lasted only shortly, this time was sufficient for some salt crystals to develop (Fig. 18). The structure of baked process Cheddar cheese is shown in Figs. 20 and 21, where fat particles widely ranging in dimensions are noticeable. The process cheese contained compact areas (Fig. 20) as well as areas having fat particles larger than 50 μm in diameter (Fig. 21). Similar to the cheeses baked in the conventional oven, TEM showed that the Mozzarella cheeses consisted of uniform protein matrices (Fig. 22) and that osmiophilic areas were present in the process Cheddar cheese (Fig. 23).

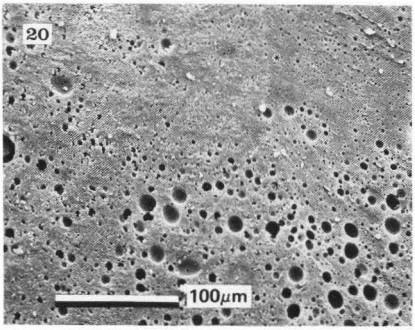
In conclusion, monitoring of the temperature in pizzas during baking showed that the cheese received only a small amount of heat compared to the heat to which process cheese may be exposed during manufacture (71-95°C [5]). The temperature in the cheeses baked in the conventional oven did not exceed 100°C and the maximum temperature found in the cheese baked in a microwave oven was 103°C. The cheeses were exposed to these high temperatures for only a few minutes. This exposure changed the microstructure of the cheeses but did not affect their amino acid composition. Low concentrations of D-Phe and D-Glu were found in the unbaked cheeses and no additional formation of these or any other D-amino acids by racemization was observed as the result of baking the cheeses in the conventional oven. By not having altered the amino acid composition of the cheese proteins, the short-time baking apparently has not affected the nutritional quality of the cheeses.

Process cheese was subjected to the least structural changes with fat globules agglomerating to a small extent. No major release of the fat from the cheese was observed. Fat particles agglomerated to a greater extent









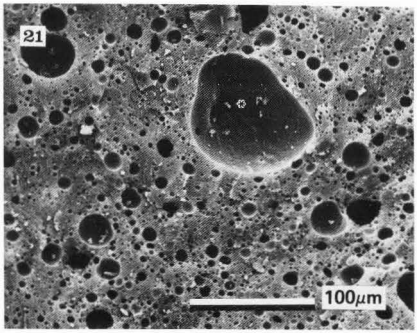


Fig. 17. Microstructure of stirred-curd Mozzarella cheese that had been baked in a microwave oven. Parallel orientation of the protein matrix suggests that the cheese was subjected to flow before cooling and fixation.

Fig. 18. A salt crystal (arrow) (probably calcium phosphate) developed in stirred-curd Mozzarella cheese baked in a microwave oven.

Fig. 19. Microstructure of stretched Mozzarella cheese that had been baked in a microwave oven. The dimensions of the fat particles fluctuate within a wide range. Large aggregations of fat have acquired irregular shapes (arrows).

Figs. 20 and 21. Microstructure of process Cheddar cheese that had been baked in a microwave oven. Fig. 20 shows a more compact area (asterisk) than Fig. 21, where fat globules larger than 50 μm in diameter (asterisk) may be seen.

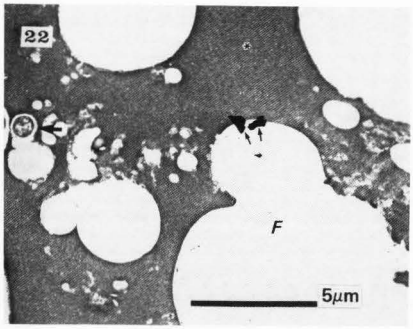


Fig. 22. TEM of a thin section of stirred-curd Mozzarella cheese that had been baked in a microwave oven. Asterisk: protein matrix; large arrow points to a bacterium and small arrows point to contaminating particles (artefact).

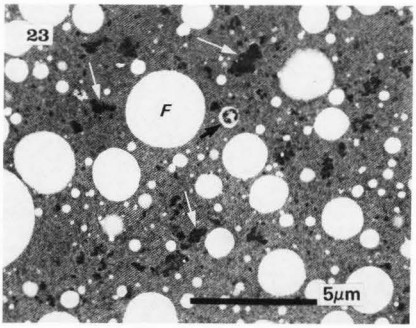


Fig. 23. TEM of a thin section of process Cheddar cheese that had been baked in a microwave oven reveals the presence of dark osmiophilic areas (white arrows). Black arrow points to a bacterium.

in low-fat stretched Mozzarella cheese and there were signs of fat release from the cheese. The most severe changes took place in high-fat stirred Mozzarella cheese. The cheese melted during pizza baking very rapidly, had a tendency to flow away from the dough, and released a considerable part of the fat.

The protein matrices of all three cheeses under study, examined by TEM, showed no alterations due to baking.

Acknowledgments

The authors thank Mrs. Vivian Agar for baking the pizzas, Mrs. Paula Allan-Wojtas and Mr. John Emery for assistance with electron microscopy, and Mr. George Morris for amino acid analysis and nitrogen determination. Appreciation is expressed to Dr. H. W. Modler and Dr. L. Lloyd for useful suggestions. Electron Microscope Unit, Research Branch, Agriculture Canada in Ottawa provided facilities. Contribution 754 from the Food Research Centre.

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Discussion with Reviewers

D. N. Holcomb: The authors have stated that "in a microwave oven ... the food is heated from the interior towards the surface..." Is this really correct? Don't some foods remain cold in the center with a warmer surrounding region, after microwaving? Isn't microwave radiation limited in its penetration depth because it is absorbed before it reaches the center of the food, i.e., the energy is absorbed first nearer the outside? Thus, in a microwave oven as in a conventional oven, frozen products thaw from the "outside in". In some other instances where there is a lot of surface moisture evaporation to cool the outside of the food, one can get the impression that the inside is heated preferentially, but I think that it is incorrect to state that the "food is heated from the interior..."

Authors: According to the literature [1, 2, 33], microwave energy penetrates to a depth limited to 5-7 cm in foods and the heat generated is then conducted farther. The surface is cooled by the evaporation of moisture from it. Consequently, the surface layer is heated by both the absorption of the microwave energy and by heat conduction from deeper areas. Thus, the heat travels from the subsurface areas to the surface and the food is heated from the "inside". Compared to baking in a conventional oven, the surface of the food is not overheated as would happen in the case where the microwave energy would all be absorbed by the superficial layer.

D. N. Holcomb: Does mentioning a "contact thermometer" mean that the "surface" temperature was measured or, as seems to be indicated, was the thermometer inserted into the interior of the product for temperature measurement? It would be interesting to compare interior and surface temperatures; in a conventional air convection oven, the surface may be hotter than the interior, while in a microwave oven, the surface may be colder than the interior, because of surface evaporation. Thus, one would expect more protein damage on the surface of the food heated in a conventional oven than on the surface of the food heated in a microwave oven. Did the authors distinguish between surface and interior areas when performing the amino acid analyses and microstructural studies?

Authors: The layer of shredded cheese on the pizza dough before baking was 0.5 to 1.0 cm thick and the total mass of the cheese was 65 g per pizza, which was 18 cm in diameter. Baking caused the cheese to melt and spread over the entire pizza surface. Thus, each cm^2 of the pizza surface contained ~0.25 g of the cheese, i.e., a layer ~2 mm thick. Taking the effects of bubbling, flow, and partial absorption of some cheese fat in the underlying cloth into consideration, it was virtually impossible to separate superficial and interior cheese areas for amino acid analysis and electron microscopy.

The contact thermometer was inserted into the thin cheese layer and touched the underlying cloth barrier.

D. N. Holcomb: Were the moisture contents the same in all of the cheese samples and at all locations within a given baked sample? Did the moisture content change upon

baking? One might expect the surface of a product baked in a conventional oven to be drier than the interior of that product and just the opposite in the case of a microwaved product, if there were moisture migration toward the surface of the product in the microwave oven. If the moisture contents varied, then the amino acid content might also vary.
Authors: All cheese samples had been freeze-dried for amino acid analysis and the results listed in Table 1 relate to the dry matter content. The moisture contents in the baked cheeses were as follows:

Cheese:	Moisture content (%) in the cheeses unbaked (fresh):	baked in an oven convent.:	microwave:
Stirred-curd Mozzarella	44.4 ± 0.9	33.7 ± 0.7	37.4 ± 2.1
Stretched Mozzarella	39.2 ± 0.1	29.2 ± 1.5	29.2 ± 0.5
Process Cheddar cheese	41.5 ± 0.1	29.5 ± 0.3	30.0 ± 0.2

E. Parnell-Clunies: The authors should indicate the pH of each cheese since it will affect texture.
Authors: Regrettably, pH measurements were not included in the tests.

E. Parnell-Clunies: Is it appropriate to use the term "fibre" in describing a continuous protein network such as that shown in Figs. 6 and 7? Don't the differences in these figures arise from fracturing areas of differing degree of compactness?
Author: These figures show stretched (Italian-style) Mozzarella in which the protein is oriented in one direction in the form of fibres. The differences arise from freeze-fracturing the protein network either along the fibres (Fig. 6) or across them (Fig. 7).

E. Parnell-Clunies: Osmiophilic areas in the unbaked process Cheddar cheese and the process cheese baked in a conventional oven occur as localized bodies. When this cheese is baked in a microwave oven, osmiophilic areas are more widely distributed. Do the authors have any suggestions for this redistribution?
Authors: Osmiophilic areas are shown by TEM in unbaked process Cheddar cheese (Figs. 10 and 11 - detail), in process cheese baked in the conventional oven (Fig. 16 - detail), and in process cheese baked in the microwave oven (Fig. 23). Using these micrographs as well as the unpublished ones, we have been unable to confirm the above suggestion that the osmiophilic areas are more widely distributed in the cheese baked in the microwave oven than in the other process Cheddar cheese samples.

E. Parnell-Clunies: Do the authors have any data on relative quantities of osmiophilic bodies? If so, are these proportions in keeping with normal usage levels of "rework" commercially manufactured processed cheese?
Authors: We have no such data. In fact, the presence of the osmiophilic areas in commercial process cheese selected for these experiments is quite surprising in view of an earlier finding [16] that the development of such areas is related to an excessive heat treatment of process cheese rework, where the osmiophilic areas were observed for the first time.

E. Parnell-Clunies: Given that a microwaveable crust has been developed [23], would the authors recommend microwave heating of cheese to the pizza industry?
Authors: Yes. We would also suggest that process cheese be considered for this purpose.

G. Sarwar: What are the nutritional and/or organoleptic implications of the changes in the microstructure or natural cheeses caused by baking?
Authors: The amino acid composition and configuration in the three cheeses studied was not affected by microwave or conventional baking. Thus, the nutritional values of the cheeses remained unaltered as far as the amino acid composition is concerned. Organoleptic properties (sensory attributes) of the cheeses were markedly affected by baking as the cheeses melted. In our opinion, the changes would be acceptable with the process Cheddar and low-fat stretched Mozzarella cheeses but would not be acceptable with stirred-curd Mozzarella because this latter cheese oiled off severely during baking. The preservation of small fat globules in the baked process Cheddar cheese was associated with its resistance to excessive melting. In contrast, the microstructural studies of the two Mozzarella cheeses as carried out in our experiments before and after baking would not be sufficient to characterize the suitability of the cheeses as pizza ingredients. However, this study showed for the first time the structural changes which take place in various cheeses during baking.

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Scanning Microscopy International, Chicago (AMF O'Hare) , IL 60666 USA

CHANGES IN THE MICROSTRUCTURE OF SAINT PAULIN CHEESE DURING MANUFACTURE STUDIED BY SCANNING ELECTRON MICROSCOPY

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Abstract

The Saint Paulin cheese used in this study contains 45% fat. Changes in curd structure were followed during syneresis until the end of ripening. Two scanning electron microscope (SEM) techniques were used: the conventional technique with sample fracture after critical point drying, and cold-stage SEM after rapidly freezing samples at -160°C. The two techniques are complementary since the first leads to satisfactory observation of casein, while the other is more suitable for observing fat globules.

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Key words: Saint Paulin cheese, curd draining, ripening, conventional SEM, cold-stage SEM.

Introduction

Saint Paulin cheese (16) is used in our laboratory as a model to study the ripening process in cheese. The manufacture of Saint Paulin cheese is similar to that of Gouda cheese. Although the microstructure of the Gouda cheese has been studied by several authors (9, 12, 20), no systematic structural studies have been carried out with Saint Paulin cheese. In this study, scanning electron microscopy (SEM) has been employed for providing pertinent information on the development of cheese structure. Two modes of operation were used: chemically fixed and dried cheese samples examined by conventional SEM, and rapidly frozen samples examined by cryo-SEM. Effects of variations in chemical fixation of the cheese using glutaraldehyde and osmium tetroxide were also studied.

Materials and Methods

Cheeses

The cheeses used were small Saint Paulin, final weight about 200 g. The ratio of lipids / total dry weight was about 0.45. Figure 1 is a flow diagram of the manufacturing steps (21). Two series of samples were taken per manufacturing run. The first series included samples taken during curd formation, i.e., 10 minutes after complete coagulation (S_1), during draining (S_2, S_3, S_4), and after processing (S_5). The second series involved the ripening process: S_6 (day 11), S_7 (day 15), S_8 (day 20), S_9 (day 30), S_{10} (day 40) and S_{11} (day 60). The appearance of the cheeses was homogeneous throughout their thickness, resulting from the absence of surface flora. From the interior of the cheese blocks sample, in the form of 5 x 5 x 2 mm pieces of curd, were taken and immediately placed in the fixing agent.

Two preparation techniques were used: (1) the conventional technique involving fixation, dehydration, critical point drying and dry-fracturing, and (2) observation on a cold-stage after cryofixation.

Conventional technique of sample preparation

Different fixation methods were tested and the results compared:

1. OsO_4 (1% w/v) in 0.1 M cacodylate buffer, pH 7.2, containing 10 mM $CaCl_2$ was used for 18 h at 4°C. Following the works of Brooker (2) and Green, et al. (8), the pH value of 7.2 was chosen to obtain satisfactory fixation of casein and, to the greatest extent possible, of the protein-rich fat globule membrane.

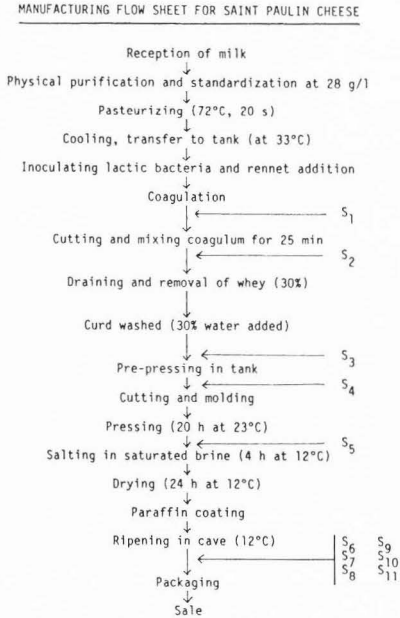


Figure 1.
Manufacturing flow diagram for Saint Paulin cheese.

2. Glutaraldehyde (2.5% w/v) in the above cacodylate buffer was used for 2 h or 30 days at 4°C. After fixation, the samples were washed three times for 10 min in the same buffer. They were then cut into small (2 x 2 x 5 mm) pieces to render them more permeable to OsO_4. Regardless of the duration of glutaraldehyde fixation, it was followed by post-fixation in OsO_4 as described above, since osmium hardens the samples and renders them conductive (4).

Dehydration: A graded ethanol series (10, 25, 50, 75, 90 and 100% v/v) was used for 20 min in each bath. The dehydrated samples were dried by the critical point method in CO_2, using a Polaron E 3100 instrument and Freon 113 as intermediate solvent. The surfaces for observation were obtained by fracturing the dried samples.

Metal coating: Fractured samples were then glued to sample holders and gold-coated in a Polaron E 5150 sputter coater. The metal coat was 15 nm thick.

Observations: A Hitachi S-450 SEM was used at 15 or 20 kV.

Cold-stage SEM

This method was used primarily for observing fat globules. Samples were obtained on the day of their observation and were cut into 1 mm^2 rods about 6 mm long. They were placed in the spaces of a metal sample holder containing a fluid kaolin paste. The holder was then rapidly immersed in Freon 22, cooled to its freezing point of -160°C with liquid nitrogen, and placed in the microscope chamber, where samples were fractured and gold-coated. The maximum temperature of the chamber did not exceed -40°C. Finally, the holder was placed on a chilled stage of the microscope, whose temperature was maintained between -90 to - 100°C.

A JEOL JSM-35 microscope with a Cryoscan device was used at 20 kV.

Results

Comparison of the fixation methods

Three different fixation methods were used with the first series of samples (from coagulation up to pressing): a) glutaraldehyde for 2 h and OsO_4 for 18 h (Fig. 2A and B); b) glutaraldehyde for 30 days and OsO_4 for 18 h (Fig. 2C); and c) OsO_4 alone for 18 h (Fig. 2D). The appearance of casein and of fat globules was practically the same with the three types of fixation (Fig. 2B-D).

The second series of samples (during ripening) were fixed in glutaraldehyde (for 2, 6, or 30 days), systematically followed by post-fixation in OsO_4 for 18 h. In all cases, the structure of casein at a given ripening time was identical. In these samples, regardless of fixation time, fat globules disappeared, leaving only their trace in the pressed casein.

Change in the structure of curd: 1. Casein structure

Curd draining: The appearance of casein in samples S_1-S_4, taken before molding, were not very different. The structure of casein in sample S_1, taken 10 min after coagulation and before the drainage of whey (Fig. 2A) was very similar to that of yogurt (11). Samples S_2 and S_3 were similar to S_1. Sample S_4, after 15 min of pre-pressing in the tank, exhibited a somewhat condensed casein network (Fig. 2B). The casein structure of sample S_5 (20 h of pressing) was more compact than the preceding samples, but otherwise was similar to them with regard to the granular structure formed by the micelle clusters (Fig. 3A). These clusters were larger than those in S_1 and S_4 (Fig. 2A, 2B). Fat globules were no longer noticeable in samples of cheese pressed for more than 20 h and only traces of the fat were found in the protein matrix.

Ripening: Samples from day 11 (S_6) to day 15 (S_7, Fig. 3B) show an arrangement of casein similar to that of S_5, obtained just after leaving the press. Between days 20 and 25 (Fig. 3C) the cheese morphology changed: micelles were more tightly clustered and the initially granular structure was transformed into a compact homogeneous structure. This transformation progressed until the end of ripening (Fig. 3D), giving rise to a compact block of cheese.

Change in the structure of curd: 2. Appearance of fat globules.

Curd draining: Using conventional SEM, fat globules were found in samples S_1 to S_4. Most fat globules were spherical and occasionally contained slight depressions in their surfaces as shown in Fig. 2A to 2C. The fat globule membranes were always smooth and were free of perforations or cracks. Cold-stage SEM images (Fig. 4-7) showed spherical and apparently intact globules, fractured globules and membrane debris attached to cavities created in the

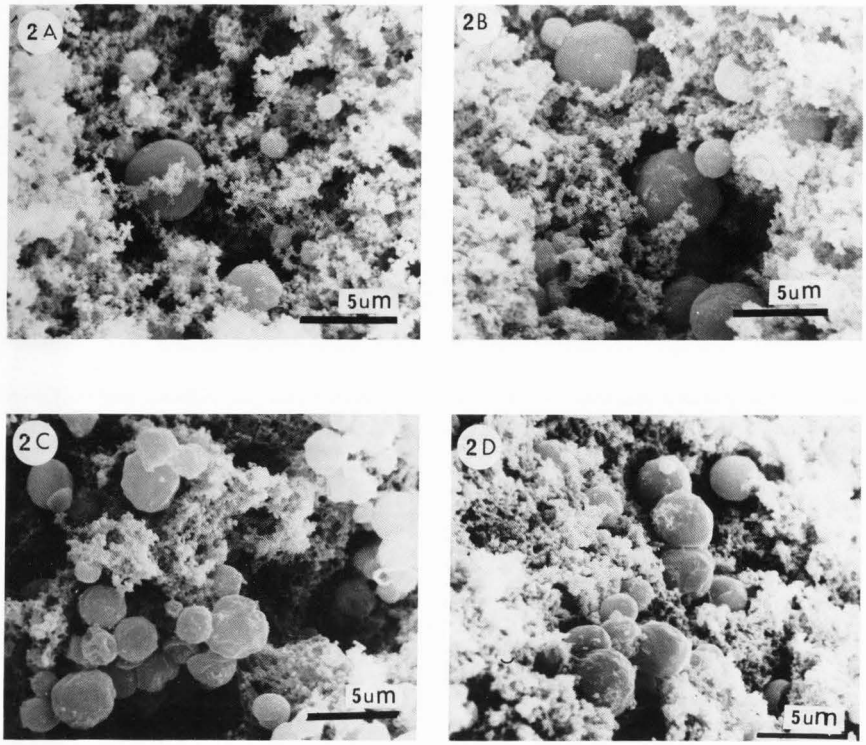


Figure 2. Curd structure before pressing. Conventional preparation method with dry-fractured samples. Influence of glutaraldehyde fixation time: Sample S_1 10 min after coagulation, fixed in glutaraldehyde for 2 h and OsO_4 for 18 h (A). S_4: sample before molding, fixed in glutaraldehyde for 2 h and OsO_4 for 18 h (B); fixed in glutaraldehyde for 30 days and OsO_4 for 18 h (C); fixed in OsO_4 alone for 18 h (D). No bacterial cell is visible in these micrographs. Only fat globules are noticable, lodged in the casein.

casein during pressing. Totally empty cavities were not observed.

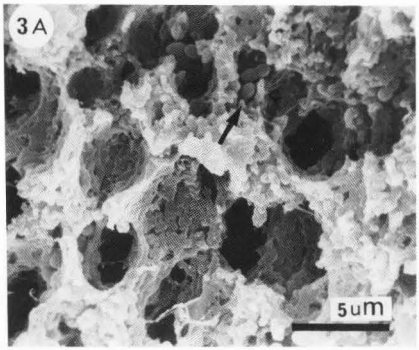
Ripening: Once the cheese was pressed, fat globules totally disappeared from samples treated using the first technique and only the cavities they occupied subsisted (Fig. 3A-D). SEM examination on the cold-stage (second technique), however, showed that fat globules were still present in the matrix of casein. This resulted from the absence of any chemical treatment and the freezing of structures in their in situ state at the moment of sampling. The fat globules presented the same morphological types (Fig. 4-7) until the end of ripening.

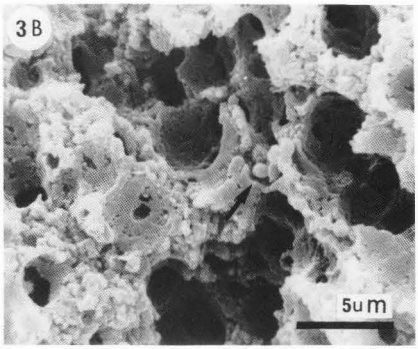
Discussion

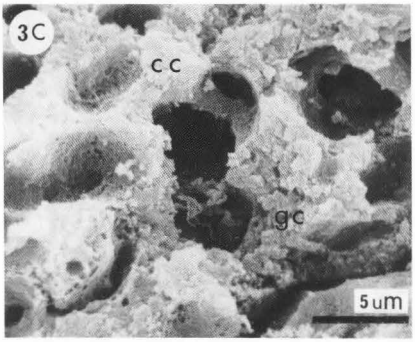
Structure of casein

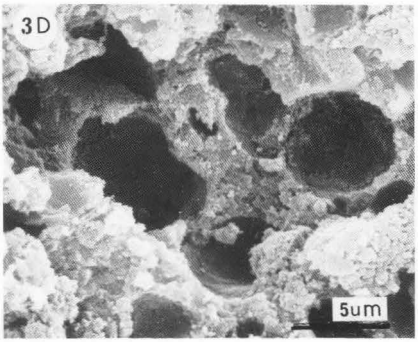
It has been shown that the true structure of the casein network in dairy products may be shown by conventional SEM, i.e., by the examination, at ambient temperature, of chemically fixed and dried samples (2, 11-15, 19). Proteins are well fixed by glutaraldehyde and are practically insensitive to the solvents used after fixation. Although this method gives a good image of objects in space, it nevertheless removes all free water.

This technique supplies interesting information on the arrangement of micelle clusters in the cheese during draining and ripening. The results show that









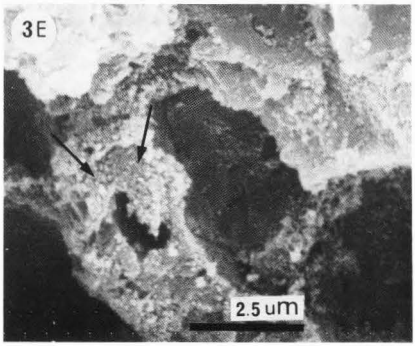


Figure 3. Curd structure during ripening. Conventional sample preparation method with dry-fractured samples. Sample S_5 taken at the end of pressing: granular casein structure and presence of streptococci (arrows). The fat globules maintained in the curd before pressing have totally disappeared from this sample and from those taken during ripening. Only the cavities formerly occupied by fat are seen (A). Sample S_7, 15 days of ripening, structure similar to S_5, presence of streptococci (arrows) (B). Sample S_8, 20 days of ripening: zones of compact casein begin to appear; compact casein (cc), granular casein (gc) (C). Sample S_9, 30 days of ripening: casein more compact (D). Detail of figure 3D showing the fine structure of compact casein (arrows) (E).

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although the treatment of the curd favors syneresis (cutting, mixing, washing), its basic appearance does not change (Fig. 2A, 2B). At the end of ripening, the homogeneous, less granular but finely divided structure of casein (Fig. 3E) undoubtedly arises from proteolysis and chemical modifications: pH, salt

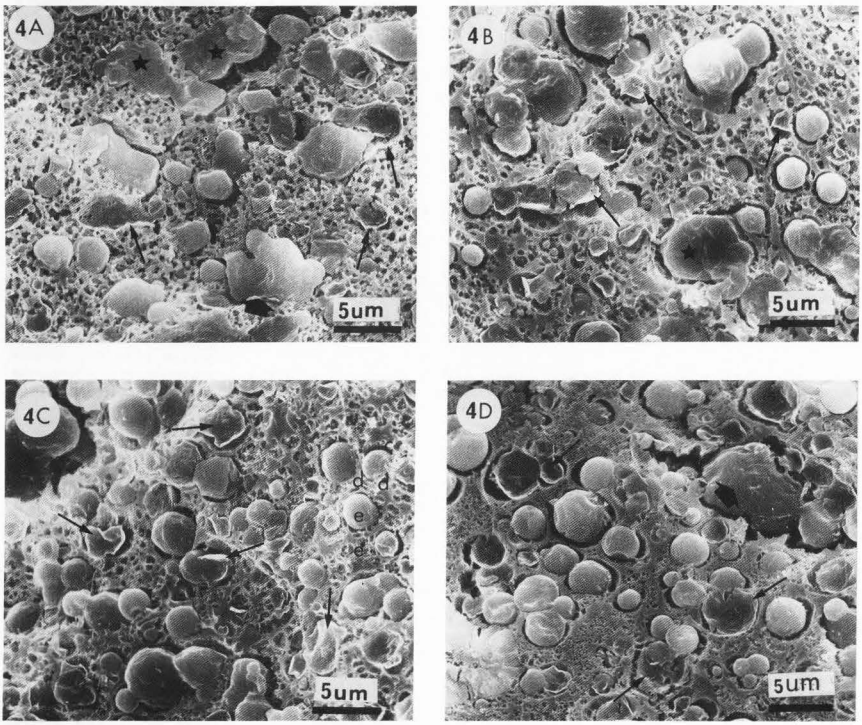


Figure 4. Appearance of fat globules during ripening using the cold-stage SEM observation technique. Fractured fat globules (asterisks), fat globule membrane debris (arrows), coalescent fat globules (large arrows) are observed. Sample S_4 before molding and pressing (A). Sample S_7: 15 days of ripening (B). Sample S_9: 30 days of ripening (C). Sample S_{11}: 60 days of ripening (D). At the relatively low SEM magnifications, the appearance of the fat globules remains the same throughout ripening. Casein has a more compact structure in sample S_{11} (D).

concentration, moisture content (10), which occur during this period. Proteolysis is moderate (18% soluble nitrogen at the end of ripening (5)) and the pH change is only 0.2 unit in 60 days. These stable values are explained by the absence of surface microflora (see Materials and Methods) and by the limited production of lactic acid by starter bacteria as a result of washing the curd. In addition, proteolytic activity of the milk protease was low and was further suppressed by keeping the cheese at 12°C. Only rennet has a pronounced action. All these phenomena (21) are thus in agreement with the slow changes of casein and its relatively unchanged appearance up to 20 days of ripening.

In the second preparation technique using frozen samples, a portion of unbound, vitrified water is sublimed during sojourn under vacuum in the microscope chamber, between fracturing and gold coating. This is shown by a spongy casein structure (Fig. 4A-C). The sample obtained at the end of ripening (Fig. 4D) shows considerably fewer empty spaces left by the sublimation of vitrified water in comparison to the earlier samples. Casein is more highly altered, including a disaggregation of micelles and its partial hydrolysis, leading to a more intimate rearrangement of water and proteins (6) than in samples S_4 (Fig. 4A), S_7 (Fig. 4B) and S_9 (Fig. 4C). A more detailed study of casein structure could not be carried out on these samples, since the microscope used was not equipped with a device for measuring sojourn time of the object in the fracture chamber. This precluded any quantitation of the sublimation occurring

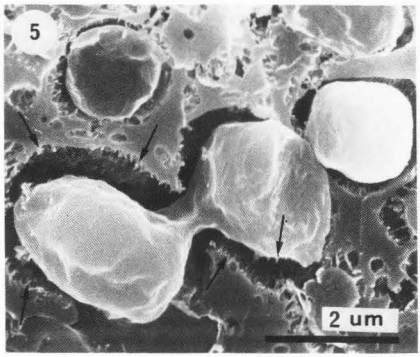
between fracturing and metal-shadowing. It was thus impossible to establish a precise relationship between the appearance of casein and its real structure, or to determine the degree of adherence of casein particles to fat globules. We observed only a tendency toward a more compact casein structure at the end of ripening (Fig. 4D), similar to the images observed using the first technique.

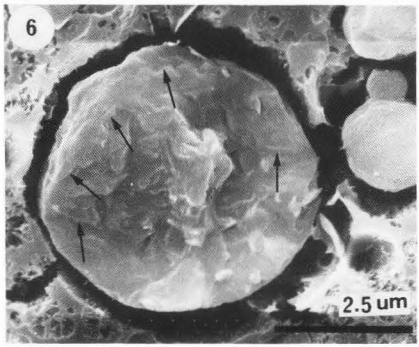
In summary, the first preparation method furnishes images in three dimensions, valuable for studying casein, while the freezing method is more appropriate for the study of fat globules.

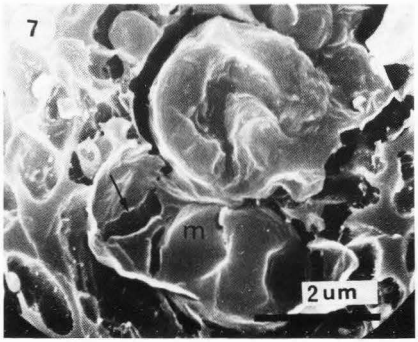
Structure of fat globules

The usual technique for preparing samples for SEM (the first technique here) is useful for observing fat globules during curd draining but not ripening. The solvents used in this technique do not drastically change the fat globules in the early curd samples (S_1 to S_4), but eliminate them totally in the ripening stage. This suggests that the globule membrane could be more permeable to solvents after pressing than before. A certain disorganization of membrane structure may be due to a change in pH, the mechanical action of pressing, or to possible enzymatic action during the 20 h at 23°C in the press. The relatively low SEM magnification does not enable fine structure modification of the fat globule membrane to be detected. Freeze-fracture or higher resolution SEM may supply additional data on this subject.

Numerous spherical globules with apparently intact membranes are observed in frozen fracture faces examined on the cold-stage. Similar observations have been reported in the case of refined Gouda cheese (20). Other globules contain surface depressions, which may have resulted from an in-process crystallization of fat, favored by the ripening temperature (12°C). Observations of coalescing fat globules were frequent in all samples, both during curd draining and ripening. Fractured globules (Fig. 7) do not exhibit a smooth or finely granular internal structure as does a totally liquid fat (Fig. 1 in (3)), but neither do they appear to be as structured as the fat globules present in butter (Fig. 2c in (18)), or in milk chilled for a long time (Fig. 2a and b in (3)).







Figures 5, 6, 7: Examples of morphological types of fat globules observed using cold-stage SEM.

Figure 5. Coalescing fat globules. Note the continuity between the membranes of the two fat globules. Sublimation of ice immediately after the fracture causes retraction of the casein, which detaches from the globule surface. The fat globule membrane-casein attachment points are still visible, especially on the casein (arrows).

Figure 6. Fractured fat globule. The internal structure of this globule seems to present a certain degree of crystallization (arrows) as compared to a frozen face of a liquid fatty material (Fig. 1 in (2)). The highly accentuated relief observed in the center of the globule may have been caused by stripping at the moment of fracture.

Figure 7. Fractured fat globule and membrane debris. Lipid material inside the fat globule appears retracted, undoubtedly during the crystallization process, leaving an empty space in the center. In the bottom of the globule, membrane debris (m) with rips (arrow) is observed.

It should be noted that sample obtained by freeze-fracture (3, 18) received a finer coating (evaporated metal thickness of 4-8 nm) than those examied by cold-stage SEM (evaporated metal thickness of 10-20 nm) and so the structure of fat in the globules is shown to be not as fine as in freeze-fracture.

The internal structure of fractured globules is closer to some published images (Fig. 3 and 4 in (1)) but the preparation and observation techniques were different and so comparisons should be made with caution. The fat of the globules appears to be formed from crystallized masses with differing orientations inside the globules, thus fracturing along different planes. This could be explained by the heterogeneous chemical composition of lipids (triglyceride mixtures with different points of solidification). The type of crystallization of the fat may be very different according to its composition and the temperatures to which it is exposed before sample preparation (3, 17).

The fat globules in these samples appear in cavities contracted away from the casein. This is seen as early as in S_4, before pressing (Fig. 4A) but less clearly than after pressing and until the end of ripening. Several hypotheses may be advanced, including a preparation artifact due to a partial stripping at the moment of fracture. In addition, sublimation of ice after fracture causes a slight retraction of the casein and the globules; the numerous attachment points existing between the casein and the fat globule membranes are still visible (Fig. 5, arrows). Finally, it is possible that the water concentration near the globule membranes is higher than in the neighboring casein. It is generally admitted that high fat cheeses retain more water than low fat cheeses (7). It may be hypothesized that a part of this water is preferentially localized around the globules.

The presence of membrane debris inside the cavities initially containing the globules may attest to a change in the membrane during the ripening period, but their frequency of appearance was the same in S_5 and in S_{11}. Thus, their existence appears to be a direct consequence of the fracture. The fracture faces never exhibited cavities lacking membrane debris. Thus, the zone of rupture at the moment of the fracture is localized, in our experimental conditions, between the membrane and the lipid matter of the globule, and not between the membrane and the casein lining the globule cavity. This is apparently contradictory to the overall appearance of globules detached from the casein. This lack of adherence of the globule membrane to casein probably exists only on the surface, over a depth less than 1 micrometer, due to the sublimation of water and to resulting contraction phenomena. The lower part of the globules located in the bottom of the cavities would thus remain strongly attached to the casein via the membrane.

In conclusion, casein undergoes slow structural changes during the process of cheese ripening. Twenty to 25 days are necessary for its granular structure to be transformed into a compact mass.

Fat globules, observed with SEM on a cold-stage persisted in situ with their membrane until the end of ripening.

Glutaraldehyde fixation for several hours to several weeks did not change the appearance of casein.

Acknowledgements

I thank J.-P. Accolas, M. Desmazeaud, J.-C. Gripon, L. Vassal and S. Kuzdzal-Savoie for their helpful suggestions during the drafting of this manuscript; and J. Berrier and R. Prost, Director of the "INRA Station des Sciences du Sol" (Versailles, France) for the use of the cold-stage SEM. I am also grateful to C. Le Gallo for expert technical assistance in the laboratory and to G. Pitel for the preparation of the cheese samples used.

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Discussion with Reviewers

M. Ruegg: SEM techniques are also useful for visualizing curd granule junctions and to follow the fusion of curd granules (e.g. M Ruegg et al., Milchwissenschaft 35:329 (1980)). How did curd granule junctions look like in St. Paulin cheese and what was the mean diameter of curd granules in this cheese variety?

Author: The size of the samples fractured after the critical point drying was very small and precluded the observation of curd-granule junctions, whose volume was greater than that of the sample. Curd-granules were about 3-8 mm on a side, while our samples were cubes 2-3 mm per side.

M. Ruegg: Cheese samples shrink after fixation and dehydration. How much did the St. Paulin samples shrink after the different fixation techniques tested and was shrinkage identical in all directions?

Author: Sample contraction after fixation and dehydration was not studied. Only one isolated experiment was performed with sample S_4 (before molding and pressing), after fixing in glutaraldehyde for 15 months at 4°C and observed with the cold-stage. The appearance of the casein was less homogeneous than in cold-fixed sample S_4, was more contracted and presented zones of rupture in its mass. The degree of contraction appeared to be the same in the two directions observable on the fraction plane.

M. Kalab: The author assumes that the absence of fat globules in presses curd was caused by a change in the permeability of the fat globule membranes to organic solvents, which led to the subsequent extraction of the fat. Will the author please comment on an alternate explanation, namely: fat was fixed and fat globules were thus retained for electron microscopy in the highly porous milk coagulum. The loss of fat in the pressed curd was probably caused by its extraction due to incomplete fixation because of the slow penetration rate of the large compact samples (2 x 2 x 5 mm) with the osmium tetroxide solution, which means that the outside was fixed and the inside was not.

Author: The suggestion made as to the different rates of penetration of osmium tetroxide into a curd still very watery and one pressed and salted is correct. But in pressed and salted curds there is always a peripheral zone penetrated by the osmium tetroxide which, in our case, is about 300 micrometers wide. This zone is black and the center of the sample remains white. This appears after fracturing dehydrated samples. Figures 3A-E were taken in this fringe penetrated by the osmium and where lipids were nevertheless solubilized. Figure 8 shows the edge of a Saint Paulin cheese sample ripened for 45 days, fractured after dehydration and impregnated with osmium tetroxide. Sample thickness here is 35 micrometers while the thickness penetrated by osmium tetroxide is about 300 micrometers. This image is thus inside the fixed zone and most of the fat globules have been extracted by the solvents.

In curd undergoing syneresis, osmium tetroxide diffuses in the aqueous phase and, when it comes in contact with the fat globules, passes through the membrane and appears to fix fatty material properly. In pressed and salted curd, OsO_4 also passes through the aqueous zone at sample periphery (the black color of this zone shows its presence) but is apparently incapable of fixing globule lipids, since they are subsquently extracted by solvents during dehydration.

How can we explain this differential action of solvents on the lipids in the globules of these two types of curd? Consider two things: the possibility of solvent action, and the state of lipid fixation by osmium tetroxide.

The action of solvents depends on their free circulation throughout the entire thickness of the sample and their capacity to pass through the fat globule membrane. These two properties are verified, since lipids are extracted from the entire sample mass. Thus, their possibility of action now depends only on the state of fixation of lipids by osmium tetroxide.

The response of lipids to solvents differs in curds before and after pressing and salting. What are the changes occurring in the curd during these operations? The volume of the aqueous curd medium bathing casein and fat globules diminishes and its ionic concentration increases, notably by the addition of sodium chloride. These changes of the ambient medium may change the properties of the fat globule surface and slow down or prevent the penetration of osmium tetroxide to contact triglycerides. It is to be noted that the fixation obtained by Allan-Wojtas and Kalab with osmium tetroxide in the presence of imidazole occurred in the very water-rich medium of a yoghurt (text reference 1) or on fat globules of fresh cream encapsulated in an agar gel (M Kalab, J. Dairy Sci., 1985, 68:3234-3248, Fig. 1A), i.e., in very dilute and very permeable media in relation to the residual whey of a pressed and salted curd.

This attempt to explain the differential action of osmium tetroxide towards lipids depending on whether it diffuses in a highly aqueous or a pressed and salted curd is still a hypothesis.

In another framework, the argument that the state of crystallization of fat can prevent the action of osmium tetroxide is not valid. Crystallization of triglycerides during cheese manufacture is never total (according to Walstra (text ref. 17), crystallization of cow milk lipids is total at -40°C). In addition, unsaturated triglycerides, the most reactive towards osmium tetroxide, are less easily crystallized at ambient temperature than saturated triglycerides (Hayat, Fixation for Electron Microscopy (1981), Academic Press, p. 156). It may be imagined that at the periphery of the globules there exists a layer of totally crystallized lipids which prevents the entry of

osmium tetroxide. Fixation images of lipids by osmium tetroxide and imidazole (text ref. 1) show that crystallization images vary and they do not appear to form a layer impermeable to the fixing agent, since in Figs. 2 to 6 (text ref. 1), noncrystallized lipids of fat globules are well contrasted by osmium.

It is thus evident that even in the presence of osmium tetroxide, the lipids of a pressed and salted curd are not fixed. A study of the physicochemical state of the whey in contact with fat globules may lead to a better understanding of the factors preventing osmium tetroxide from reacting with lipids in curds during ripening.

M. Kalab: Will the author please explain in more detail the nature of Saint Paulin cheese?
Author: The history of Saint Paulin manufacture is described by J.G. Davis (Cheese, volume III, (1976). Churchill Livingstone, pages 660-662). Prof. F. Kosikowski also described the manufacture of Saint Paulin cheese (text ref. 16). The process we used is described in Fig. 1.

Reviewer 2: Does the author have independent proof that the indicated structures in Figure 5 are "fat globule membrane - casein attachment points"?
Author: The author has no other proof for affirming that there exist attachment points between casein and fat globules. These junctions may be found by using cold-stage SEM in which the sublimation of fractured sample faces can be controlled.

Reviewer 2: Has the author proven that "m" in Figure 7 is "membrane debris"? Does the author have other evidence that lipid material retracts "during the crystallization process"?
Author: On Fig. 7, "m" designates membrane debris as do the arrows in Figs. 4A, 4B, 4C and 4D. In the special case of Fig.7, this membrane remnant seems to correspond to the envelope of two coalescing fat globules.

Regarding the retraction of lipids during crystallization, Precht et al. (18) and Buchheim (3) have shown (Figs. 2a-2c, in ref (18) and Fig. 3 in ref. (3)) that lipids retract during the crystallization process. Passage of the lipid material from the liquid phase to the crystallized solid phase causes a volume decrease resulting in deformation of the globules.

D.G. Schmidt: I wonder whether the applied specimen preparation techniques might have introduced artefacts. The author has observed surfaces obtained either by cutting or by fracturing. Cutting leads to artefacts such as smeared fat, and may damage the structure in the casein matrix. Were the samples cut or fractured?
Author: Half the length of 6mm long cheese rods were immersed in the kaolin filling the lodgings in the sample holder. The shock caused by the advance of the razor blade resulted in fractures slightly above the sample holder surface or even slightly below. In the latter case it is impossible to have a section of the sample. Several of our observations were in these conditions. Sectioned (cut) samples are easily identified by the scratches caused by the passage of the blade, purposely utilized in a worn state. All surfaces appearing partially or totally sectioned were eliminated.

Samples taken at various times during the manufacturing process never presented totally empty cavities. When the globules were stripped, pieces of their membranes remained attached to the casein (arrows on Figs. 4 A-D).

D.G. Schmidt: With Figs. 4 - 7 it is stated that they were made with specimens fractured in the frozen hydrated state. If this were really true then the fractured surface would also show holes, where fat globules have been broken out (as, e.g., visible in the micrographs of ref. 20) and no cross-sections of fat globules. These features are not observed (at least not in the micrographs shown), but Figs. 4B, 4C, 6 do show cross-sectioned fat globules and, in the casein matrix, structures similar to that shown in Fig. 7, which author now ascribes to alteration in the casein during ripening. However, I like to ascribe these features as artefacts caused by the fact that the sample was cut rather than being fractured. What is authors's opinion about this?
Author: The fractured globules indicated on the figures are truly fractured as they are in freeze fracturing. Samples frozen to -160°C were transferred in 2-3 seconds to the fracture chamber whose temperature was maintained below -40°C. Fracturing is possible even though it occurs at a higher temperature than in the freeze-fracture technique, and can portray the internal structure of the fat globules. Finally, if the samples were sectioned with a razor blade, we would have seen far fewer intact spherical globules remaining in place, and many more sectioned globules.

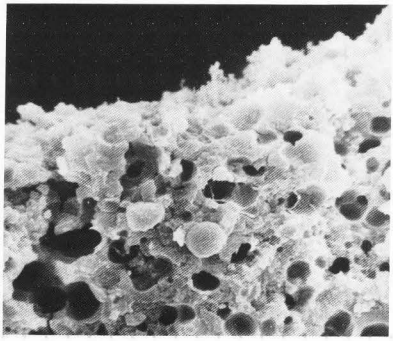

Figure 8. Edge of a Saint Paulin cheese sample ripened for 45 days, fractured after dehydration, and impregnated with osmium tetroxide.

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SAMPLE HOLDERS FOR SOLID AND VISCOUS FOODS COMPATIBLE WITH THE HEXLAND CRYOTRANS CT 1000 ASSEMBLY

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Abstract

A brass block, 3.7 mm high and 10 mm in diameter, which has three openings to accommodate rivet-type or plain tubular specimen holders for scanning electron microscopy at low temperature, has been designed to fit the Hexland Cryotrans CT 1000 assembly in place of the original aluminum disc. Viscous food samples are placed in two-piece tubular holders (0.9 mm inner diameter, 1.2 mm outer diameter) made from sterling silver, and rapidly frozen. The holders are inserted into the brass block under liquid nitrogen and tightened with individual setscrews. A handle screwed into the central hole of the block facilitates manipulation of the block. The samples are fractured inside the Cryotrans CT 1000 assembly by knocking off the part of the sample located in the upper tube. The subsequent operations are the same as those suggested by Hexland.

A second simple type of holder has been developed for low-moisture foods, such as cheese, which are resistant to ice crystal formation during freezing. This holder consists of a Hexland aluminum sample disc drilled with a single opening (4.0 mm in diameter) temporarily closed at the bottom with sticky tape. The food is sampled with a cork borer and the sample plug is then inserted into the 4.0 mm opening, with a rivet covering the part of the sample protruding from the disc. Thermal contact between the sample, the disc, and a rivet that is used to cover the part of the sample protruding from the disc, is provided by Tissue Tek. The sample with the disc is rapidly frozen in nitrogen slush, mounted in the Hexland Cryotrans CT 1000 assembly, and inserted into the prechamber of the cold stage attachment where the rivet is knocked off. From that point on, the regular procedure recommended by Hexland is followed.

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KEY WORDS: Cold-stage scanning electron microscopy, Cryo-SEM, Hexland Cryotrans CT 1000 assembly, Sample holders, Tubular sample holders, Viscous foods.

Introduction

Examination of biological samples by scanning electron microscopy performed at low temperature (cryo-SEM) [10, 15, 16] is convenient for several reasons. One of them is that no chemical fixation is required as the sample is physically fixed by rapid freezing and, therefore, artefacts associated with chemical fixation are avoided. This is particularly important with foods which contain ingredients such as fat or gelatinized starch that are difficult or impossible to fix [18]. Another reason is the rapidity of specimen processing. Cryo-SEM allows for the sample to be frozen, inserted into the cryo-attachment, fractured, etched if necessary, coated with metal, and examined in the microscope within 15 min from the time the specimen is obtained [2].

Some samples are easier to prepare for examination than others. Whole small plant organs can serve as examples of superb preservation of structures by cryo-fixation [3, 4]. Microorganisms present in foods, such as fungi in mould-ripened cheeses and on surface-ripened salami, can also be preserved without developing artefacts provided that appropriate precautions are taken. Such precautions involve handling the samples in a high-humidity chamber before freezing, rapid freezing, and sputter-coating inside the cold-stage attachment in short bursts rather than in a single long interval [1]. Difficulties may be encountered with high-moisture foods (>65% moisture) such as cheese curd, soybean curd (tofu) [9], yoghurt, and cream which are more susceptible to the development of ice crystals in the aqueous phase during freezing [13] than low-moisture products (<65% moisture), such as cream cheese and other cheeses [21] or whipped cream [22]. With regard to biological samples, Moor [17] concluded that the lower the content of free water in living cells, the narrower the critical temperature zone in which crystallization of ice may take place. Apparently, this principle may also be applied to foods. Cryoprotective agents such as glycerol or dimethyl sulfoxide reduce the interval that exists between the freezing point and the recrystallization temperature and diminish the risk of ice crystal development in the sample during freezing, but incorporation of these agents into the food samples under study alters the original structure of the foods.

Ice crystal formation is related to the rate of freezing [17], which, in turn, is affected by the dimensions of the samples. The larger the sample,

the lower its freezing rate. Thus, the reduction in the size of the sample is one way of reducing the development of artefacts due to ice crystal formation [19, 20]. In practice, the size of the sample depends, to a great extent, on the sample holder used [8].

The objective of this paper is to describe the design of a brass disc compatible with the Hexland Cryotrans CT-1000 assembly which accommodates small sample holders and to describe some sample preparation aids. The accessories can be employed when using the Hexland system for food samples without any need to modify the original equipment and with only a slight modification of the procedure used.

Materials and Methods

Sample holder for high-moisture foods

Sets of two-piece open-ended tubes made from sterling silver are used as holders for viscous foods such as cream and stirred yoghurt. The lower tube is longer (2.7 mm) than the upper tube (1.2 mm). The outer diameter of both tubes is 1.2 mm and the wall thickness is 0.15 mm. A brass block has been designed to accommodate 3 samples (Fig. 1) and to fit the standard specimen holder of the Hexland Cryotrans CT-1000 assembly (Fig. 2) (Hexland Electron Microscopy Division, Oxford Instruments Ltd., Eynsham, Oxford OX8 1TL, UK). In order to achieve compatibility, the brass block (Figs. 1 and 3) has the same dimensions (3.7 mm high and 10 mm in diameter) as the original 10-mm diameter aluminum disc supplied with the attachment. The characteristic feature of the block is three openings, each 1.2 mm in diameter and 2.7 mm deep, to accommodate the silver tubes. Small orifices (0.6 mm in diameter) are drilled through the bottoms of the openings to facilitate mounting of the sample-filled tubular holders and for cleaning. Shafts containing setscrews have been drilled in at a 122° angle to the radial direction. This design allows for longer shafts, which provide a better anchoring system for the setscrews than the shorter shafts that would result from their drilling in the radial direction. The setscrew shafts are accessible through grooves machined in the brass block. In addition, the block has a central hole into which a handle can be screwed. The handle is used to manipulate the block during sample loading (Fig. 4) and also to insert the loaded block into the Hexland Cryotrans CT 1000 assembly (Fig. 5).

In preparation for the cold-stage work, the handle is screwed into the brass block and together with a pair of insulated tweezers and the screwdriver, which will be used to tighten the setscrews, pre-cooled by immersion in liquid nitrogen.

A sample of the viscous food destined for examination by cryo-SEM is placed in the bottom silver tube which is held with a pair of forceps. The tube may be filled with the sample using a Pasteur pipet (which has been drawn out into a fine tip) until the sample protrudes at one end. Using a second pair of forceps, the shorter tube is placed over the protruding sample. This operation may also be performed using only one pair of special forceps designed by Sleytr and Umrath [22]. The filled tubes, held together in a vertical position by the sample, are individually frozen in Freon 12 cooled to its freezing point of -150°C with liquid nitrogen and transferred into liquid nitrogen in a shallow insulated container. There, the tubes are mounted into the pre-cooled brass block using the pre-cooled tools. Each pair of tubes is pushed down into one of the three holes in the block with the longer tube inserted first so that the junction in the coupled tubes is flush with the top of the block and the short tube projects above. The tubes are then fixed in place by tightening the setscrews while the brass block is held by its handle. The brass block is then inserted into the Hexland specimen carrier assembly (Fig. 5), which had been pre-cooled to -196°C, and is secured with a setscrew. The handle is then removed from the block and the Hexland assembly is lifted out of liquid nitrogen and inserted into the pre-chamber of the attachment.

Fracturing is effected by using the back edge (not the blade) of the cooled scalpel to knock off the top silver tube from the bottom one. After examining the fracture face in the microscope and etching it if required, the sample may be withdrawn into the pre-chamber, sputter-coated with gold, returned into the microscope, and photographed.

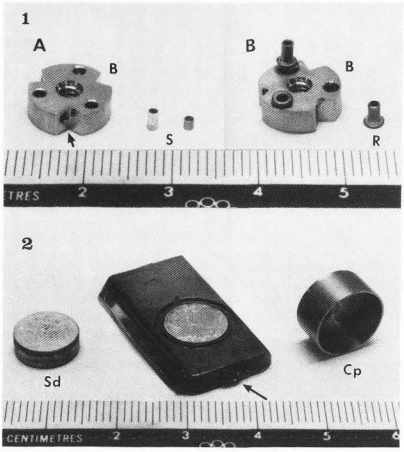
A similar brass block accommodating slightly wider (outer diameter, 1.5 mm, inner diameter, 1.1 mm) brass rivet-type holders of the same length as the silver holders and open at both ends but having a lip (rim) at one end, has also been made and tested (Fig. 1B). It may be used to examine food samples by cryo-SEM without fracturing. The sample is loaded into the rivet allowing the convex meniscus to protrude onto the lip. Without covering it with another rivet, the sample is rapidly frozen by plunging the rivets individually into Freon 12 cooled to -150°C as described above. All steps from here are the same as described earlier but the fracturing step is omitted.

Sample holder for low-moisture foods

The original Hexland design of a central blind-ended rivet sunken into the aluminum disc has been replaced with a more robust open-ended rivet (inner diameter of 4.0 mm, wall thickness of 1.0 mm), also sunken centrally in the disc. The bottom orifice is temporarily covered with a disc of sticky tape cut to size and is removed after the completion of cryo-SEM for cleaning the holder.

For semi-solid low-moisture food samples such as mayonnaise, whipped cream, or thick salad dressings, the bottom orifice of the sunken rivet is covered and the aluminum disc secured to the specimen carrier of the Hexland assembly. The sample is loaded into the rivet in the aluminum disc with a spatula until the sample bulges slightly above the top of the rivet. Another rivet is placed on top and filled with the sample (Fig. 6). The whole specimen carrier, including the sample, is then plunged into nitrogen slush in accordance with the instructions by Hexland, inserted into the pre-chamber of the attachment where fracturing is effected by knocking the top rivet off from the bottom one as described above. The procedure from here on follows the manufacturer's instructions.

For solid low-moisture food samples such as cheese, the same double-rivet set-up is used, again with the bottom orifice covered with a sticky tape. A drop of Tissue Tek (Hexland) is placed in the bottom rivet and the other rivet is placed on its



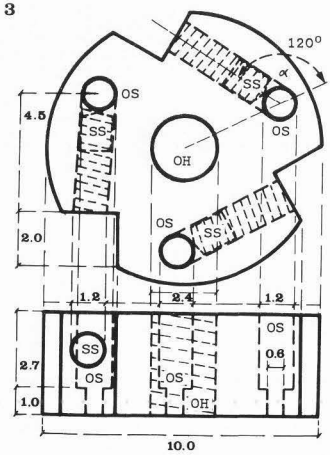
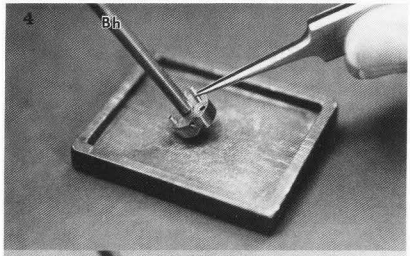
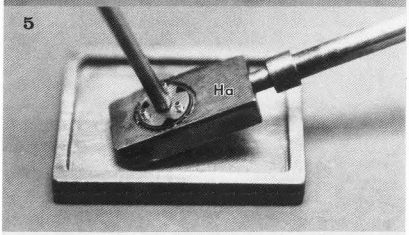


Fig. 1. Brass blocks (B) with concentric holes for three 2-piece silver tubes (S) with an outer diameter of 1.1 mm (Fig. 1A), or open-ended rivets (R in Fig. 1B) with an outer diameter of 1.5 mm, used as sample holders. The central hole in the brass block accommodates the block handle which facilitates tightening of the setscrews (arrow). Numbers on the ruler indicate centimeters.





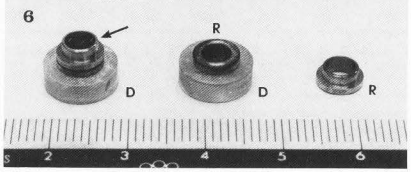


Fig. 2. Hexland standard aluminum sample disc (Sd) used as a holder in the Cryosystem CT 1000 assembly. After insertion, the disc is tightened using a setscrew (arrow). Cap (Cp) is used to cover samples that are sensitive to moisture loss [1]. Numbers on the ruler indicate centimeters.

Fig. 3. Diagram of the brass block. Dimensions are shown in millimeters. OS = openings for sample holders, OH = opening for block handle, SS = setscrews (the shafts have been drilled at an angle α of 122° from the radial direction).

Fig. 4. Mounting of the silver tubes with the sample frozen inside into the brass block. The brass block is manipulated by its handle (Bh). In this demonstration, liquid nitrogen was not used in order to obtain clear photographs of the block.

Fig. 5. Insertion of the brass block with the samples in place into the Hexland assembly (Ha).

Fig. 6. A rivet (R, 4.0 mm inner diameter) sunken in a hole drilled in the original aluminum disc (D) is used as a holder for low-moisture samples. Another rivet (arrow) is placed on top of the sunken rivet.

top. The solid food is sampled using a #1 cork borer (inner diameter of 4.0 mm) with the plunger raised. Then, using the plunger, the sample is extruded into the rivets, frozen in nitrogen slush, and fractured as described above.

Results

High-moisture food samples

The reduction in size of the samples held in small tubular silver holders makes it possible to freeze viscous food samples, such as cream (Fig. 7), more rapidly than in larger holders and to obtain good micrographs by using cryo-SEM. It is easier to freeze the samples individually in cooled Freon than in nitrogen slush. Yet, despite the small size of the sample, ice crystals occasionally develop even when precautions are taken. There are either no ice crystals in the area adjacent to the silver tube or, if they are present, they are small. Larger ice crystals may be found at the centre of the sample. This indicates that the freezing rates are different in different areas of the sample. Usually there are, however, some areas unaffected by the crystal growth which are suitable for photography.

Hexland makes available a wide range of specimen holder blocks (discs) including locking and non-locking types, to accommodate different types of samples placed in rivet-like holders. With regard to the locking holders, the manufacturer employs two methods for securing the rivets to the holder, either with a screw set into the top of the disc or with a pivoting ring which anchors 3 rivets at the same time. With the modified design based on a similar type of holder described earlier [12], loading of the samples into the brass block and manipulating the block is made easy by using a handle designed to be temporarily screwed into the block and removed afterwards. In contrast, using the commercially available holders, the manipulation of the samples and the adjustment of the ring to lock or free the rivets under liquid nitrogen may not be as easy. The rivets in the original specimen holder are placed farther away from the thermocouples than the tubes in the design described here. In addition, using the original holder, it is difficult to fracture all three samples by knocking off the top rivets from the bottom ones using the scalpel. The difficulty is caused by the central pin, which is part of the mechanism that allows the locking of the rivets; it stands in the way of the scalpel and is permanently attached there. Only two fractures can be effected easily even when three samples are mounted in the disc. The lack of a special tool to be used for pivoting the commercially available discs under liquid nitrogen constituted another reason for developing an easier way to accomplish that goal.

It was found that freeze-fracturing may not always be required to show the microstructure of the sample. It is possible to freeze-etch samples, such as stirred yoghurt, thus allowing examination of a large area of the superficial layer of the frozen sample which is free from any ice crystals [7], because it was in direct contact with the coolant and thus frozen more rapidly than the interior of the sample. A yoghurt sample that was allowed to protrude slightly from the single brass rivet (1.5 mm in diameter) was used. The protruding surface, in direct contact with the cryogen upon freezing, had negligible ice crystal development at the sample surface. Casein micelle chains and clusters seen in the sample (Fig. 8) are similar to those seen in yoghurt by conventional SEM [14], i.e., examination at ambient temperature of chemically fixed samples that had been dried. Cryo-SEM thus provides useful confirmation of the reliability of conventional methods.

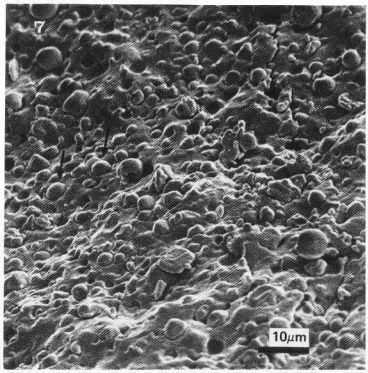
Low-moisture food samples

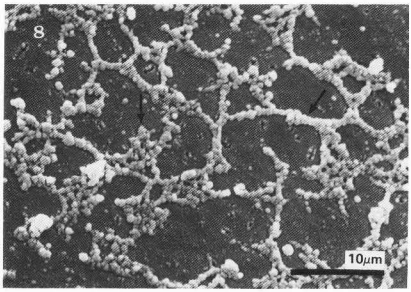
The reason for using the more robust (4.0 mm in diameter) sample holders for low-moisture foods is a higher resistance of low-moisture foods to ice crystal formation during freezing. Thus, the freezing rate is not as critical as with high-moisture foods. The larger holders are easier to handle and fill with the sample than the narrow silver tubes. Fig. 9 shows a commercial salad dressing frozen in the holder. There is no ice crystal damage to the structure of the sample, as is apparent from the appearance of the aqueous phase in which fat globules and other ingredients are dispersed. Starch is noticeable in another salad dressing sample that had been extensively freeze-etched (Fig. 10). The image of the starch is in agreement with the micrographs of starch in cooked pasta [11].

Discussion

Many of the specimen holders available for cold-stage work today, including those available from Hexland, are patterned on the basic design by Echlin and Burgess [6]. These authors have demonstrated a support for 4 samples in rivet-type holders made from silver, but these holders were not individually secured. The use of "fine-bore" silver tubes, 1 mm in diameter, was suggested earlier [12] for freeze-fracturing followed by replication with platinum and carbon in a Polaron freeze-fracturing module. Silver has high thermal conductivity and even thin-walled tubes made from this metal have sufficient mechanical strength [19]. Open-ended tubular holders are easy to fill with liquid samples whereas air bubbles are usually trapped in "blind" holders. By accommodating 3 holders in the block (similar to the design put forth by Echlin and Burgess [6], as well as the design of the Hexland holders), time is spent efficiently on the instruments and the chances of obtaining artefact-free zones are increased compared to working with only one sample per run. This is important particularly with high-moisture food samples, which are susceptible to ice crystal damage.

Even with the refinements suggested, the study of high-moisture food products is not easy. Problems may develop when freeze-etching is carried out. The aqueous phase in most milk products, particularly freshly coagulated curd and yoghurt, contains varying concentrations of solutes such as lactose, mineral salts, and whey proteins. These substances appear as a fine efflorescence on the freeze-etched surface after pure water is removed by sublimation of the ice. In such cases, where an insight into the protein matrix is required and where possible, cold stage SEM should be complemented by SEM carried out at ambient temperature and using chemically fixed samples which had been dehydrated, freeze-fractured, and critical point dried [5, 13]. In other cases,





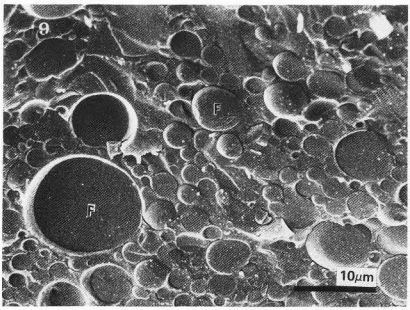
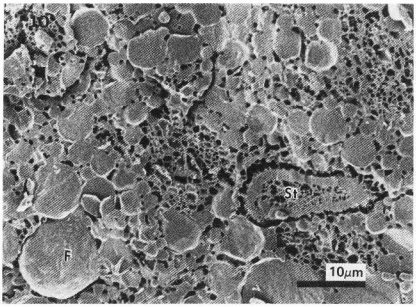


Fig. 7. Cryo-SEM of a whipping cream sample (35% fat) individually frozen in tubular silver holders in Freon 12 at -150°C. After fracturing at -150°C, the temperature was gradually increased to -83°C for freeze-etching at



$1x10^{-5}$torr; 15 min after fracturing, the temperature was decreased to -150°C and the sample was coated with gold and examined at 10 kV. Freeze-etching exposed fat globules (arrows).

Fig. 8. A stirred yoghurt sample placed in a brass rivet, 1.5 mm in diameter, was examined by cryo-SEM without fracturing following freeze-etching of the superficial layer. Casein particle chains and clusters (arrows) are in agreement with the microstructure of yoghurt as seen by conventional SEM [14].

Fig. 9. Cryo-SEM of a commercial salad dressing placed in the single rivet holder reveals numerous fat globules (F) of various dimensions.

Fig. 10. Cryo-SEM of another salad dressing sample shows fat globules (F) and starch (St).

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e.g., low-moisture foods, an examination of the freshly freeze-fractured but unetched surface may provide the required information.

Acknowledgements

The authors thank Mr. Al Bingham, SEMOptics, Ltd., Nepean, Ontario, Canada, for assistance with the design and manufacture of the brass blocks, the holders, and the handle. Appreciation is expressed to Dr. G. H. Haggis for useful suggestions. Electron Microscope Unit, Plant Research Centre, Agriculture Canada in Ottawa provided facilities. Contribution 765 from the Food Research Centre and 1117 from the Plant Research Centre.

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Discussion with Reviewers

J. A. Sargent: It would be helpful to give the address of the supplier of the sterling silver tubes.
Authors: The tubes were obtained from local jewellers' supplies and findings stores. In Canada, they may be ordered from Imperial Smelting and Refining Co., Ltd., 451 Denison, Markham, Ontario.

J. A. Sargent: Is there not a possibility of altering the structure of the sample through shearing effects at the neck of a drawn pipette?
Authors: The only samples which are handled with a drawn pipette are the ones which are liquid, *e.g.*, milk or cream. These samples consist basically of particulate matter suspended in a liquid. The small diameter of the tip of the drawn pipette (approx. 0.5 mm in diameter) is considerably larger than the particles in suspension and so shearing effects in this kind of high-moisture samples should not be a problem.

J. N. A. Lott: The filling of small diameter tubes without getting air bubbles is often a problem. The discussion could probably be extended as to how this was done.
Authors: The fact that the tubes are open-ended, we believe, is the most important single factor which allows the samples to be prepared without the inclusion of air bubbles. As a precaution, the first bit exiting from the drawn pipette, which may contain bubbles, is blotted away and only the sample which is bubble-free is used to fill the tubes. In addition, the tubes are filled to overflowing and only then is the top tube added.

D. J. Gallant: In my experience, samples are not always completely preserved even by rapid freezing. I observed, *e.g.*, that potato cells which did not show ice crystals immediately after freezing, suddenly developed large ice crystals when the temperature of the Hexland Cryotrans assembly was increased to -85°C. This finding has been reproducible. Have you observed such a phenomenon and could you outline the temperature variations which occurred with the various steps of your experiments and which may explain the drying effect observed with the swollen starch granule in Fig. 10?
Authors: Yes, we have observed this phenomenon also, *e.g.*, with celery and parsnip (but not with processed foods such as yoghurt) following the increase of their temperature to -85°C. The temperature profile of the vegetables and the processed foods was identical: the samples were frozen in nitrogen slush, inserted onto the cold stage at about -190°C, and maintained at -160°C except for freeze-etching which was carried out at -85°C.

BIOTECHNOLOGY AND BIOAPPLICATIONS OF COLLOIDAL GOLD

Compiled by Ralph M. Albrecht and Gisele M. Hodges. Published by Scanning Microscopy International, P.O. Box 66507, AMF O'Hare (Chicago), IL 60666, USA, 1988. 312 pp. $39.00 (US delivery), $43.00 (elsewhere).

This soft-cover book, introduced by Marc Horisberger, contains 32 papers which have been published earlier in Scanning Microscopy, Vol. 1 (1987), Scanning Electron Microscopy 1981/II and 1986/IV, and in the Proceedings of the Fourth Pfeffercorn Conference 1985. The source is given for each paper. Publication of the papers in one volume makes it easy to learn about the merits of this relatively new microscopical procedure.

The papers are divided into the following sections, each containing a number of papers (shown in parentheses): Review/Biotechnology of Labelling (4), Backscattered Electron Imaging (2), Cell Surfaces (8), Extracellular Matrix (2), Cytoskeleton (3), Chromosomes/In situ Hybridization (1), Replicas and Low Temperature Procedures in Colloidal Gold Labelling (5), Biomaterials - Protein/Cell Interactions (4), Cell Movement (1), and Labelling of Plant Material (2). Discussion with reviewers is part of most papers unless the authors have incorporated the reviewers' comments into the text. The book has a subject index with over 160 items, a list of reviewers, and an author index. The text is profusely illustrated with excellent black-and-white micrographs, each showing the magnification using micrometer markers. Structures of interest are marked and explained in the legends. The book is printed on glossy paper and the reproduction of the micrographs is of high quality.

The introductory section (Review/Biotechnology of Labelling) provides the background needed to realize the investigative potential of the colloidal gold technique. It is explained why gold markers, first applied as a technique in transmission electron microscopy, can now also be used in scanning electron microscopy (SEM) and in light microscopy, particularly in fluorescence microscopy. The gold particles are obtained by reducing gold salts. Depending on the reducing agent used, the dimensions of the particles may be controlled to lie within the range of 5 to 150 nm. The gold particles are then used to label antibodies, lectins, and other macromolecules such as proteins, polysaccharides, glycoproteins, lipoproteins, etc. In immunological studies, the location of antigens is detected by their reactions with labelled antibodies. In other studies, however, it is also possible to follow the changes which labelled macromolecules undergo as a result of various reactions.

The next section (Backscattered Electron Imaging) explains that using SEM, gold particles can be detected by either secondary electron imaging (SEI) or backscattered electron imaging (BEI). In addition, the gold particles may be identified by X-ray microanalysis from their characteristic X-ray signals.

These first two sections are very important even for an uninitiated microscopist because they contain reviews of instrumentation, specimen preparation, marker systems, labelling procedures, and practical applications complete with numerous references.

Although there are no direct food applications dealt with in the subsequent text, a paper is included on the labelling of sweet taste binding sites in the bud-bearing foliate papillae of Rhesus monkey tongue using thaumatin as a sweet-tasting protein. Papers on immuno electron microscopy of muscles, studies of plant cells and of plant enzymes, visualization of proteins adsorbed on polymers, and low temperature embedding may also be of interest to food microscopists.

This book is a welcome addition to the library of microscopists who are looking for new methods to characterize the structure of foods. The wide variety of applications already documented indicates that this technique could be also useful in food science.

M Kaláb

Food Biotechnology (Food Science and Technology Series, Volume 21). Edited by Dietrich Knorr. 1987. 632 pages, bound, illustrated. $99.75 (U.S. and Canada); $119.50 (all other countries). ISBN: 0-8247-7578-3. Published by Marcel Dekker, Inc., New York.

From the Preface of their book, one reads, "... only 20% of the food products that will be available in 1990 were known in 1982 and ... a major portion of them will be produced via biotechnology." Such hyperbole has been typical of the promotions of biotechnology. In case the preceding prediction does not materialize, it has been pre-excused: "there is a question as to whether the scientific community is willing to accept the challenges presented by these new technologies. Are the resources to be made available?"

Some 40 contributors in 22 chapters of this book provide a "working definition" of biotechnology and lend credibility to the prediction. Applications of biotechnology to foods include sweetener development, waste treatment, nutritional improvement, single cell protein development, enzyme technology for bioconversion, plant tissue culture and many others. Each such application is described in a chapter in this book.

The economic feasibility of biotechnology applications in the food area is touched only lightly in this book; perhaps that is appropriate since meaningful economic analysis in many applications cannot be done until processes are developed. Certainly protein yield comparisons (ranging from 24,000 kg dry weight/ha per year for algae to 60 kg dry weight/ha per year for meat from cattle on grass land) present biotechnology in a favorable light; still the processes have to be developed.

Micrographs are used for illustrative purposes in Chapter 16 on "Bioconversions: Enzyme technology", but this is not a book on food microstructure. Of course, applications of microscopy to conventional foods would be expected to be useful also with foods derived from biotechnology.

This book achieves its aim of providing "a more concentrated source of information on biotechnology as it relates to food production and processing." The book has been edited skillfully, and there is a natural flow of material from one chapter to the next - no mean accomplishment in a book with 40 authors. It is probably a book about the future of the food industry and should be read by all food scientists.

David N. Holcomb

HPLC in Food Analysis, edited by R. MacRae, published by Academic Press, London U.K.. 2nd Edition, 1988.

The thirteen chapters of this monograph address chromatographic theory; HPLC instrumentation; determination of carbohydrates; lipids; amino acids; vitamins; food additives and flavors; natural and synthetic colorants; and analysis for mycotoxins and other food contaminants. A concluding chapter sheds light on possible applications of liquid chromatography/mass spectrometry to food analysis, particularly as the long-sought "universal" HPLC detector. The authors are predominantly affiliated with the food industry or academic food science programs and give a balanced, readable, generally practical account of mature separation techniques available for food analysis.

Major strong points of the book as a whole are emphasis on real-world sample preparation techniques and liberal inclusion of illustrative chromatograms. The chapters covering analysis of lipids and plant pigments are particularly well-organized and informative. Major omissions include no mention of high-performance size exclusion chromatography (either for clean-up or determination) and no discussion of ion chromatography or HPLC techniques for analysis of complex proteins (the latter two omissions are acknowledged by the editor).

This book is recommended to neophytes and those wishing to survey proven HPLC separations for common food components.

K.A. Meyer

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Papers for publication in the international journal Food Microstructure are invited. Papers can cover all types of foods, including vegetables, grains, sea foods, meat, dairy products and others. Topics of interest are: 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. Techniques used may include transmission and scanning electron microscopy, light microscopy, x-ray microanalysis, or other related microscopy/microanalytical methods.

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