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Pascale Mentré
Centre de Biologie Cellulaire

Sylvain Halpern
Institut Gustave Roussy

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APPLICATION OF THE PYROANTIMONATE METHOD AND ELECTRON PROBE MICROANALYSIS TO THE STUDY OF GLYCOGEN METABOLISM IN LIVER

Pascale Mentré[1*] and Sylvain Halpern[2]

[1]Centre de Biologie Cellulaire, 94205 Ivry-sur-Seine.
[2]Laboratoire de Microscopie Ionique
Institut Gustave Roussy, 94805 Villejuif, France.

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Abstract

Glycogen distribution in the liver of mouse under different metabolic conditions was studied by the pyroantimonate (PA) method combined with semi-quantitative electron probe microanalysis (EPMA). In the liver of animals subjected to a sugar-rich diet, glycogen granules were abundant and electron transparent. In fasted animals, they were less numerous and stained by PA, which indicates the presence of a complexed cation. This cation was identified as calcium by EPMA. In both cases, adjacent cytoplasmic areas contained "masked" calcium not revealed by PA but detected by EPMA, which is characteristic of a neutral complexed form; but in the case of the fasted animals, the calcium concentration was significantly lower. If the liver of fasted animals was dissected in 0.2% glucose-containing medium, the glycogen areas dramatically released calcium and lost their stainability by PA, whereas mitochondria and adjacent cytoplasm contained many PA precipitates rich in calcium and sodium, suggesting a sudden increase of intracellular [Ca^{2+}]. In mitochondria, the sodium:calcium ratio was relatively constant, which suggests a process involving a coupling between these two elements.

Our results could be explained in the light of physiological and biochemical data. We particularly noted that diffusible cations as calcium and sodium did not appear to be displaced over long distances from their likely source. This observation agrees with recent theories on the state of water and ion mobility in the cell.

Keywords: glycogen, liver, pyroantimonate, calcium, sodium, electron probe microanalysis.

*Address for Correspondence:
P. Mentré, Centre de Biologie Cellulaire, 67 rue Maurice-Gunsbourg, 94205 IVRY-SUR-SEINE CEDEX, France
Phone No. (1) 46.72.18.00

Introduction

In two previous papers, we have described an adaptation of the pyroantimonate (PA) method combined with microanalysis that permitted the localization of calcium and sodium at the ultrastructural level [23,24]. Free cations can be visualized as PA precipitates. Chelated cations (i) can be liberated and precipitated; or (ii) can bind PA anions so that the structures in which they are present appear finely stained; or (iii) can be masked by an anion that PA cannot displace. We proposed that staining of glycogen granules observed in skeletic muscle could be considered due to the presence of chelated [Ca^{2+}]. We have in effect detected significant quantities of calcium in these stained glycogen areas. We have related this presence of calcium to the activity of phosphorylasekinase, an enzyme which is required in the glycogenolysis process, and which is associated to the glycogen granules. To be activated, this enzyme needs addition of [Ca^{2+}] to its calmodulin subunit [1].

In order to verify this interpretation, we applied the PA method to glycogen-rich material in glycogenolysis or in glycogenosynthesis. Muscle was not considered suitable for such a study because of the rapidity of its metabolism. In this paper, results obtained with liver are presented. A study of the amphibian oocyte at different stages of oogenesis and maturation will be presented in another paper.

Materials and Methods

Cytochemistry

Liver tissue was obtained from two groups of C57B2/6 inbred mice. The first group was subjected to a sugar-rich diet during two days and was sacrificed 30 minutes after ingestion of 2M glucose; the second was deprived of food during two days but was provided with water ad libitum. Samples were rapidly dissected into little pieces in Eagle (MEM IX with Hank's salts without L-glutamine; Gibco, Grand Island N.Y.). Several pieces of liver from the second group were dissected in glucose-enriched Eagle (0.2% glucose).

In all cases, material was prepared for the localization of calcium and sodium according to

the PA method and for the localization of glycogen by a classical aldehyde-osmium double fixation followed by the periodic acid-thiocarbohydrazide-silver-protein method (PATAG) described by Thiéry [34].

Pyroantimonate Method. Material was fixed 2 hours at 4°C with the following solution:

4% potassium pyroantimonate (Merck)
2% paraformaldehyde
0.04M potassium phosphate buffer
1% phenol
final pH : 7.8

The specific procedures have been described in detail previously [24]. After fixation, the tissue was rapidly rinsed in a large volume of distilled water and dehydrated in a graded series of alcohols starting at 70% ethanol. Ethanol was replaced with propylene oxide and embedding was in Araldite. Light gold sections for conventional transmission microscopy (TEM) and dark gold sections for electron probe microanalysis (EPMA) were cut with a diamond knife. As previously described [24], sections were floated as briefly as possible on the microtome trough containing 0.01M K_2HPO_4 in double-distilled water freshly collected in plastic vessels.

Thiéry's PATAG Method [32]. Tissue was first fixed 1 hour at 4°C in 2% glutaraldehyde in 0.01M sodium cacodylate buffer at pH 7.4 and postfixed 1 hour at 4°C in 1% osmium tetroxide in veronal buffer at pH 7.4. Then, according to the PATAG method, ultrathin sections were successively (i) floated on 1% periodic acid, 30 minutes; (ii) rinsed on water; (iii) floated on 0.2% thiocarbohydrazide in 20% acetic acid, 1 hour; iv) rinsed on 10%, 5%, 2% and 1% acetic acid; v) rinsed on water; vi) floated, in darkness, on silver proteinate, 1 hour; vii) rinsed on water. All steps were carried out at room temperature.

Electron Microscopy and Microanalysis

Transmission Electron Microscopy (TEM). Observations were performed with a Philips EMU 300 at 60 or 80kV. Sections of PA fixed material were either unstained or lightly contrasted by lead citrate or uranyl acetate.

Electron Probe Microanalysis (EPMA). Microanalysis was performed under the conditions described previously [24] with a computer-aided Cameca (CAMEBAX BMX) fitted with two wavelength dispersive spectrometers mounted on a transmission electron microscope. The probe diameter was 500nm at a beam intensity of 150nA at 45kV. Kα-1 lines of calcium and sodium, respectively, were analyzed. Each X-ray count was integrated over 50 seconds. For each analyzed area of the specimen, the background was estimated according to a method adapted from [29], as the average of the counts measured on each side of the peak. This value was subtracted from the average of two measurements of the peak. The statistical error of the characteristic signal (peak − background) was estimated by $\sigma = (\text{peak} + \text{background})^{\frac{1}{2}}$ [32]. Signals less than 2σ were regarded as non-significant (confidence level of 95.5%). Each EPMA measurement consisted of simultaneous Na_count, Ca_count and included the ratio Na_count:Ca_count. For each group of mice, three animals were studied. In each animal at least 8 representative areas of the different structures, as similar as possible, were analyzed. The standard deviations were calculated by $(\Sigma (\text{measure} - \text{mean})^2 / n)^{\frac{1}{2}}$ where n was the number of measurements.

Results

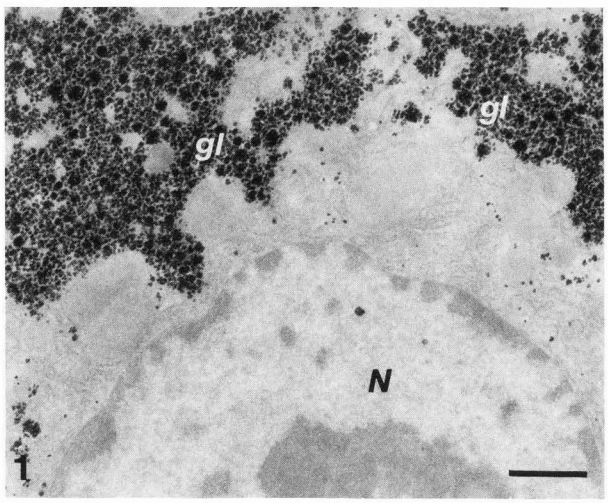
Cytochemistry

In hepatocytes of the animals subjected to a sugar-rich diet, the PATAG method demonstrated very large areas of glycogen α-granules deeply contrasted by silver (Fig. 1). In PA fixed material observed without any other staining, glycogen granules were electron transparent (Fig. 2), but they could be intensely stained by lead citrate (Fig. 3).

In hepatocytes of animals subjected to a 48 hour fasting, the PATAG method revealed reduced areas of glycogen (Fig. 4). After fixation by the PA method, glycogen granules appeared finely stained (Figs. 5 and 6).

In these two cases, small PA precipitates (less than 0.1μm) were occasionally observed in endoplasmic reticulum areas and mitochondria (Figs. 2, 3, 5 and 6).

FIGURES 1 to 3. Hepatocytes of animals subjected for two days to a sugar-rich diet. Glycogen areas occupy a substantial part of the cells. Circles (0.5μm diameter) indicate examples of the regions examined by EPMA probe.
Fig. 1: Glycogen as revealed by PATAG method. Granules are intensely contrasted by silver.
Figs. 2 and 3: PA method. Granules are not stained by pyroantimonate (Fig. 2) but are not extracted as can be verified after lead citrate staining (Fig. 3). Small PA precipitates are occasionaly observed in endoplasmic reticulum areas (arrow-heads) and mitochondria. Intranuclear precipitates (Figs. 2 and 3) are quite variable (but the two nuclei of the same hepatocyte always display an identical distribution).
FIGURES 4 to 6. Hepatocytes of animals fasted for two days.
Fig. 4: Glycogen as revealed by PATAG method. Granules (reduced in number) are intensely contrasted by silver. Figs. 5 and 6: PA method. Glycogen granules are finely stained by pyroantimonate. Occasional PA precipitates are observed in the cytoplasm. Figure 6 also illustrates a frequent aspect of the intercellular space, with aligned PA precipitates; finely delineated plasma membranes (arrows) and a bile canaliculus are recognizable.
Bar = 1μm (Figs.1 to 5) and 0.5μm (Fig. 6).
bc: bile canaliculus; gl: glycogen; *: adjacent cytoplasm; L: lipid; m: mitochondrion; N: nucleus.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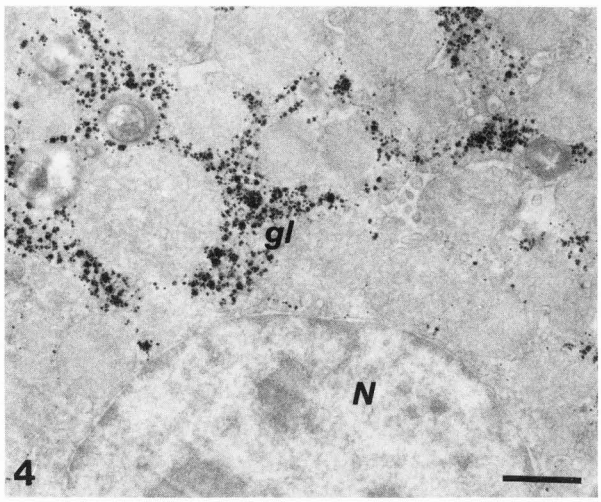
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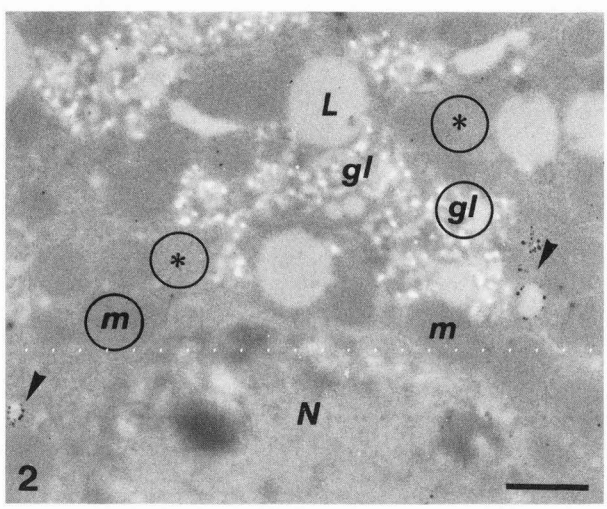
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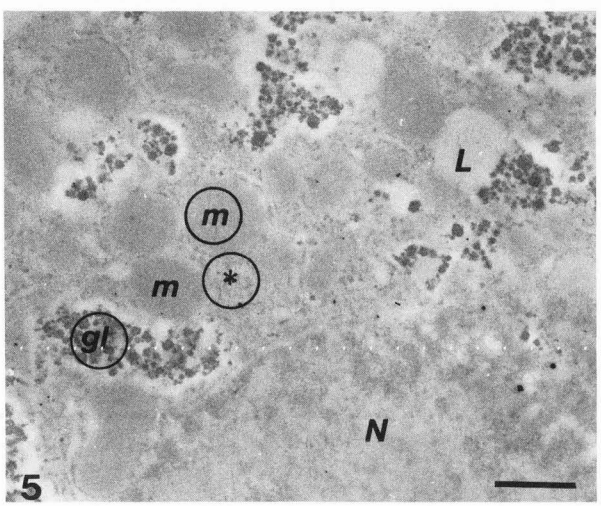
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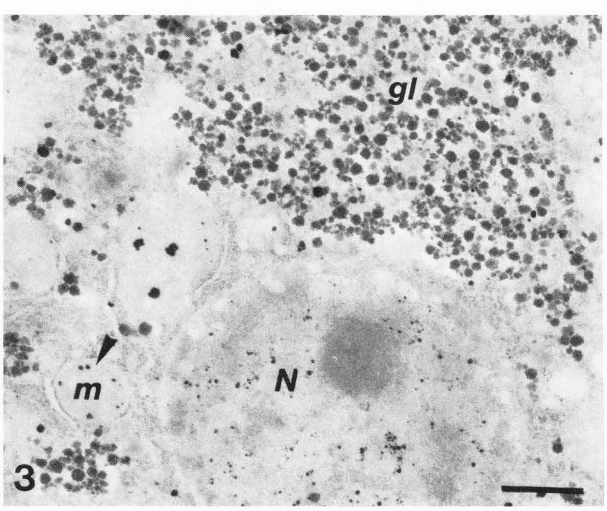
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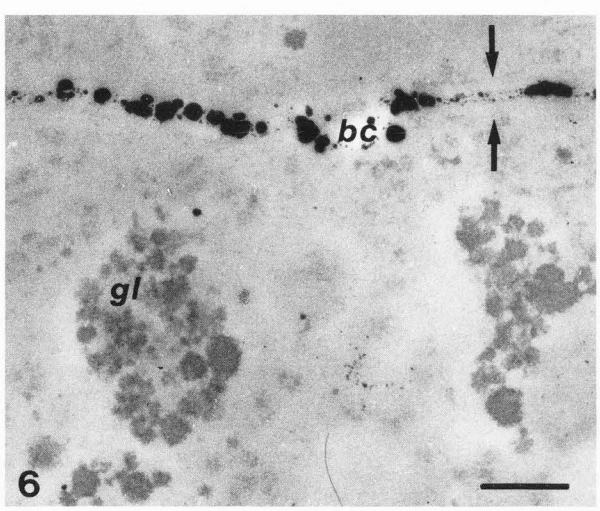
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If the liver of the fasted animal was exposed to Eagle containing 0.2% glucose, glycogen granules could not be stained anymore by pyroantimonate (Fig. 7). On the other hand, three distinct populations of PA precipitates were observed: 0.02-0.05µm in the peripheral part of mitochondria, and 0.1-0.2µm or less than 0.02µm in the adjacent cytoplasmic areas (Figs. 7 and 8).

In all cases, the nuclei contained very variable amounts of PA precipitates, without any evident relation to glycogen metabolism (Figs. 2 and 3). These precipitates were located in the dispersed chromatin. Less frequently, tiny precipitates were visible in nucleoli. But, in spite of this variability, the two nuclei of the same hepatocyte (a generally binucleated cell) always displayed a remarkable similar distribution of precipitates.

Electron Probe Microanalysis

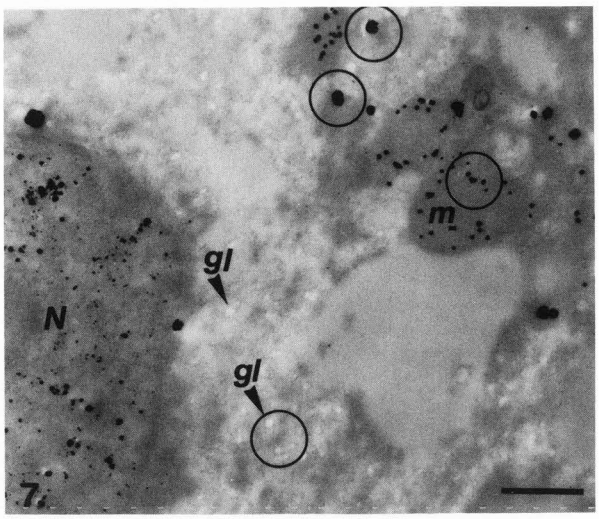
Glycogen areas, adjacent cytoplasm and mitochondria were analyzed. Circles, 0.5µm in diameter, in Figures 2 to 8 illustrate the probe outline in each type of measurement. The Araldite background was also analyzed in order to verify that it contained no significant amounts of calcium and sodium. Results are given in Tables 1 and 2.

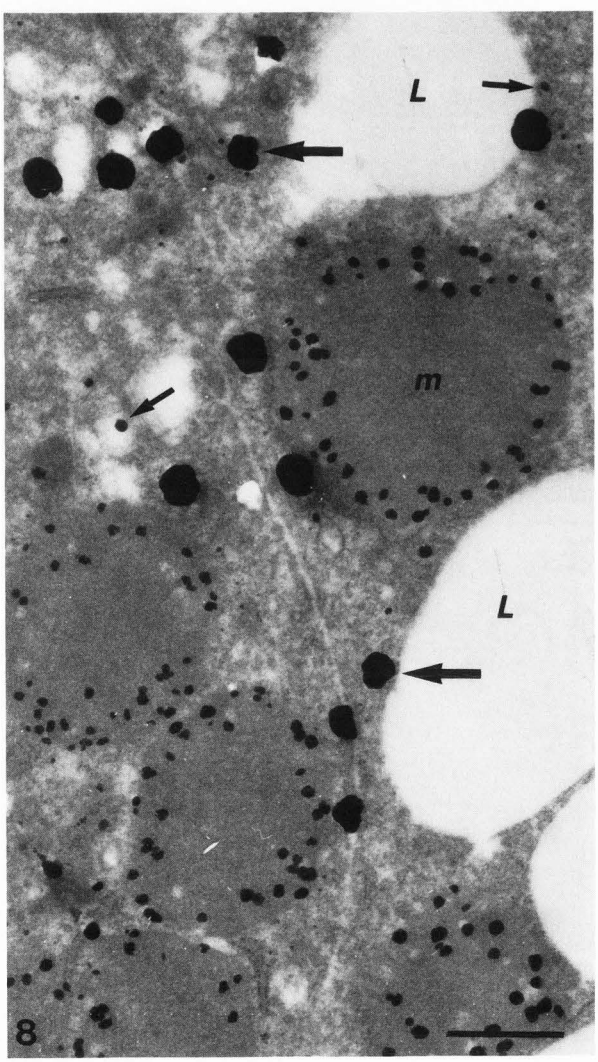
In the fasted animals (Table 1), calcium counts were significantly higher in the glycogen areas (visualized by PA) than in the paired cytoplasmic areas (Student t-test, α risk < 0.05, β risk ≈ 0). In the well fed animals, they were not significantly different (α risk $= 0.05$, β risk > 0.50). The comparison of the means of the calcium counts of the glycogen areas demonstrated that they were significantly higher in the fasted animals than in the well fed ones. On the contrary, calcium counts of the surrounding cytoplasm were lower (α risk < 0.05, β risk ≈ 0).

On the other hand, intramitochondrial calcium which was not detectable in well fed animals, reached significant values in fasted animals even though it was not combined with pyroantimonate.

The small precipitates observed on the ER cisternae did not appreciably affect the counts.

Sodium was not detected in these two cases.





FIGURES 7 and 8. Hepatocytes in fasted animal liver dissected in Eagle containing 0.2% glucose. Fig. 7: Glycogen granules are not stained. Numerous precipitates are present in mitochondria and in adjacent cytoplasmic areas. Fig. 8: Same material slightly contrasted with aqueous uranyl acetate. Three populations of PA precipitates can be distinguished: one, intramitochondrial, 0.02–0.05µm, located at the periphery of mitochondria; the two others, cytoplasmic, 0.1-0.2µm (large arrows) and less than 0.02µm (small arrow).
Bar = 1µm (Fig. 7) and 0.5µm (Fig.8).
gl: glycogen; L: lipid; m: mitochondrion; N: nucleus.

TABLE 1. Results of PA method and EPMA Ca-counts (means ± standard deviation). Liver dissected in Eagle. No = number of measurements. ST = stained.

structure	Animals in sugar-rich diet			Fasted animals		
	No	PA	EPMA	No	PA	EPMA
glycogen areas	25	--	305±72	24	ST	491±140
adjacent cytoplasm	24	--	340±59	26	--	164±45
mito-chondria	20	--	--	28	--	148±35

After exposure to 0.2% glucose (Table 2), calcium dramatically decreased in glycogen areas which were not stained any more by pyroantimonate. But an evident accumulation of both calcium and sodium was observed in two of the three precipitate populations (Figs. 7 and 8): the big 0.1-0.2µm cytoplasmic precipitates and the 0.02-0.05µm intramitochondrial precipitates. For analysis of large precipitates, counting was performed on precipitates of similar size (about 0.2µm; one per probe area). Very variable results were obtained. For the intramitochondrial precipitates, we chose mitochondrial areas representing a similar PA distribution. Sodium and calcium counts, though equally variable showed a fairly coherent ratio of Na-count:Ca-count. It was not possible to analyze the tiny precipitates of the third population.

Table 2. Results of PA method and EPMA Na and Ca-counts (means ± standard deviation). Liver dissected in Eagle containing 0.2% glucose. No = number of measurements. PT = precipitates (*Only about 0.2µm precipitates were analyzed).

structure	No	PA	EPMA Na-count	EPMA Ca-count	Na-count count
glycogen areas	26	--	--	94 ±32	--
adjacent cytoplasm	26	PR*	168 ±87	485 ±180	0.42±0.31
mito-chondria	26	PR	113 ±42	240 ±97	0.54±0.10

Discussion

The principles of the use of the PA method have been previously considered [23,24]. Pyroantimonate forms electron opaque precipitates with free cations (either initially free or liberated during fixation). It combines with chelated cations producing a fine staining of the structures containing these cations. If cations are neutralized by anions which cannot be displaced by PA anion, they remain "masked" in TEM but can be detected by EPMA.

A priori, for most authors, the PA method does not present a total guarantee against ion diffusion and therefore against erroneous interpretation. Indisputably, precipitates contain non-biological concentrations of calcium and sodium and must be considered as artefacts resulting from cation migration. Nevertheless, we never observed precipitates far from probable source and the PA precipitates offer the advantage that they allow measurements by EPMA when the initial element distribution is below the sensitivity of this technique. On the other hand, the detection of stained or masked complexed cations could be ascribed to sequestration after migration over a greater or lesser distance. But, also in this case, all our results were consistent with known ion distribution in living cells.

In the present work, we observed that the glycogen granules, though electron transparent in the case of well fed animals (Fig. 2), were not extracted since they were visualized by lead citrate (Fig. 3). In fasted animals, the glycogen granules were stained by pyroantimonate (Figs. 5 and 6). This result suggests the presence of a cation immobilized under a chelated form. Microanalysis confirmed that the glycogen areas were calcium-richer in the fasted animals than in the well fed ones (Table 1).

Our result could not be explained by the conclusion of Clark and Ackerman [8] who proposed that staining of glycogen by pyroantimonate could be ascribed to free hydroxy groups of glycogen and not to calcium since glycogen is a non polar molecule. Our result is consistent with the role of calcium during glycogenolysis and with the fact that the principal enzymes of glycogen metabolism constitute the external layer of glycogen granules. Indeed, glycogenolysis can occur only if phosphorylasekinase is activated by addition of Ca^{2+} to its calmodulin subunit [1, 3, 9, 12, 16, 19, 31]. Moreover, calcium indirectly contributes to inactivate glycogen synthase by forming a complex with the calmodulin-dependent synthasekinase [14, 30]. On the other hand, the presence of these enzymes in the protein layer of the glycogen granules is corroborated both by cytological investigations demonstrating the existence of this layer [27] and by numerous biochemical studies [1, 22, 25, 26].

Moreover, calcium in the cytoplasm adjacent to glycogen areas appears less abundant during glycogenolysis than glycogenosynthesis (Table 1), which suggests that the calcium necessary for glycogenolysis came from these contiguous cytoplasmic areas. This calcium which is not revealed by the PA method is likely to be chelated in a stable form "masked" by an anion preventing precipitation with pyroantimonate. This assumption is in agreement with the fact that nearly all intracellular calcium is in a bound form [5, 7, 17, 21, 28].

The presence of "masked" calcium in the mitochondria of fasted animals (Table 1) is in agreement with the studies of Denton et al. [11] and Hems et al. [20] who established involvement of Ca^{2+} in the regulation of intramitochondrial metabolism. They studied the production of ATP in tissues requiring high amounts of this compound: cardiac muscle and vasopressin perfused liver (vasopressin triggers glycogenolysis of which the second step, glucose-1-P phosphorylation, is ATP-

dependent). They established that calcium activates the intramitochondrial pyruvate-dehydrogenase-enzymatic-complex which increases ATP production by feeding the Krebs cycle with pyruvate. Therefore, we suggest the possibility that, in our material, during fasting induced glycogenolysis, calcium could accumulate into mitochondria in response to an increased need of ATP to form a high affinity complex, which would be "masked" for PA. In well fed animals, the calcium content of mitochondria is markedly lower than in the rest of the cytoplasm, as it is not even detected by EPMA. This observation is in agreement with the results obtained by Somlyo et al. [33] and Bond et al. [2] in liver rapidly frozen in vivo. But the presence of calcium in the mitochondria of fasted animals is in contradiction with the observations of these authors who postulated that the calcium concentration must always be low in mitochondria. This contradiction may be only apparent: the calcium distribution in the cell compartments is dependent on the metabolic state. In particular, as it is expounded below, glycogen content and glycogen metabolic state would be determinant.

Glycogen areas in well fed animals, though less calcium-rich than in fasted animals and not stained by pyroantimonate, contain non-negligible amounts of calcium (Table 1). That suggests that a relatively stable neutral form of calcium is present in the glycogen granules.

After incubation with 0.2% glucose, the glycogen granules could no longer be stained by pyroantimonate. This result is consistent with the fact that glucose enters into hepatocytes (in such a way that intracellular and extracellular concentrations remain practically equal), inhibits glycogenolysis and stimulates glycogenosynthesis [13]. Indirectly, this entry of glucose produces an increase of cytosolic [Ca^{2+}] and [Na^{+}]. Firstly, the entry of glucose implies an entry of sodium into the cell since glucose enters by a glucose-sodium symport [1]. This entry of Na^{+} reduces the extracellular-intracellular Na^{+} gradient and, consequently, inhibits the Ca^{2+} exit normally carried out by a calcium-sodium antiport. Secondly, the entry of glucose, by inhibiting glycogenolysis, induces the liberation of Ca^{2+} which was associated to phosphorylasekinase and synthasekinase.

The numerous sodium and calcium rich precipitates observed in the cytoplasm (Figs. 7 and 8) are in agreement with such a sudden increase of free cations. The difficulty to choose identical volume precipitates probably explains the dispersed character of the sodium and calcium counts. But the incoherence of the sodium/calcium ratio also suggests the absence of correlation between these elements. Our results did not suggest any accumulation of excess cations into endoplasmic reticulum, permitting [Ca^{2+}] regulation as it has been advanced by different authors [6, 7, 10, 33].

Mitochondria also have often been proposed as another possible exit for excess [Ca^{2+}] although Hems et al. [20] estimated that the accumulation of calcium into mitochondria was necessary to increase the ATP production rather than a way to preserve the cytosol homeostasis and Somlyo et al. [33], and Bond et al. [2] did not observe any accumulation in vivo. Numerous authors observed changes of the calcium levels in mitochondria isolated from liver perfused with β-adrenergic agonists or glucagon. These hormones act via cAMP but generally they also involve, as a secondary effect, an influx of calcium into hepatocytes and a stimulation of the mitochondrial Ca^{2+} uptake (Williamson et al. [36]). It has been established in vitro that calcium which entered into mitochondria was slowly released by a sodium-dependent efflux [4, 15, 18, 37, 38]. Wingrove and Gunter. [37, 38] proposed a model in which both calcium and sodium must be bound simultaneously for this efflux transport. However, Bond et al. [2] contested the existence of this process in the mitochondria in vivo because they did not consider the slight increase of intramitochondrial calcium observed in liver perfused with these hormones to be significant. It is difficult to draw any firm conclusions in view of the multiplicity of the processes involved in intracellular ionic regulation. In particular, glycogen granules, especially when so abundant as in animals fed ad libitum (Bond et al. [2]) can be sufficient to trap Ca^{2+} taken up by hepatocytes after stimulation with glucagon and or β-adrenergic agents. Moreover, these glycogenolytic hormones trigger calcium binding to the calmodulin of the glycogen associated enzymes. For these reasons, we do not exclude the model proposed on the basis of biochemical data by Wingrove and Gunter [37, 38] to explain the simultaneous presence of calcium and sodium which we observed inside the mitochondria after the transfer of glycogen-deficient liver to a glucose-rich medium, i.e., in a condition producing a sudden increase of cytosolic [Ca^{2+}] and [Na^{+}] and inhibition of glycogenolysis.

The spread of the values for Na and Ca evidenced by the large standard deviations (Table 2) is probably mainly due to the difficulty to find identical sections of mitochondria. But it is interesting to notice that the sodium:calcium ratio is relatively coherent, which supports the existence of a relation between intramitochondrial sodium and calcium.

In conclusion, we think that the PA method, combined to EPMA, could constitute an efficient tool to study glycogen metabolism. It appears to support biochemical results concerning different mechanisms of control of cytosolic [Ca^{2+}] and [Na^{+}].

Whenever we detected calcium and sodium in this study and previously [23, 24], there localization was very close to if not identical to their supposed initial localization. We therefore consider the cytosol, even during fixation, like a kind of obstacle course rather than a field of free diffusion for the strongly electrostatically charged ions. Indeed, a number of authors claims that water in the cell is in a special state. Water molecules behave like dipoles and form concentric hydration layers around strongly charged ions and cytomatrix macromolecules. Such

bound water is responsible for the decreased motional properties of a substantial portion of ions [5, 21, 28]. Moreover, studies of detergent-permeabilized cells have established that the integrity of the membranes does not seem to be essential for the retention of diffusible ions such as Ca^{2+}, Mg^{2+} and Na^{+} [17].

Ionic movements in living cells are likely to be a cascade of short distance exchanges in a "solid" world essentially made of ions, macromolecules and bound water. Most of these exchanges involve high affinity bindings which only can be broken in living cells by enzymatic processes (for example, calmodulin with calcium [35]). It seems reasonable to assume that, during PA fixation, free cations (liberated or initially free) would be trapped by various sequestering molecules in the environment or by PA anions. As a consequence, the observed localization may correspond to a chemically erroneous but topologically acceptable configuration and valid conclusions could be drawn from the data.

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References

1. Alberts B, Bray D, Lewis J, Raff M, Roberts K., Watson JD (1983) Muscle contraction. In: Molecular Biology of the Cell, Garland, New-York, London, pp. 297,327,745.

2. Bond M, Vadasz G, Somlyo AV, Somlyo AP(1987) Subcellular calcium and magnesium mobilization in rat liver stimulated in vivo with vasopressin and glucagon. J. Biol. Chem. 262, 15630-15636.

3. Bromstom CO, Hunkeler FL, Krebs EG (1971) The regulation of skeletal muscle phosphorylasekinase by Ca^{2+}. J. Biol. Chem. 1961-1967.

4. Bygrave FL, Reinhart PH, Taylor WM (1982) Hormonal control of calcium fluxes in rat liver. In: Membranes and Transport, Martonosi AN (ed), Plenum Press, New York & London, vol 1, 617-622.

5. Cameron IL, Fullerton GD, Smith NKR (1988) Influence of cytomatrix proteins on water and on ions in cells. Scanning Microsc. II, 275-288.

6. Carafoli E (1982) The transport of calcium across the inner membrane of mitochondria. In: Membrane Transport of Calcium, Carafoli E (ed), Acad. Press, 109-139.

7. Carafoli E, Penniston JT (1985) The calcium signal. It mediates a broad array of cellular functions. Protein pumps and other structures turn it on or off. Scientific American 253, 5, 50-89.

8. Clark MA, Ackerman GA (1971) A histochemical evaluation of the pyroantimonate-osmium reaction. J. Histochem. Cytochem. 19, 727-737.

9. Cohen P (1982) The role of protein phosphorylation in neural and hormonal control of cellular activity. Nature 296, 613-620.

10. de Meis L, Inesi G (1982) The transport of calcium by sarcoplasmic reticulum and various microsomal preparations. In: Membrane transport of calcium, Carafoli E (ed), Acad Press, 141-186.

11. Denton RM, Mc Cormack JG, Edgell NJ (1980) Role of calcium ions in the regulation of intramitochondrial metabolism. Effects of Na^{+}, Mg^{2+} and ruthenium red on the Ca^{2+} stimulated oxidation of oxoglutarate and on pyruvate dehydrogenase activity in intact heart mitochondria. Biochem. J. 190, 107-117.

12. Fischer EH, Krebs EG (1955) Conversion of phosphorylase b to phosphorylase a in muscle extracts. J. Biol. Chem. 216, 121-132.

13. Freychet P (1978) Les hormones pancréatiques. In: Hormones, Baulieu EE (ed), Herman, Paris, 359-412.

14. Garrison JC, Borkland MK, Florio VA, Twible DA (1979) The role of calcium ions as a mediator of the effects of angiotensin II, catecholamines and vasopressin on the phosphorylation and activity of enzymes in isolated hepatocytes. J. Biol. Chem. 254, 7147-7156.

15. Goldstone TP, Duddridge RJ, Crompton M (1983) The activation of Na^{+}-dependent efflux of Ca^{2+} from liver mitochondria by glucagon and β–adrenergic agonists. Biochem. J. 210, 463-472.

16. Haschke RH, Heilmeyer LMG, Meyer F, Fischer EH (1970) Control of phosphorylase activity in a muscle glycogen particle. III. Regulation of phosphorylase phosphatase. J. Biol. Chem. 245, 6657-6663.

17. Hazlewood CF, Kellermayer M (1988) Ion and water retention by permeabilized cells. Scanning Microsc. 2, 267-273.

18. Heffron JJA, Harris EJ (1981) Stimulation of calcium-ion efflux from liver mitochondria by sodium ions and its response to ADP and energy state. Biochem. J. 184, 925-929.

19. Heilmeyer LMG, Meyer F, Haschke RH, Fischer EH (1970) Control of phosphorylase activity in a muscle glycogen particle. II. Activation by calcium. J. Biol. Chem. 245, 6649-6656.

20. Hems DA, McCormack JG, Denton RM (1978) Activation of pyruvate dehydrogenase in the perfused rat liver by vasopressin. Biochem. J. 176, 627-629.

21. Ling GN (1988) A physical theory of the living state: application to water and solute distribution. Scanning Microsc. 2, 899-913.

22. Luck DJL (1961) Glycogen synthesis from uridine diphosphate glucose. The distribution of the enzyme in liver cell fractions. J. Biophys. Biochem. Cytol. 10, 195-209.

23. Mentré P, Escaig F (1988) Localization of cations by Pyroantimonate. I. Influence of fixation on distribution of calcium and sodium. An approach by analytical ion microscopy. J. Histochem. Cytochem. 36, 49-54.

24. Mentré P, Halpern S (1988) Localization

of cations by pyroantimonate. II. Electron probe microanalysis of calcium and sodium in skeletal muscle of mouse. J. Histochem. Cytochem. 36, 55-64.

25. Mersmann HJ, Segal HL (1969) Glucocorticoid control of the liver glycogen synthetase activating system. J. Biol. Chem. 244, 1701-1704.

26. Meyer F, Heilmeyer LMB, Haschke RM, Fischer E.H. (1970) Control of phosphorylase activity in a muscle glycogen particle. I. Isolation and characterization of the protein-glycogen complex. J. Biol. Chem. 245, 6642-6648.

27. Minio F, Lombardi L, Gautier A (1966) Mise en évidence et ultrastructure du glycogène hépatique. Influence des techniques de préparation. J. Ultrastructure Res. 16, 339-358.

28. Negendank W (1988) The state of water in the cell. Scanning Microsc. 2, 21-32.

29. Nicholson WAP (1974) Experience of diffractive and non-diffractive quantitative analysis in the CAMECA microprobe. In: Microprobe analysis as applied to cells and tissues, Hall T, Echlin P, Kaufman R (eds), Academic Press, New York, 239-248.

30. Payne ME, Soderling TR (1980) Calmodulin-dependent glycogen synthase kinase. J. Biol. Chem. 255, 8054-8056.

31. Rubin CS, Rosen OR (1975) Protein phosphorylation. Ann. Rev. Biochem. 44, 831-887.

32. Ruste J (1983) Microanalyse X quantitative en Biologie. In: Microanalyse X en Biologie, Quintana C, Halpern S (eds), Société Française de Microscopie Electronique, Paris,37-54.

33. Somlyo AP, Bond M, Somlyo AV (1985) Calcium content of mitochondria and endoplasmic reticulum in liver frozen rapidly in vivo. Nature 314, 622-625.

34. Thiéry JP (1967) Mise en évidence des polysaccharides sur coupe fine au microscope électronique. J. Microscopie 6, 987-1018.

35. Waterson DM, Harrelson WG, Keller PM, Sharief F, Vanaman T.C. (1976) Structural synthesis between the Ca^{2+}-dependent regulatory proteins of 3':5' cyclic nucleotide phosphodiesterase and actynomyosin ATPase. J. Biol. Chem. 251, 4501-4513.

36. Williamson JR, Cooper RH, Hoek JB (1981) Role of calcium in hormonal regulation of liver metabolism. Biophys. Biochem. Acta 639, 243-295.

37. Wingrove DE, Gunter TE (1986a) Kinetics of mitochondrial transport. I. Characteristics of the sodium-independent calcium efflux mechanism in liver mitochondria. J. Biol. Chem. 261, 15159-15165.

38. Wingrove DE, Gunter TE (1986b) Kinetics of mitochondrial calcium transport. II. A kinetic description of the sodium dependent calcium efflux mechanism of liver mitochondria and inhibition by ruthenium red and by tetraphenylphosphonium. J. Biol. Chem. 261, 15166-15171.

Discussion with Reviewers

W.C. de Bruijn: Have the authors performed model experiments with Ca chelated to known matrixes (e.g. ion exchange materials) to substantiate their belief that the precipitates do contain calcium removed from quite nearby sites?

Authors: We did not. Good models, undoubtably, would be very informative. We feel that the largest difficulty is not to find substances with a suitable cation affinity, but it is to realize a three-dimensional configuration of these substances and a water distribution similar to those of the cell matrix. We think that the space configuration of the free water between the solid particles is a determinant parameter of the ion migration.

We think that our unpublished results can provide a partial answer, at least concerning sodium. Amphibian oocytes were subjected to sodium currents using a 3M NaCl electrode (100 to 500nA, during 10 min) by Dr. Raymond T. Kado (Laboratoire de Neurobiologie Cellulaire, 91190 Gif-sur-Yvette). As soon as the electrode was removed, the oocytes were fixed according to the PA method. Morphology was well preserved and the electrode trace was recognizable. Contrary to our expectations, the sodium content was not dramatically increased in the whole oocytes. It was similar to that of the controls except in a limited jet-shaped area, containing numerous sodium PA precipitates and localized between the electrode tip and the nuclear envelope. The nuclear envelope appeared to be a barrier. This result supports the assumption that cell matrix and nuclear envelope prevent the free diffusion of sodium ions, even if as in our experiment, there is a very sharp gradient.

W.C. de Bruijn: Have the authors considered application of a glycogen selective contrasting procedure with OsO_4 plus complex cyanides (e.g., $K_4Fe(NC)_6,3H_2O$, proposed by our laboratory) which happen also to immobilize EPMA detectable Ca [see e.g. de Bruijn et al., Histochem. J. 16:37-50, 123-136, 1984, and the literature cited there].

Authors: We have not. We have rejected the use of osmium tetroxide in our PA method (see text ref. 24) in particular because osmic fixation does not preserve sodium in the tissues (Van Iren et al., 1979). In the light of a recent study, a substantial part of the calcium observed in glycogen after osmic fixation may be an artefact: Blaineau et al. (1988) demonstrated by energy dispersive X-ray analysis that the apparent Ca concentration was increased in tissues postfixed with osmium tetroxide solutions prepared in glass but not with solutions prepared in plastic. They concluded that care must be taken when osmium fixed tissues are to be subjected to X-ray microanalysis of calcium.

W.C. de Bruijn: Will the authors compare the results of their experiment 3 with experiments previously performed by Burger and de Bruijn (Calcif. Tissue Int. Suppl. 27: A5, 1979) on deliberately introduced tissue damage and

metabolic inhibitors?

Authors: After pyroantimonate-osmium fixation, you observed that, in intact cells shortly before intracellular matrix mineralization, calcium PA precipitates were accumulated alongside the mitochondria and plasma membranes. In mechanically damaged cells, calcium accumulates in large granules located in mitochondrial matrix, whereas treatment by metabolic inhibitors produced a random distribution of precipitates over all cellular compartments.

We think that the thixotropic equilibrium (gel <==> sol) is likely to be disturbed by mechanical damage (pressure, stretching) with local liberation of bound water and ion dissolving.

The accumulation of the excess calcium into mitochondria is admitted by many authors (see Trump et al., 1980). The energy-dependent presence of calcium alongside membranes may be related to various processes requiring ATP as Na-dependant Ca exchanges, Ca^{2+} pumps. The metabolic inhibitors could therefore inhibit ion exit processes with, as a consequence, the increase of free cations inside cell.

In our 3 experiments, mechanical damage was identical, caused by the dissection of the tissue into small pieces; but we did not demonstrate any random distribution of PA precipitates in the cells which were not mechanically injured in the experiments 1 and 2. As to the experiment 3, we do not think that the exposure to 0.2% glucose could be considered as a metabolic damage: in normal animals, after a carbohydrate-rich meal, the glucose concentration can reach 0.17% in the general circulation (and is higher in the portal vein).

W.C. de Bruijn: Will the authors elaborate on the idea that the rather large standard deviatons are caused by the variable amounts of (Ca-containing) cytoplasm between the glycogen granules, and even explain the differences between the two experiments?

Authors: The Ca-counts of the glycogen areas are equal to:

$$GA = G + Cg$$

where G and Cg represents the Ca-counts of the glycogen granules and the cytoplasm located between the granules, respectively.

If we assume that this cytoplasm is identical to the cytoplasm adjacent to the glycogen areas (AC), then:

$$GA = G + AC$$

The EPMA counts being proportional to the concentrations, it could be concluded that (i) in experiment 1 (glycogen-adjacent cytoplasm pairs not significantly different), calcium concentration would be similar in the glycogen granules and the cytoplasm located between the granules; (ii) in experiment 2 (glycogen-adjacent cytoplasm pairs significantly different) the glycogen granules would be richer in calcium than the GA value would lead us to believe (GA = 491 whereas AC = 164).

This hypothesis, therefore, strengthens the significance of our results. We have no information to advance the hypothesis that the cytoplasm located between the granules would be very different from the cytoplasm adjacent to the granule areas.

G. Nicaise: Could the end of the abstract be more specific, omitting the last sentence and stating for example 'the results suggests that the variant of the PA used in the present work preserves a reasonably physiological distribution of sodium and calcium, at least in the glycogen particles'?

Authors: We feel that our remarks concerning water and diffusion are more important than the visualization of the glycogen metabolism. We prefer not to delete the last sentence 'This observation agrees with recent theories on the state of water and ion mobility in the cell'. We had the opportunity to present this paper at the 'Scanning Microscopy/1989' Meeting and to discuss with several co-authors of the State of Water in the Cell' (studies compiled by Negendank and Edelmann, 1988).

Cryomethods are more appropriate for the study of the total ion contents and the stable bound forms: however, they do not permit one to distinguish chemical forms, but free ions generally represent a negligible fraction. Nevertheless, it must not be forgotten that many metabolic processes involve variation of the ratio free ions / bound ions without changing the total ion content [7]. Therefore, the precipitation methods, could be, in the case of free ion distribution study, more suitable than cryomethods.

G. Nicaise: It is not specified in the Material and Methods sections nor in ref. [24] if the counts were taken from an identified area of the grid. In the Camebax-TEM configuration, particularly with the commercial standard specimen holder, even a semiquantitative study would be impaired by the important shade and noise effects (see Blaineau S, Julliard AK, Amsellem J, Nicaise G (1987) Quantitative X-ray microanalysis of calcium with the Camebax-TEM system in frozen, freeze-substitued and resin embedded tissue sections, Histochemistry 87, 545-555).

Was the objective aperture in or retracted during the counts?

Authors: Initially, the presence of numerous backscattered electrons in the analyzing chamber of our commercial standard Camebax BMX made any quantitation impossible and involved important risks of artefacts in qualitative analysis [32]. Therefore,already in 1982, we modified several parts of our analyzer (see, for details, Quintana and Halpern, 1983). Moreover, all our analyses were performed with a bulky graphite sample holder, the grids being immobilized by beryllium clamps.

The utilization of a vertical fully-focussing wavelength dispersive spectrometer permitted to detect the X-rays emitted in the

preparation plan only in the beam area. In these conditions, there was no appreciable noise if the analysis was performed far enough from a grid bar (> 10μm). Sections were always on the side of the grid facing the detector in order to avoid bar shade.

G. Nicaise: It can be misleading in the discussion to use 'PA method' or 'PA fixation' without restriction. Would you agree that it is the modern variants of pyroantimonate methods which give these fine results, and mention that the original and often used Komnick method is known to induce rather gross displacements of cations?
Authors: When OsO_4 is added to PA, as in the Komnick method, cations are generally visualized as populations of coarse precipitates. The reason could be, as we suggested [23,24], the slow penetration of OsO_4 into the material, or/and as you clearly demonstrated (Blaineau et al. 1988), the enrichment of the material in calcium during osmic fixation. Some authors obtained a fine distribution of PA precipitates after Komnick fixation followed by a carefull rinse. But these precipitates were not very abundant and it could be assumed that a major part of them (the smallest ones) was lost during the rinse.

G. Nicaise: In your variant of the PA method, you use a phosphate buffer, in the fixative and also in the trough. I have the experience on gastropod ganglia that a glutaraldehyde fixation buffered with potassium phosphate 0.1M followed by a rinse in buffer and osmium tetroxide post-fixation (same buffer) will be sufficient to clearly stain glycogen particles, uranium and lead citrate being omitted. Could you comment on the possibility that the pyroantimonate in your case was perhaps only increasing the contrast of a calcium phosphate precipitate?
Authors: We can answer that phosphate buffer is not necessary to stain glycogen by pyroantimonate. We have also fixed our material with collidine buffer or without buffer, according to the procedure previously described [24]. For routine TEM observations, we did not always employed alkalized water in the microtome trough: the TEM results were identical in all cases (of course, the EPMA results were very dependent on the nature of the trough liquid).

Minio et al. [27] observed that glycogen is not contrasted when it is fixed by a solution containing glutaraldehyde and phosphate buffer. We think that the calcium associated to the glycogen granules can bind phosphate but the formed complex is not abundant enough to produce any contrast. Contrast seems to be obtained only if OsO_4 is present with phosphate; it is not obtained with OsO_4 in the absence of phosphate (de Bruijn et al. 1984).

J.A.V Simson: Why was Sb not also analyzed in the spots indicated (or in comparable areas)? With wave length dispersive X-ray microanalysis, the Ca and Sb spectra should be easily separable and one should be able to determine wich of the other elements present (Na and Ca were bound to pyroantimonate and which were bound to other endogenous tissue molecules.
Authors: The analysis which you propose is interesting but not easy to do. In effect, pyroantimonate can precipitate with Na^+ and Ca^{2+}, but also with Mg^{2+}, K^+, H^+, Ba^{2+}, Zn^{2+}, Fe^{2+} and Fe^{3+}, and when several cations are present, they generally coprecipitate (Van Iren et al., 1979).

J.A.V. Simson: How long were the minced tissues allowed to incubate in Eagle's medium with or without glucose prior to fixation? Was it the same amount of time for both? The reason I ask is that the distribution of pyroantimonate deposits seen with mincing in the glucose-Eagle's medium is very similar to the distribution I have seen in damaged cells, and may simply result from anoxia if they had been incubated for a fairly long time (15-20min.) prior to fixation.
Authors: It has been effectively demonstrated by several authors that cells become richer in free ions when they are damaged, but they can also become richer when their metabolic activity increases (Lederer, 1984). In our work, the minced tissues, in all the cases, were incubated 10min. The increased number of PA deposits after incubation in 0.2% glucose-Eagle's medium would therefore correspond to a specific modification of the metabolism (see, above, our answer to Dr. de Bruijn).

Additional References

Blaineau S, Amsellem J, Nicaise G (1988) Increase in the calcium content of cardiac tissue after postfixation with osmium tetroxide. Stain Technol. 63, 339-350.

de Bruijn WC, Memelink AA, Riemersma JC (1984) Cell membrane and contrast differenciation with osmium triazole and tetrazole complexes. Histochem. J. 16, 37-50.

Lederer J. (1984) Biochimie du Magnésium. In: Magnésium, Mythes et Réalités, Maloine (eds), Paris, pp 26-36.

Negendank W. and Edelmann L. (1988) The State of Water in the Cell. Scanning Microsc. Interntl., Chicago.

Quintana C, Halpern S (1983) Limites expérimentales de la microananalyse X des échantillons biologiques. In: Microanalyse X en Biologie, Quintana C, Halpern S (eds), Société Française de Microscopie Electronique, Paris, 79-82.

Trump BF, Berezesky IK, Laiho KU, Osornio AR, Mergner WJ, Smith MW (1980) The role of calcium in cell injury. A review. In: Scanning Electron Microscopy, II, SEM Inc., AMF O'Hare, IL 60666, 437-462, 492.

Van Iren F, Van Essen-Joolen L, Van der Duyn-Schouten, Boers-Van der Sluijs P, de Bruijn WC (1979) Sodium and calcium localization in cells and tissues by precipitation with antimonate: a quantitative study. Histochemistry 63, 273-294.