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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.

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 (S₁), during draining (S₂, S₃, S₄), and after processing (S₅). The second series involved the ripening process: S₆ (day 11), S₇ (day 15), S₈ (day 20), S₉ (day 30), S₁₀ (day 40) and S₁₁ (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. Different fixation methods were tested and the results compared: 1. OsO₄ (1% w/v) in 0.1 M cacodylate buffer, pH 7.2, containing 10 mM CaCl₂ 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.

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

**MANUFACTURING FLOW SHEET FOR SAINT PAULIN CHEESE**

**Reception of milk**

Physical purification and standardization at 20 g/l

Pasteurizing (72°C, 20 s)

Cooling, transfer to tank (at 33°C)

Inoculating lactic bacteria and rennet addition

Coagulation

Cutting and mixing coagulum for 25 min

Draining and removal of whey (30%)

Curd washed (30% water added)

Pre-pressing in tank

Cutting and molding

Pressing (20 h at 23°C)

Salting in saturated brine (4 h at 12°C)

Drying (24 h at 12°C)

Paraffin coating

Ripening in cave (12°C)

Packaging

Sale

![Figure 1](image)

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 OsO₄. Regardless of the duration of glutaraldehyde fixation, it was followed by post-fixation in OsO₄ 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 CO₂, 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 mm² rods about 6 mm long. They were placed in the spaces of a metal sample 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 -90°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 OsO₄ for 18 h (Fig. 2A and B); b) glutaraldehyde for 30 days and OsO₄ for 18 h (Fig. 2C); and c) OsO₄ 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 OsO₄ 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 S₁-S₄, taken before molding, were not very different. The structure of casein in sample S₁, taken 10 min after coagulation and before the drainage of whey (Fig. 2A) was very similar to that of yogurt (11). Samples S₅ and S₆ were similar to S₁. Sample S₄, after 15 min of pre-pressing in the tank, exhibited a somewhat condensed casein network (Fig. 2B). The casein structure of sample S₅ (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 in S₁ and S₄ (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 (S₅) to day 15 (S₉, Fig. 3B) show an arrangement of casein similar to that of S₅, obtained just after leaving the press. Between days 20 and 25 (Fig. 3C) the cheese morphology changed: micelles were more tightly clustered and the initial 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 S₁ to S₄. 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 S1: 10 min after coagulation, fixed in glutaraldehyde for 2 h and OsO₄ for 18 h (A). S4: sample before molding, fixed in glutaraldehyde for 2 h and OsO₄ for 18 h (B); fixed in glutaraldehyde for 30 days and OsO₄ for 18 h (C); fixed in OsO₄ alone for 18 h (D). No bacterial cell is visible in these micrographs. Only fat globules are noticeable, lodged in the casein.

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 S5 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 S7, 15 days of ripening, structure similar to S5, presence of streptococci (arrows) (B). Sample S8, 20 days of ripening: zones of compact casein begin to appear; compact casein (cc), granular casein (gc) (C). Sample S9, 30 days of ripening: casein more compact (D). Detail of figure 3D showing the fine structure of compact casein (arrows) (E).

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$^\circ$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 (S1 to S4), 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 (120°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 (arrows) is observed.
SEM and cheese microstructure

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 examined by cold-stage SEM (evaporated metal thickness of 10–20 nm) and so the structure of fat in the globules is shown, like that seen 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 S4, 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 S4, S5, and S6. The absence of these cavities 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 membranes 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.

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

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References


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 33:329 (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 S4 (before molding and pressing), after fixing in glutaraldehyde for 15 minutes at 4°C and storing with the cold-stage. The appearance of the casein was less homogeneous than in cold-fixed sample S4, 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 pressed 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 St. 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, OsO₄ 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 subsequently extracted by the 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 surfaces and slow or prevent the penetration of osmium tetroxide to contact triglycerides. It is to be noted that the fixation obtained by Allan–Woitas and Kalab with osmium tetroxide in the presence of imidazole occurred in the very water–rich medium of a yogurt (text reference 1) or on fat globules of fresh cream emulsified 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.


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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 660-682). 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 - 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. 4A-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 author'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.