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B. E. Brooker

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THE CRYSTALLIZATION OF CALCIUM PHOSPHATE AT THE SURFACE OF MOULD-RIPENED CHEESES

B.E. Brooker

AFRC Institute of Food Research, Reading Laboratory
Shinfield, Reading, Berkshire RG2 9AT, U.K.

Abstract

Samples of several different types of mould-ripened cheese were examined by light and electron microscopy for evidence of calcium phosphate crystallization near their surfaces, which, it was predicted, should result from the pH changes that take place in the rind during ripening. Transmission electron microscopy showed that characteristic convoluted crystals appeared in the rind as mould growth developed and that there was good evidence that at least some of the crystal nucleation was taking place inside effete hyphae. Light microscopy showed that this coincided with the appearance of birefringent, phosphate-rich crystals in the cheese rind which were tentatively identified as calcium phosphate. This was confirmed by a series of experiments in which frozen and fractured cheese was examined by scanning electron microscopy in conjunction with digital X-ray spectrometry. This showed that the rind contained very high levels of calcium and phosphorus which could not be attributed to surface drying because in the same area, there was no corresponding concentration of other elements, such as chlorine.

It is proposed that the high pH generated by the surface flora causes the precipitation of calcium phosphate from the continuous aqueous phase. In addition, the inhibitory effect of casein on the phase transformation to crystalline calcium phosphate is probably removed by the action of extracellular proteases from the mould. The resulting depletion of calcium phosphate in the aqueous phase establishes a gradient which is responsible for the diffusion of more of the salt from deeper parts of the cheese and the progressive concentration in the rind.

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Direct inquiries to B.E. Brooker
Telephone number: 44-734-883103x2283

KEY WORDS: Calcium phosphate, crystallization, cheese, mould-ripened, mould, ammonia, casein, X-ray analysis, freezing stage, scanning electron microscopy.

Introduction

It has been known for some time that during the maturation of cheese, microscopic aggregates of calcium phosphate crystals (up to 25 μm diameter) soon appear in the protein matrix and gradually increase in number with time (Laxa, 1926; Swiatek and Jaworski, 1959; Brooker et al., 1975). Although these aggregates appear throughout the protein matrix, the microscopical study of hard cheeses has shown that they occur in largest numbers along the curd junctions and it has therefore been assumed that calcium phosphate crystallizes from pockets of residual whey. Since milk is saturated with respect to a number of calcium phosphate salts, it can be expected that, during the development of acid in renneted milk, part of the micellar calcium phosphate will dissolve to give a supersaturated solution in the subsequent curd or cheese serum. This appears to be borne out by the results of a recent study of 1 month old Cheddar cheese by Morris et al. (1987), in which the aqueous phase was isolated from the cheese by pressing and then found on analysis to be supersaturated with respect to various calcium phosphate salts and to tricalcium citrate. However, there still exist problems in explaining the precipitation of calcium phosphate from an aqueous phase at the low pH's which predominate in young cheeses and in understanding the subsequent crystal nucleation and growth in a casein and peptide-rich environment which, according to previous observations (Termine and Posner, 1970; Termine et al., 1970; Visser et al., 1986) should inhibit such phase transformation. The observation that bacteria are often closely associated with the crystalline material in cheese, and indeed sometimes contain crystals, has suggested to some authors (Knoop and Peters, 1971; Kalab, 1980) that a local rise in pH caused either by the lysis and release of cell contents from dead bacteria or by microbial activity, is sufficient to initiate crystallization of calcium phosphate from the nucleation sites presented by the bacteria.

In the case of hard cheeses, obvious practical problems confront any attempt to demonstrate the minute local changes in pH required by this hypothesis, but it is obvious that similar yet very profound and well established changes do take place over the entire surface of mould-ripened

cheeses such as Camembert and Brie. For example, Le Graet et al. (1983) have demonstrated that pH changes take place at the surface of Camembert-like cheeses during maturation. According to these authors, the pH of the original cheese curd is 4.6 but by day 6 a sharp rise takes place at the cheese surface such that at day 17 the pH has reached 7.6.

On the basis of what has been said above, it can be predicted that in a zone near the surface of soft, mould-ripened cheeses where there is a sharp rise in pH as the flora develops, extensive crystallization of calcium phosphate from the aqueous phase can be expected to occur. In the present work, a number of mould-ripened cheeses were examined for evidence of such changes. This was done using transmission electron microscopy (TEM) and by exploiting the benefits of the low temperature stage of the scanning electron microscope (SEM) which, in addition to preserving natural structural detail, allows the quantification and spatial distribution of relevant elements to be determined in fully hydrated specimens using X-ray spectrometry.

Materials and Methods

Coulommier cheeses were made from pasteurised milk according to the factory method given by Scott (1986) and their surface sprayed with an aqueous suspension of spores of Penicillium candidum (supplied by Eurozyme, U.K.). At least 2 days were allowed to elapse between salting and the earliest microscopical examinations. Mature and immature samples of French Camembert, German and English (Lubborn cheese) Brie and white Lymeswold (Dairy Crest) were purchased from retail outlets.

Light microscopy

Slices of cheese 3 mm thick and normal to the surface were taken from mould-ripened cheeses and frozen by immersion in iso-pentane cooled with solid carbon dioxide. Frozen sections, 3 µm thick, were cut using a Reichert sledge microtome fitted with a Peltier cooling stage and attached to slides. After drying, they were immersed in xylene, mounted unstained in a neutral medium and examined with a Zeiss WL microscope between crossed polarizer and analyzer filters. Other sections were stained by the von Kossa technique (after Carleton and Drury, 1957) for phosphate groups and examined by bright field microscopy. Using these methods, the volume fraction of birefringent and phosphate-rich particles in the rind of ripe Coulommier cheese was determined using a modification of the point counting method of Glagoleff (1933).

Transmission electron microscopy

Samples of each of the above soft cheeses were taken from near the surface to include the layer of mould together with 2 - 3 mm of the underlying cheese matrix. They were fixed for 3 - 4 h in unbuffered 2% glutaraldehyde and then washed for 1 h in 0.1 M cacodylate-HCl (pH 6.8) buffer before transfer to 1% osmium tetroxide buffered with 0.1 M cacodylate-HCl to pH 6.8. After 2 h, they were washed in water and dehydrated in a graded series of acetone-water mixtures and 100% acetone before embedding in Araldite. Sections were cut on a Reichert OmU3 ultramicrotome and stained in lead citrate before examination in a Hitachi 600 transmission electron microscope at an accelerating voltage of 100kV.

Scanning electron microscopy and X-ray analysis

All specimens were examined by SEM whilst still fully hydrated and were prepared and handled using an EMscope SP2000 cryo-preparation unit with transfer device and microscope cold stage. A slice of soft cheese, 2 mm thick, was taken at right angles to the surface so that it included the whole thickness of the layer of mould and some 3 - 4 mm of the underlying protein matrix. It was then placed in a grooved copper holder (or carbon if X-ray analysis was to be performed) where, if necessary, it was secured using a solution of carboxymethyl cellulose and frozen by plunging into nitrogen slush. The sample was transferred to the preparation unit under vacuum, fractured using a pre-cooled blunt rod and then coated in situ either with gold, when purely morphological information was required, or with carbon when X-ray spectrometry was to be performed in conjunction with the structural observations. When coating had been done, the frozen sample was transferred to the cold stage (maintained at -185°C) of a Hitachi S570 SEM and examined. In some cases however, where more detail of the organic matrix was required, the samples were etched by subliming some of the surface ice before coating with gold. This was done in a controlled fashion by observing the uncoated specimen in the microscope at low accelerating voltage (1 - 2 kV) whilst raising the temperature of the sample by means of a stage heater. Sublimation was allowed to occur for 5 min at temperatures as high as -50°C until sufficient detail could be seen. When this was completed and the sample had once more been cooled down to liquid nitrogen temperature, it was transferred to the preparation unit and coated with gold before examination once again in the SEM at higher accelerating voltages.

All X-ray microanalysis was done using a Link Analysis AN 10/85 spectrometer with a standard windowed detector fitted to the specimen chamber of the SEM. When areas of frozen specimens had been selected for elemental analysis, the lowest possible accelerating voltage consistent with the excitation potentials of the selected elements was used to reduce unnecessary penetration of the specimen. The spatial distribution of a number of elements including calcium (Ca), phosphorus (P) and chlorine (Cl) was determined using a semi-quantitative digital X-ray mapping procedure (Digimap) with a screen resolution of 128 x 128 pixels. Fully quantitative determination of the levels of elements in different parts of the cheese was performed using the ZAF-PB analyser software for rough surfaces (Link Analysis) on multiple site (>30) spot analysis with standards (Statham and Pawley, 1978; Statham, 1979).

Results

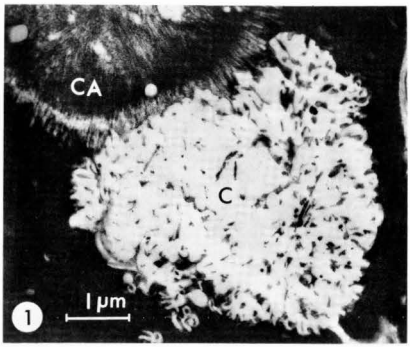
Light microscopy and TEM

The ultrastructural appearance of Coulommier cheese before its surface had been inoculated with mould spores was similar to that previously described by Knoop and Peters (1971). Uniformly distributed throughout the entire cheese in small numbers, aggregates of needle-like crystals (up to 20 µm diameter) were found which were identical to those reported elsewhere from a variety of soft and hard cheeses (Knoop and Peters, 1971; Brooker et al., 1975; Kalab, 1977,1980; Bottazzi et al., 1982). These crystal aggregates were birefringent when examined in polarized light and when stained by the von Kossa technique, appeared intensely black. When the spatial distribution of Ca and P was determined in frozen specimens by digital X-ray mapping, the coordinates of focal concentrations of both elements coincided and it was therefore concluded that the material at these points was composed of calcium phosphate. Because the distribution and numbers of these bodies was similar to that of the crystalline aggregates, it was concluded that the latter were composed of calcium phosphate, as indeed did Bottazzi et al. (1982) in their study of Grana cheese. Detected levels of Ca and P in the surrounding protein matrix were relatively uniform and low.

When the rind (surface 150 µm) was examined from the same cheese on which the inoculated mould had been growing for 1 week, it was evident from TEM that large convoluted crystals had started to appear alongside the existing crystal aggregates (Fig. 1). This coincided with an obvious increase in the number of birefringent particles which were also found to be rich in phosphate groups from their intense staining after the von Kossa technique (Fig. 2). Examined by TEM, the morphology of these particles was quite distinct from that of the crystal aggregates, but it was tentatively concluded that they also were composed of crystalline calcium phosphate in view of their optical activity and their high phosphate content.

In the case of Coulommier cheese, there was a gradual and noticeable increase in the number of these crystals during ripening. Similarly, in all of the other varieties of ripe cheese examined, enormous numbers of calcium phosphate crystals were observed by light microscopy but there was also a gradual reduction in their diameter, the deeper in the cheese they were found (Fig. 2). As expected, the mean volume fraction of birefringent particles in the surface 150 µm of ripe Coulommier was 21.81% ± 7.92 (n = 4000) compared well with 24.50% ± 8.34 (n = 4000) for the phosphate-rich bodies visualised by the von Kossa technique. In comparable areas of the cheese surface, the corresponding values for uninoculated cheese which had no mould growth were 0.06% ± 0.04 for birefringent particles and 0.09% ± 0.03 after von Kossa staining.

By TEM, crystals were usually found associated with the surface of the closely packed hyphae in the rind (Fig. 3) and on very many occasions, crystal growth could be seen to extend across the cell wall into the dispersed contents of a dead hypha (Fig. 4). However, in such cases it was not clear whether crystal growth had originally started within the hyphae or from the aqueous phase of the cheese. Profiles such as those given in Fig. 5 demonstrate that in many instances, the nucleation site for crystal growth was inside the moribund hyphae. Since it is impossible to infiltrate crystals with resin in order to section them,it is important to note that the structures viewed by TEM and referred to here as crystals, actually refer to the spaces vacated by crystalline material during processing. Such spaces were visible by negative contrast because of the presence of the surrounding protein.



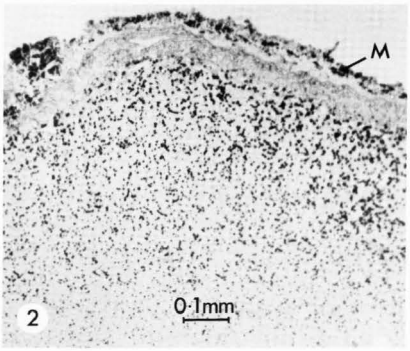
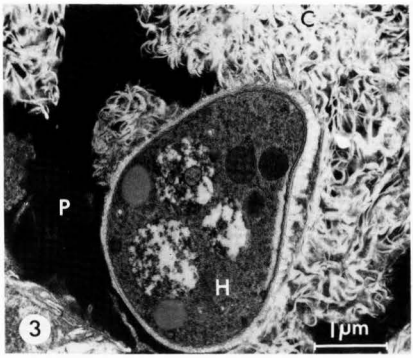
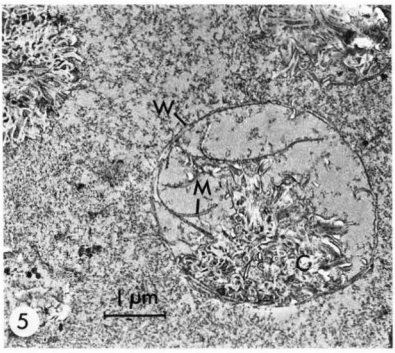
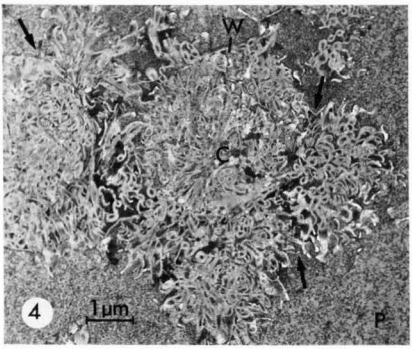


Fig. 1. A convoluted crystal (C) in the rind of a 3 week old Coulommier cheese lying adjacent to a crystal aggregate (CA). TEM.
Fig. 2. Frozen section through the surface of 3 week old Coulommier cheese stained by the von Kossa technique. Beneath the mould (M), numerous phosphate-rich particles are visible. Light microscopy.







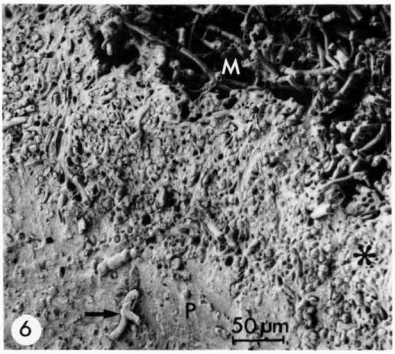


Fig. 3. Transverse section of a hypha (H) of Penicillium candidum in the rind of Camembert cheese surrounded by several convoluted crystals (C). P = protein matrix. TEM.
Fig. 4. Convoluted crystals (C) growing inside the cell walls (W) of two effete hyphae in Brie cheese. The crystals extend through the cell walls (at arrows) into the surrounding cheese matrix (P). TEM.

Fig. 5. Transverse section of a single moribund hypha in which only a few membrane remnants remain (M). The growth of a convoluted crystal (C) has not yet extended beyond the cell wall (W). From the rind of Brie. TEM.
Fig. 6. Ten day old Coulommier cheese rind frozen and fractured showing the growth of mould (M) on the protein matrix (P). The most superficial layer (*) of the cheese contains a tangled mass of hyphae. Below this (arrows), hyphae are less common. SEM cryo stage.

SEM and X-ray analysis

When frozen cheese samples were fractured normal to the mould covered surface and examined by SEM, a clearly defined superficial layer (150 - 250 µm thick depending on cheese variety) was visible in which a tangled mass of mould hyphae had burrowed into the cheese matrix (Fig. 6). This corresponded to the layer seen by TEM but because of the tight packing of structures, and the lack, at this resolution, of any characteristic morphology on the part of the calcium phosphate crystals, the latter could not be positively identified in this type of preparation. Digital X-ray mapping of a fracture face such as that shown in Fig. 7 showed the individual crystal aggregates of calcium phosphate in the region below the cheese rind as described above (Figs. 8,9,10 and 11) but it also showed very high levels of Ca and P in a layer 75 - 250 µm thick (depending on age and variety of cheese) which always corresponded to that part of the rind containing the tightly packed

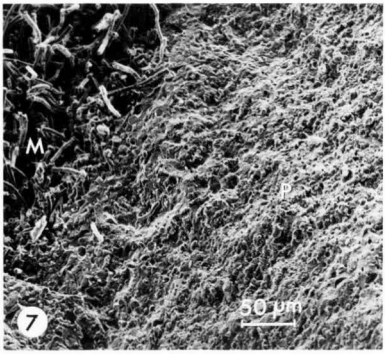


Fig. 7. Camembert cheese rind frozen and fractured showing the growth of mould (M) on the surface of cheese matrix (P). Figs. 10 and 11 are digital X-ray maps of this same area. SEM cryo stage.

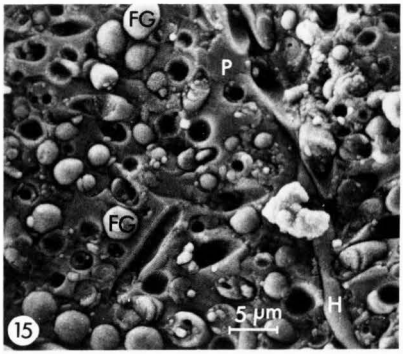


Fig. 15. Lymeswold cheese frozen and fractured. In the lower part of the rind (500 µm from surface) numerous fat globules (FG) protrude from the surface. Note the exposed hyphae (H). P = protein matrix of the cheese.

hyphae and convoluted crystals (Figs. 10 and 11). The distribution of Ca and P was continuous in this zone so that the X-ray emission from individual crystals could not be separated from that of their neighbours. X-ray maps of the same area viewed at low magnification (up to x30) showed, in addition, gradually diminishing levels of Ca and P from the rind to the deeper parts of the cheese matrix (Figs. 12 and 13). This gradient could not be regarded as an artefact caused by the surface topography of the specimen because a digital X-ray map of Cl in the same area showed a comparatively uniform distribution both in the rind and in the much deeper parts of the cheese (Fig. 14). This important observation also demonstrated that the very high levels of Ca and P in the cheese rind were not caused by drying of the cheese surface, for such desiccation would also have led to a concentration of the sodium chloride in the aqueous phase and, correspondingly, high levels of Cl in the X-ray map.

Similar observations were made with all of the other mould-ripened cheeses examined. However, Lymeswold was unlike any other cheese because when frozen and fractured, the fracture plane passing through the rind showed many spherical objects projecting from the surface whose dimensions were similar to those of the convoluted calcium phosphate crystals observed by TEM (Fig. 15). However, when multiple spot analyses were done on these objects, they contained no more Ca and P than background and therefore probably represented fat globules embedded in the protein matrix.

Quantitative data on the relative levels of Ca and P in the surface rind and the deeper areas of Camembert and Coulommier cheeses were obtained using spot analysis of at least 30 randomly assigned points in each zone by X-ray spectrometry. The results presented in Table 1 are those obtained from Camembert cheese and show that the average concentration of Ca and P in the rind was almost 20 times greater than in the body. Similar figures were obtained using Coulommier cheese. In some places, there was a 100 fold difference between the highest values of Ca (4.94 g%) and P (4.12 g%) in the rind and their minimum values in the cheese body (Ca 0.04 g%: P 0.03 g%). The wider spread of values obtained for Ca and P concentrations in the body of the cheese (Table 1) is not entirely unexpected because a proportion of these random point analyses would be expected to impinge on some of the many crystalline aggregates of calcium phosphate and can thus be expected to give much higher values than those derived solely from the background protein matrix.

Discussion

In many surface ripened cheeses, the deamination of amino acids by the mould and its associated flora produces ammonia (Hemme et al., 1982) which diffuses into the rind and raises the pH (Le Graet et al., 1983), thereby creating conditions in which soluble calcium phosphate might be expected to precipitate and then crystallize from the aqueous phase. However, because casein and peptides have an inhibitory effect not only on the formation of amorphous calcium phosphate but also on its transformation to the crystalline form (Termine and Posner, 1970; Termine et al., 1970), the extracellular proteases produced by the mould which degrade cheese protein to peptides and amino acids, probably have a part to play in crystallization by removing the local source of inhibition.

Figures 8 - 14 (color plate) are on pp. 32-33.

The results of the present study show that during the maturation of mould-ripened soft cheeses, calcium phosphate crystallizes and becomes concentrated in a thin layer near the cheese surface. Since crystalline, phosphate-rich bodies appear in the rind at the same time, it is concluded that they are composed of calcium phosphate and from their numbers, that they are largely responsible for the observed high levels of Ca and P near the cheese surface. However, it cannot be excluded that in the digital X-ray maps, there is a significant contribution from amorphous calcium phosphate dispersed throughout the aqueous phase of the rind which has not yet crystallized. This would help explain the near continuous distribution of Ca and P seen in the X-ray maps. It seems clear from the number and distribution of calcium phosphate crystal aggregates observed throughout the cheese that they contribute little to the total levels in the rind. Profiles such as that in Fig. 5 are reminiscent of those obtained by Kalab (1980) in the case of lactic acid bacteria and demonstrate clearly that nucleation and growth of crystals frequently takes place inside hyphae and that their cell walls are freely permeable to the soluble calcium phosphate of the cheese. Such nucleation sites have the advantage that the pH is high enough to initiate precipitation of calcium phosphate whilst, at the same time, being remote from the inhibitory effect of casein on its subsequent crystallization. In spite of this, it is still doubtful how much of the total crystal growth is initiated in this way compared with other sites in the rind. However, it appears highly likely that the escape of cell contents from moribund hyphae contributes not only to the rise in pH of the cheese surface but that hyphae also release cell organelles which may act as nucleating centres in the aqueous phase.

Although the concentration of Ca and P in the surface of Camembert has been noted by other authors using atomic absorption spectrometry (Metche and Fanni, 1978; Le Graet et al., 1983), the values for Ca and P in the rind given by these workers were considerably lower than those obtained in the present study. This was because the sampling procedure of removing relatively large pieces of cheese for analysis necessarily included the deeper zone of Ca and P - depleted cheese which therefore had the effect of lowering the average levels throughout the sample.

Whilst these considerations explain crystallization of the calcium phosphate that already exists in the aqueous phase near the cheese surface, the pronounced concentration of this salt, apparent from the digital X-ray mapping, requires a further explanation. A diagrammatic representation of the events that could be involved in this process are shown in Fig. 16. It can be seen that as the pH of the cheese surface begins to rise and crystallization begins, the aqueous phase becomes depleted and a chemical gradient is established with respect to calcium phosphate. Soluble calcium phosphate in the aqueous of deeper parts of the cheese then diffuses towards the surface where, eventually, that too is precipitated by the prevailing conditions of high pH. In this way, calcium phosphate gradually accumulates in the cheese rind where it reaches (and can probably surpass) the high levels detected in this study. Although diffusion rates for calcium phosphate through the aqueous phase of cheese have not been measured, Geurts et al. (1974) have determined the effective diffusion coefficient for sodium chloride and found it to be in the order of 0.2 cm^2/day. Since similar rates of diffusion can be expected for calcium phosphate, concentration to the observed levels is quite feasible by this mechanism.

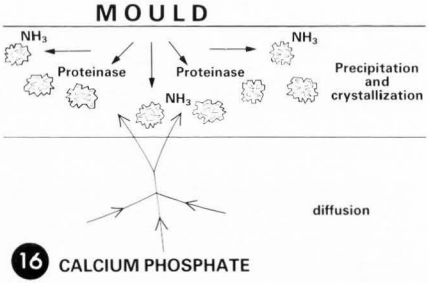


Fig. 16. Diagrammatic representation of the events involved in the crystallization and concentration of calcium phosphate in the rind of mould-ripened cheeses.

Table 1. Mean concentrations of Ca and P (g%) ± SD in 30 randomly assigned points of Camembert rind and cheese body in cheese of 53% water content. Determinations by X-ray spectrometry.

	Ca	P
Rind	3.50 ± 0.77	2.86 ± 0.64
Cheese body	0.19 ± 0.17	0.16 ± 0.15

Acknowledgements

The author extends grateful thanks to Mrs. Jill Webb for her technical assistance in parts of this work.

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Discussion with Reviewers

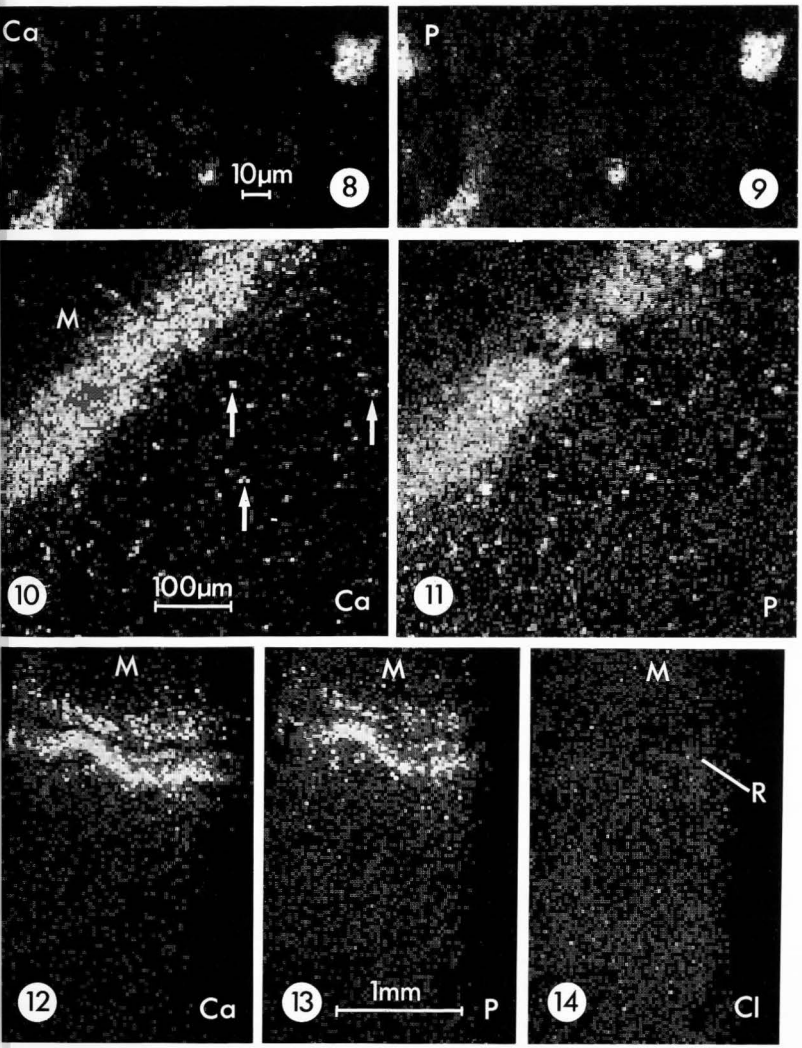
M. Kalab: Did not the relatively high temperature of $-50^{\circ}C$ used during freeze-etching lead to ice crystal formation ?

Author: Ice crystal growth certainly does occur at this temperature but in practice this presents no great problem. Specimens of the size used in this study always contain some ice crystals but the time for which they are held at this temperature does not allow significant growth to occur. It is interesting to note that such high temperatures are required with some specimens in which the water is tightly bound.

M. Kalab: What was the source of the ZAF-PB Analyser software ? Are references available ?

Author: This is a standard software package available from Link Analysis, High Wycombe, U.K. It is especially useful for the quantitative determination of elements in samples with a 'rough' surface and its special feature is the measurement of peak to background (PB) ratios to do this accurately. Of the many references available, I have given two by Statham.

Figs. 8 - 14. Digital X-ray maps of frozen ar
fractured samples of Camembert cheese showing
distribution of various elements. SEM. M = mo
Fig. 8. Distribution of Ca in a zone just ber
the rind. The focal concentrations represent
tal aggregates.
Fig. 9. Distribution of P in the same area as
in Fig. 8. Focal concentrations correspond in
position to those in Fig. 8.
Fig. 10. Distribution of Ca in the area shown
Fig. 7 near the cheese surface. Very high lev
occur in a layer of the rind. Note also the m
tiple focal concentrations (arrows) in deeper
which correspond to aggregates of crystalline
calcium phosphate. There is little or no acti
in the area occupied by the surface mould.
Fig. 11. Distribution of P in the same field
Fig. 10. A similar layer in which levels are
high can be seen in the rind. The co-ordinate
the focal concentrations in the deeper areas
cide with those for Ca in Fig. 10. Note the r
sonably high levels of P in the mould.
Fig. 12. Low magnification elemental map show
the distribution of Ca. The high concentratio
in the rind is clearly seen but the individua
crystal aggregates are invisible at this magn
cation. Note the zone of reduced Ca levels be
the rind and beneath this, lower levels still
Fig. 13. Distribution of P in the same field
Fig. 12. A gradient similar to that in Fig. 1
is less clear but the high levels in the rind
obvious.
Fig. 14. Distribution of Cl in the same field
Figs. 12 and 13. There is no concentration of
in the rind (R) and no indication of the grad
seen in Figs. 12 and 13.



INCREASING CONC.→