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ELECTRON DENSE GRANULES IN YOGHURT: CHARACTERIZATION BY X-RAY MICROANALYSIS

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Abstract

A study was undertaken to investigate the influence of buffer composition, pH and glutaraldehyde fixation time on the appearance of electron dense granules in yoghurt. Yoghurt particles were fixed in 3.5% glutaraldehyde and postfixed in 2% osmium tetroxide in veronal acetate or phosphate buffer. Thin sections were examined unstained with an electron microscope equipped with a scanning transmission electron microscope module and energy dispersive X-ray analyser.

Electron dense granules appeared whenever glutaraldehyde and osmium tetroxide were utilized sequentially, irrespective of the type of buffer, pH (5.0 vs 6.75), or glutaraldehyde fixation time (2 or 24 h). Granules were absent if glutaraldehyde was used alone. Granules were generally located around the outer edge of casein particles, fat globules and bacteria. X-ray microanalysis of these granules detected the presence of 89-100% osmium (Os) and 0-11% chlorine (Cl) on a weight percent basis. Granules were removed by treatment with periodic acid or hydrogen peroxide. It appears that the presence of osmium tetroxide is a prerequisite for granule formation and their appearance is not always dependent on the use of phosphate buffer as has been suggested in previous research. The small quantity and variability in Cl content precluded this element from being considered a factor in granule formation. The most probable source of Cl is the embedding medium. Results from this study suggest that granules are fixation artifacts consisting of a complex of glutaraldehyde and osmium tetroxide, however the structure of the complex and mechanism of formation are still unknown.

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Key Words: Yoghurt, electron dense granules, artifacts, X-ray microanalysis, double fixation, osmium fixation.

Introduction

Double fixation utilizing glutaraldehyde and osmium tetroxide in sequence is widely recognized as an effective method of preserving biological tissue for electron microscopy. During a microstructural study of yoghurt, we consistently observed electron dense granules in thin sections of yoghurt fixed with glutaraldehyde and osmium tetroxide. This phenomenon was previously observed in yoghurt by Tamime et al. (1984) but no explanation of its presence was given. Kalab (1977) noted the presence of small, dark grains concentrated around the fat globule membrane in yoghurt fixed with glutaraldehyde containing lead acetate.

Similar granules have also been documented in non-food tissue including lung (Gil and Weibel, 1968), heart and kidney (Kuthy and Csapo, 1976) and lymph and thymus tissue (Hendriks and Eestermans, 1982). The above studies concluded that an influential factor in granule formation was the presence of phosphate buffers during a double fixation procedure. This study reports: (1) preliminary observations on electron dense granules in yoghurt, and (2) additional experiments aimed at characterizing them.

Materials and Methods

Preparation of Yoghurt

Yoghurt was prepared from ultra-high-temperature (UHT,140 C for 2 s) processed and homogenized (105 kg/cm^2) milk, or commercially pasteurized milk further heat treated at 85 C for 20 min. Milk was cooled to 45 C, inoculated with 3% mixed culture Streptococcus thermophilus and Lactobacillus bulgaricus (1:1) and incubated at 43 C until pH 4.6 + 0.05 was attained. Yoghurt was stored at 5 C and examined 1 day later.

Electron Microscopy

Yoghurt particles of approximate size 2 X 2 X 5 mm were excised from 200 ml cups about 10 - 15 mm below the surface and immersed in 3.5% aqueous glutaraldehyde (unbuffered, pH 3.6) for 24 h at room temperature. Samples were then cut into approximate 1 mm cubes, washed several times (minimum of 3 washes of 20 min each or overnight wash) in distilled water, postfixed in 2% osmium tetroxide (veronal acetate buffer pH 6.75) for 2 h at room temperature and washed in postfixative buffer. Samples were dehydrated in graded ethanol series

and embedded in Spurr's resin. Additional experiments were conducted to determine the influence of buffer composition (veronal acetate versus phosphate), pH (5.0 versus 6.75) and glutaraldehyde fixation time (2 versus 24 h), as outlined in Table 1. Between fixatives, samples were washed in the prefixative vehicle. Following osmium fixation, where applicable, (Table 1), samples were further processed as outlined above.

Thin sections (90 nm) were examined either unstained, or stained at room temperature with uranyl acetate (2% in 50% ethanol for 30 min) followed by 5 - 10 min in lead citrate (Reynolds, 1963). Where applicable, sections were treated with 2% periodic acid or 3% hydrogen peroxide for 15 - 30 min (Ellis and Anthony, 1980). Sections were examined with a Philips EM 300 or Philips EM 400T transmission electron microscope (TEM) at 60 or 100 kV, respectively.

X-ray Microanalysis (EDX)

For elemental analysis, sections were mounted on formvar-carbon coated aluminum grids and examined with a Philips EM400T TEM equipped with a Philips PW6585 scanning transmission electron microscope (STEM) module and an EDAX PV9100/400 semi-quantitative energy dispersive X-ray analyser. Static probe analysis was performed at 100 kV in the TEM mode with a probe diameter of 40 nm for a count time of 100 live time s. Total counts were calculated as counts per second (CPS) X 100. Elemental distribution maps (X-ray dot maps) of osmium (Os), using the M_α, M_β, L_α and L_β energy lines, were collected in STEM mode by the EDX detector for 2000 s using a 20 nm convergent scanning beam.

Reagents

Glutaraldehyde (Can-Em Chemical Distributors) solutions were prepared from 70% sealed ampoules stored at 4 C. Osmium tetroxide (Fisher Scientific) was prepared from crystal form. All buffer reagents were analytical grade.

Results

Preliminary Observations

Electron dense granules (Fig. 1) were first observed by the authors while studying the effects of heating milk on the ultrastructure of yoghurt. Initially it was assumed that these granules were probably lead carbonate precipitate - the result of lead citrate contrasting of thin sections. However, the presence of granules in unstained sections eliminated this hypothesis. Preliminary examination of granules by EDX showed an extremely high concentration of the element Os and small amounts of chlorine (Cl). From a total of six spectra obtained from different sections, Os accounted for 83 - 90% and Cl for 10 - 17% of granule composition on a weight percent basis.

Effect of buffer, pH and glutaraldehyde fixation time

Results are summarized in Table 1. Electron dense granules were present in all cases where osmium tetroxide was used as a postfixative. There was no apparent difference in granule appearance or density when samples were fixed in 3.5% glutaraldehyde for 2 or 24 h. Type of buffer (veronal versus phosphate, (Table 1)) did not affect the incidence of granules but affected the size and distribution of granules. Postfixation in veronal acetate buffer (0.05M) at pH 5 or pH 6.75 produced discrete, irregular shaped granules of diameter 40 nm and less (Fig. 2). Granules were usually located on the periphery of casein micelles and to a lesser extent around fat globules. The use of sodium phosphate buffer (0.13M) in both the glutaraldehyde and osmium fixation steps yielded smaller (5-20 nm) granules which were distributed throughout casein micelles as well as being present on exterior edges (Fig. 3).

In thin sections of yoghurt containing a bacterium, there was a definite concentration of electron dense granules around the cell wall (Fig. 4). This localization was observed in both veronal and phosphate buffer systems. In samples fixed in aqueous glutaraldehyde only, there were no granules present even after 24 h fixation time (Fig. 5).

X-ray Microanalysis (EDX)

Os and Cl were detected in granules with Os being the major element on a weight percent basis. Cl was present in trace amounts. The percentage range for each element was 89 - 100% for Os and 0 - 11% for Cl. A typical EDX spectrum showing the M_α (1.91 keV), M_β (1.98 keV), L_α (8.90 keV) and L_β (10.35 keV) lines of Os, and the K_α (2.62 keV) line of Cl is given in Fig. 6. The presence of aluminum is due to the grid.

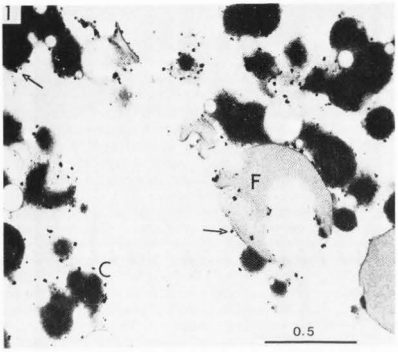
X-ray dot maps for Os (Fig. 7) clearly demonstrate a high density of this element in corresponding electron dense areas. Immersion of grids for 15 min in 2% periodic acid was usually sufficient in removing a large percentage of granules, however 30 min provided complete removal. When mapped, sections treated with periodic acid displayed Os deficient areas where granules were previously

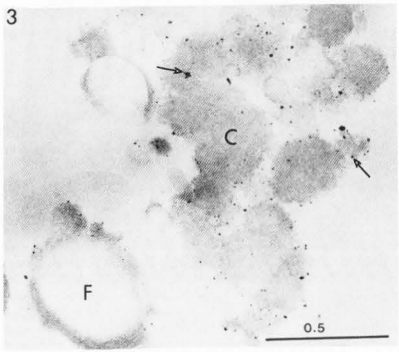
Fig. 1. TEM micrograph of yoghurt fixed with 3.5% aqueous glutaraldehyde 24 h and 2% osmium tetroxide in 0.05M veronal acetate buffer pH 6.75, 2 h. Section stained with uranyl acetate and lead citrate. C = casein; F = fat globule; arrows point to electron dense granules. Bar = 0.5 μm.

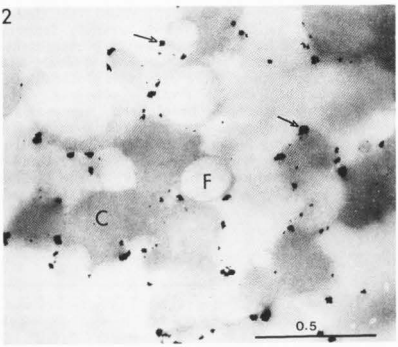
Table 1. Appearance of electron dense granules in yoghurt as influenced by buffer composition, pH and fixation time.

Fixation Schedule	Glutar-aldehyde	Fixation Time
	2 h	24 h
3.5% Glut(aq), 2% OT (VAB pH 5) 2 h	(+)	(+)
3.5% Glut(aq), 2% OT (VAB pH 6.75) 2 h	(+)	(+)
3.5% Glut(PB pH 6.75), 2% OT (PB pH 6.75) 2 h	(+)	(+)
3.5% Glut(aq)	(-)	(-)

Glut = glutaraldehyde; aq = aqueous; OT = osmium tetroxide; VAB = veronal acetate buffer 0.05M; PB = sodium phosphate buffer 0.13M.
(+) = granules present; (-) = granules absent







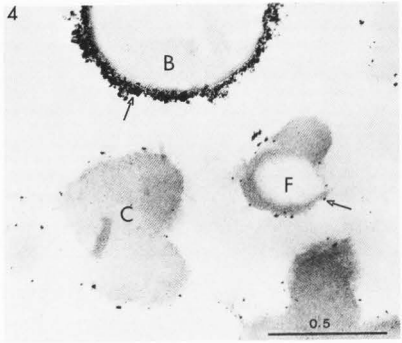
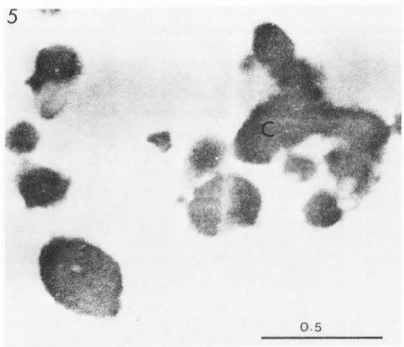


Fig. 2. TEM micrograph of yoghurt fixed in 3.5% aqueous glutaraldehyde 2 h and 2% osmium tetroxide in 0.05M veronal acetate buffer pH 5.0, 2 h. Unstained section. C = casein; F = fat globule; arrows point to electron dense granules. Bar = 0.5 μm.

Fig. 3. TEM micrograph of yoghurt fixed in 3.5% glutaraldehyde in 0.13M phosphate buffer pH 6.75, 2 h and 2% osmium tetroxide in 0.13M phosphate buffer pH 6.75, 2 h. Unstained section. C = casein; F = fat globule; arrows point to electron dense granules. Bar = 0.5 μm.

Fig. 4. TEM micrograph of yoghurt fixed as outlined for Fig. 3. C = casein; F = fat globule; B = bacterium. Arrows point to electron dense granules. Note concentration of electron dense granules around bacterium. Bar = 0.5 μm.

Fig. 5. STEM micrograph of yoghurt fixed in 3.5% aqueous glutaraldehyde 24 h. Unstained section. C = casein. Bar = 0.5 μm.



localized (Fig. 8). Hydrogen peroxide was not as selective in granule removal, with granules still partially present after 30 min in peroxide.

Discussion

Characterization of electron dense granules

A continuing concern in microstructural studies is the interpretation of structures, such as electron dense granules, as genuine components of the substrate under investigation or as artifactual material. Although the microstructure of yoghurt has been studied (Kalab et al., 1976; Davies et al., 1978; Tamime et al., 1984), there have been few documented reports of the occurrence of electron dense granules.

The two major components of milk readily identifiable by electron microscopy are casein micelles and fat globules. Casein micelles exhibit a range of size; a representative micellar diameter of 100 nm, and 500 - 1000 nm for fat globules was recently cited (Kalab, 1985). Casein micelles are made up of smaller subunits or submicelles, 10 - 20 nm in diameter, and held together by calcium phosphate bridges (Morr, 1967). In view of the subunit structure of casein micelles, it was not inconceivable to initially consider the electron dense granules as potential submicelles, based on size similarity. However, based on results in this study, there are several contributing factors on which to classify electron dense granules in yoghurt as artifacts.

From Table 1 it is evident that granules appear only when glutaraldehyde fixed samples were postfixed in osmium tetroxide. The absence of granules when glutaraldehyde was the only fixative is strongly indicative of an artifact. These findings are in agreement with other studies on organ tissue (Gil and Weibel, 1968; Kuthy and Csapo, 1976; Hendriks and Eestermans, 1982) who also observed the absence of granules if samples were singularly fixed in glutaraldehyde or osmium tetroxide. There appeared to be an influence of the tissue being fixed in relation to the size of granules observed. Granules in organ tissue ranged in size from 20 - 1000 nm compared to those in yoghurt which were generally less than 40 nm in diameter.

As well as determining elemental composition of granules, EDX was applied to casein micelles (Fig. 9) to assess whether there was a similarity in uptake of osmium tetroxide. The ratio of Os in granules to Os in micelles was greater than 2:1. Thus the components of granules have a much greater affinity for osmium tetroxide than casein.

Factors affecting the occurrence of electron dense granules

Our results indicate that the appearance of electron dense granules was independent of buffer, pH or glutaraldehyde fixation time within the ranges investigated. It seems conclusive that osmium tetroxide is required for granule formation, however granule formation is not solely dependent on the presence of phosphate buffers as suggested by other researchers. Phosphate was suggested as a requirement for granule formation at concentrations greater than 0.1M (Hendriks and Eestermans, 1982). Our results with veronal acetate buffer dispute the exclusive role of phosphate buffers in granule formation. To gain further insight into the possible role of phosphate in granule formation, milk was fortified with 10mM calcium chloride and 10mM sodium phosphate (pH 6.6) and processed into yoghurt as previously outlined. This action was an attempt to increase the incidence of calcium phosphate particles which would normally be soluble at the pH of yoghurt. McGann et al. (1983) showed that calcium phosphate precipitate in bovine milk consisted of sphere-like granules approximately 2.5 - 5 nm in diameter. It was speculated that calcium phosphate particles may have been the point of nucleation or an intermediary in granule formation in yoghurt. However, examination of calcium fortified yoghurt showed granules of similar composition to those observed in regular yoghurt. Calcium was not consistently detected in granules from calcium fortified yoghurt (less than 1.5% calcium or none detected). Thus it is likely that solubilization of calcium occurred during acidification of milk to form yoghurt, and was subsequently leached away during fixation and washing steps. Unfortunately, the proximity of the K_α line of phosphorus (2.01 keV) to the M_α line of Os (1.914 keV) prevented positive identification of phosphorus in samples by EDX.

It has been noted (Gil and Weibel, 1968; P. Allan-Wojtas, personal communication) that there is an increased tendency towards granule formation when samples have been stored in glutaraldehyde for extended periods. Granules were, however, found in the current study after only 2 h glutaraldehyde treatment with a double fixation schedule. It is worth noting that we have been successful in producing micrographs of yoghurt free of electron dense granules by following the fixation procedure of Allan-Wojtas and Kalab (1984). Yoghurt fixed by their procedure is shown in Fig. 10. Those variables that differed in their fixation method include: a lower concentration of glutaraldehyde (1.4% for 24 h), lower concentration of osmium (0.5% for 24 h), a cacodylate buffer for glutaraldehyde fixation and an imidazole-veronal acetate buffer mixture for osmium

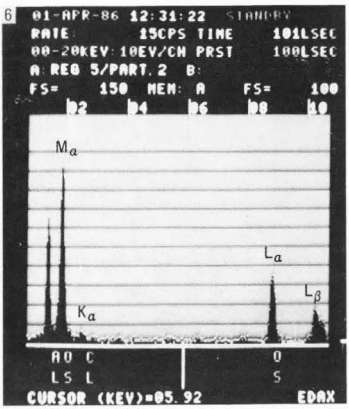
Fig. 7. (A) STEM micrograph of yoghurt fixed in 3.5% aqueous glutaraldehyde 2 h and 2% osmium tetroxide in veronal acetate buffer pH 5.0, 2 h. (B) STEM X-ray dot map for Os corresponding to (A). Unstained section. Bar = 0.5 μm.

Fig. 8. (A) STEM micrograph of yoghurt fixed as outlined in Fig. 7 and treated with 2% periodic acid 15 min. (B) STEM X-ray dot map for Os corresponding to (A). Unstained section. Bar = 0.5 μm.

Fig. 9. EDX spectrum of casein particle in yoghurt showing M_α, L_α, L_β lines for Os and K_α line for Cl. Al peak is from the grid.

Fig. 10. TEM micrograph of yoghurt fixed in 1.4% glutaraldehyde in 0.1M cacodylate buffer pH 7.4 and 0.5% osmium tetroxide in imidazole-veronal acetate buffer 24 h (Allan-Wotjas and Kalab, 1984). C = casein; F = fat globule. Section stained with uranyl acetate and lead citrate. Bar = 0.5 μm.

Fig. 11. TEM micrograph of yoghurt fixed in 3.5% aqueous glutaraldehyde 2 h, and 2% osmium tetroxide in veronal acetate buffer pH 5.0, 2 h. C = casein; F = fat globule. Section treated with 2% periodic acid 15 min and stained with uranyl acetate and lead citrate. Bar = 0.5 μm.



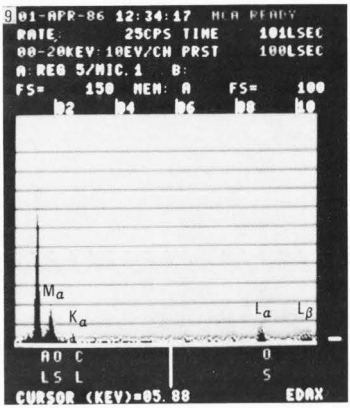
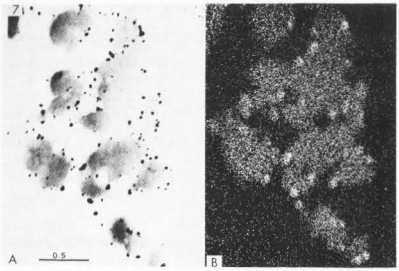
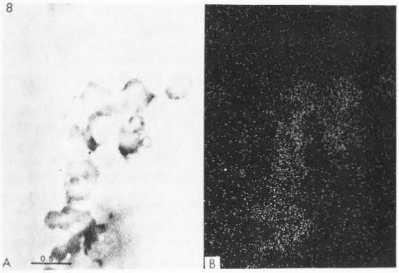
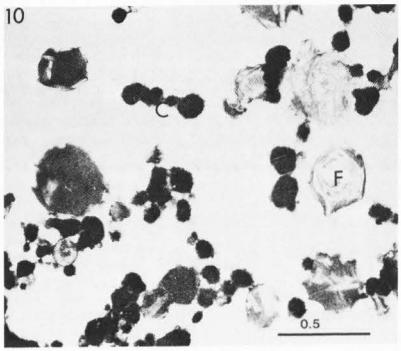
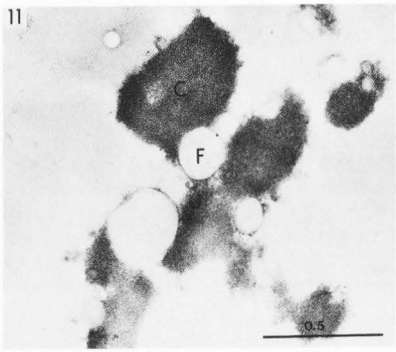


Fig. 6. EDX spectrum of electron dense granule in yoghurt showing Mα, Lα, Lβ lines for Os, and the Kα line for chlorine. Al peak is due to the grid.









fixation. The lower concentration of osmium tetroxide may be an influential factor in preventing granule formation and deserves further study. Granules were found by other researchers using 1.5% glutaraldehyde (Hendriks and Eestermans, 1982), and also the presence of sodium cacodylate buffers have been implicated in granule formation (Kuthy and Csapo, 1976).

Composition of electron dense granules

X-ray microanalysis identification and the removal of granules by oxidizing agents established that the major element present in granules is Os. Periodic acid and hydrogen peroxide have been recommended as agents for the removal of Os (Knight, 1977). Since osmium tetroxide also acts as an electron stain (Hayat, 1981), its removal greatly lowered contrast on micrographs (Fig. 8). For this reason, periodic acid treated sections were stained with uranyl acetate and lead citrate and reexamined. Following staining (Fig. 11), there were still no underlying structures, proteinaceous or otherwise, which were visible in areas previously dispersed with granules.

Due to the low atomic number of oxygen (8), this element could not be detected by EDX. Therefore, it is difficult to speculate which lower oxidation state of osmium was prevalent in the granules. The formation of electron opaque compounds is typical of a reduced state of Os, and is in fact the basis of fixation of unsaturated fatty acids in lipids. The scheme proposed for the oxidation of a double bond in lipids has been summarized by Hayat (1981). The reaction between glutaraldehyde and osmium tetroxide to form "osmium black" is well known. The term "osmium black" refers to the lower oxides and polymeric compounds of Os which are also formed during the reaction of osmium tetroxide with unsaturated lipids (Hayat, 1981). Hopwood (1970) found that the formation of "osmium black" was higher at increased concentrations of glutaraldehyde and osmium tetroxide. Formation of "osmium black" was temperature dependent, but was possible at room temperature (25 C) within 2 h. The chemical composition of "osmium black" was investigated by White et al. (1976) who confirmed the existence of a mixture of Os(VI), Os(IV) and Os(III) compounds. While it seems unlikely that "osmium black" was formed from residual glutaraldehyde - osmium tetroxide interaction in this study (due to washing between fixatives), this possibility cannot be entirely disqualified.

Chlorine does not appear to have a role in granule formation. Originally, it was thought to originate from hydrochloric acid used as a pH adjusting agent for veronal buffers, but it was also present in phosphate buffered samples. Cl was detected in the plastic background as well as within casein micelles (Fig. 9). The amount of Cl in granules was extremely low and was never encountered at levels found in our preliminary studies (less than 17%). The most probable source of Cl is the Spurr's resin used for embedding. Cl is often a contaminant in epoxy resins in variable amounts (Ingram and Ingram, 1980; Roomans, 1979). Thus Cl should be classified as an embedding artifact since any Cl originally present in the yoghurt would have been removed during the preparative steps.

It is possible to conclude from our results that electron dense granules are composed of a complex of glutaraldehyde and osmium tetroxide, but the mechanism of complex formation is still not known. We disagree with the critical role of certain buffer salts, particularly phosphate as suggested earlier (Gil and Weibel, 1968; Kuthy and Csapo, 1976; Hendriks and Eestermans, 1982), in granule formation. The higher (3.5%) than normal (1.5%) concentration of glutaraldehyde may have promoted complex formation of the "osmium black" category. Additional studies aimed at isolating electron dense granules which could be subject to structural analysis are required. The tendency of electron dense granules to concentrate around bacteria could be useful in obtaining sufficient amounts for further study.

Acknowledgements

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Discussion with Reviewers

M. Ruegg: The authors should explain the reason for choosing rather high 2% OsO_4 concentration. Did 1% solutions also produce granules?
Authors: Solutions of 2% OsO_4 have been utilized effectively for fixation of yoghurt (Harwalker and Kalab, 1981; Kalab, 1977; Tamime et al., 1984), therefore they were chosen for this study. 1% solutions were not tried in this study, but were utilized by other researchers (Kuthy and Csapo, 1976; Hendriks and Eestermans, 1982) who also observed electron dense granules.

M. Ruegg: The pH values of the buffers are arbitrary and not adapted to that of the yoghurts. What happens if the pH is closer to that of the original sample?
Authors: The fixation of yoghurt followed procedures in the literature where pH 6.75 is commonly cited (Harwalker and Kalab, 1981; Kalab, 1981; Tamime et al., 1984). The lowest pH tried was pH 5.0 which did not reduce the incidence of granulation (see also the next answer).

R.J. Carroll: Why were the yoghurt samples fixed at pH 6.75 and 5.0 rather than pH 4.6?
Authors: The published works (Kalab, 1977; Davies et al., 1978; Harwalker and Kalab, 1981; Alan-Wojtas and Kalab, 1984; Tamime et al., 1984) on yoghurt fixation for electron microscopy have extensively utilized pH 6.75, 7.2 or 7.4 in various buffers. Glauert (1978) suggests that fixative pH within the 6.5 to 8.0 pH range is adequate for most tissues. We used pH 6.75 due to literature recommendations above, and also pH 5.0 which is within 1 pH unit of the pH of yoghurt. pH 5.0 was also recognized as being a more effective buffering pH for veronal acetate.

M. Ruegg: The artifact has been observed after fixation at pH 5.0 and 6.75. The original samples had a pH around 4.6. Could the pH shift during fixation and the difference in ionic strength influence the nucleation of the electron dense granules?
Authors: As mentioned in the discussion, a possible point of nucleation may have been insoluble calcium phosphate. Micellar calcium phosphate is solubilized at pH 5.0 (Heertje et al., 1985). Granules were observed at both pH 5.0 and 6.75, even when milk was fortified with additional calcium and phosphate prior to making yoghurt, therefore calcium phosphate is not implicated. This is not surprising considering the low pH of yoghurt. Had the sample been of a higher pH (for example, a chymosin gel), then insoluble calcium phosphate may have been involved. We are unable to suggest any significant ionic changes in the pH range 4.3 (average pH of yoghurt) to 5.0 (pH of fixative buffer) which would influence nucleation.

R.J. Carroll: The authors suggest that granules are a complex of glutaraldehyde and osmium, but no direct evidence for glutaraldehyde is present.
G.M. Roomans: What is your evidence that the granules contain, apart from Os, glutaraldehyde?
Authors: Although there is no direct evidence confirming the presence of glutaraldehyde in the granules, both fixatives are required in order for granules to appear. When either fixative was used singularly (see question regarding primary OsO_4 below), no granules were observed. We consider this indirect evidence sufficient to implicate the involvement of glutaraldehyde in granule formation. Kuthy and Csapo (1976) suggested that impurities or breakdown products of commercial glutaraldehyde may be the active form in complex formation.

R.J. Carroll: Do granules form when OsO_4 is the only fixative, buffered or unbuffered?
G.M. Roomans: Have you tried fixing the specimen with Os alone?
Authors: Yoghurt samples were also fixed in 2% OsO_4 in veronal acetate buffer (0.05M, pH 5.0 and 6.75) and phosphate buffer (0.13M, pH 6.75). Electron dense granules did not form when OsO_4 was the primary fixative irrespective of the buffer or pH. Fig. 12 shows a sample of yoghurt fixed in 2% OsO_4 (veronal acetate buffer pH 5, 2 h at room temperature) and is completely free of electron dense granules.

If Fig. 12 (primary OsO_4) is compared to other figures (Figs. 2 and 3) which were double fixed (i.e. glutaraldehyde followed by OsO_4) on the basis of appearance and stability of the casein and fat components, both types of fixation seem adequate. This raises the question of whether double fixation is really necessary in all cases. In the case of yoghurt, double fixation proved to be a complicating factor which led to formation of electron dense granules. We therefore suggest that a primary OsO_4 fixation be considered for this tissue in addition to the widely advocated double fixation in an attempt to reduce the incidence of artifacts such as described in this study.

G.M. Roomans: Have the authors considered the following alternative explanations for their findings:

(1) the granules are present in specimens fixed in glutaraldehyde alone but cannot be seen in the electron microscope unless Os is present;

(2) the granules were present in specimens after glutaraldehyde fixation but lost during further processing that did not include Os postfixation?

Authors: (1) Although there was the possibility of not visualizing electron dense structures such as granules in TEM mode if osmication was omitted, samples fixed in glutaraldehyde only (Fig. 5) were examined in STEM mode for that reason. If electron opaque structures were present with the singular glutaraldehyde fix (Fig. 5), we expected to visualize them in STEM mode as was the case for casein. STEM mode offers the capability of increased signal to noise ratio due to: (i) electron optical design and, (ii) electronic signal enhancement.

(2) The most significant processing step following singular glutaraldehyde fixation (i.e. no osmication) is dehydration in ethanol series. Substances most likely to be lost in dehydration are lipids. Gil and Weibel (1968) extracted lipids from lung tissue using chloroform-methanol followed by osmication in phosphate buffer and noted no difference in granule formation when compared to a tissue sample that was not lipid-extracted.

P. Allan-Wojtas: Were other dehydration agents (such as acetone or 2,2 dimethoxypropane) tried in addition to ethanol? Hayat (1981, p 157) explains that the secondary blackening of which the authors speak is believed to take place during dehydration, so the dehydration system may influence the formation of the granules.

Authors: The only dehydrating agent used in this study was ethanol. The possibility of granules being reduced Os compounds or "osmium black" which we referred to was in relation to a residual glutaraldehyde-OsO_4 compound. We are aware of similar compounds being formed from reaction with OsO_4 and ethanol, however, granules were not formed when samples fixed in 2% OsO_4 was followed by ethanol dehydration (see Fig. 12).

P. Allan-Wojtas: Was simultaneous fixation with glutaraldehyde and osmium tried? Hayat (1981, pages 201-206) has suggested that it be tried in certain cases where sequential fixation does not work well.

Authors: Simultaneous fixation was not attempted therefore we are unable to comment on the significance of a glutaraldehyde-OsO_4 mixture on granule formation.

G.M. Roomans: In the interpretation of X-ray maps it should be remembered that the data represent not only characteristic X-rays but also continuum X-rays. This means that they are sensitive to mass and density variations. In general, X-ray maps can only be used with a very high signal to noise ratio. While Fig. 7 is a good example of the correct use of an X-ray map, Fig. 8, which intends to show the absence of Os, is not. A spectrum would be more adequate.

Authors: In Fig. 8 we are attempting to show removal of Os from a large area. It would have been difficult to analyze the exact spot of where a granule had been located after periodic acid treatment, using the stationary probe in TEM mode. This would have entailed a subjective decision as to positioning the probe to collect a spectrum. The purpose of Figs. 7 and 8 was to show Os distribution before and periodic acid treatment. We realize that Fig. 8 is inferior in appearance but removal of Os is expected to reduce overall contrast.

Additional References

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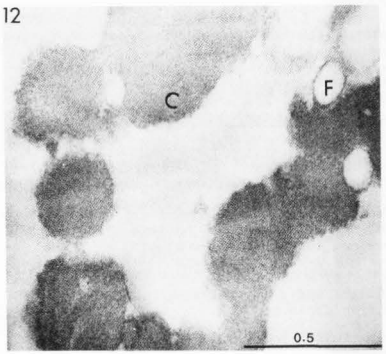

Fig. 12. TEM micrograph of yoghurt fixed in 2% osmium tetroxide in veronal acetate buffer pH 5.0, 2 h. Unstained section. C = casein; F = fat globule. Bar = 0.5 μm.