

ROLE OF INTRACELLULAIRE Ca^{++} IN THE CONTROL OF MOTILITY OF DUNALIELLA

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ABSTRACT :

During the past few years, it has been shown that Ca^{++} plays an essential role in the control of ciliary beat and cell motility and it has been suggested that the ciliary membrane modulates the internal Ca^{++} level in cilia and flagella. The present work demonstrates that the motility of *Dunaliella* a biflagellate green algae, remains constant during 15 days in a Ca^{++} free-medium. This result suggests that large intracellular reserves of Ca^{++} could be mobilized by *Dunaliella* to control its motility. Pyroantimonate detection technique followed by X-ray microanalysis show that these reserves are localized in large intracellular vesicles and could be involved in control of the ciliary beat in *Dunaliella*.

KEY WORDS : Cell motility, ciliary beat, Ca^{++} control

1 - INTRODUCTION

Previous studies have suggested that Ca^{++} ions play an essential role in the regulation of ciliary beat (16). In *Paramecium*, the reversal of the ciliary beat is dependent on the transient entry of Ca^{++} through the plasma membrane and its subsequent depolarisation (7; 19; 20). In *Chlamydomonas*, an increase in intracytoplasmic Ca^{++} concentration induces a modification of the form of the flagellar beat of

isolated flagella and of the beating frequency during photophobic response (9, 21); Kamiya and Whitman (12) also showed in demembranated models of *Chlamydomonas* differential responses of the axonema to submicromolar concentrations of Ca^{++} , which could form the basis of phototactic response. On ciliated epithelial cells, an increase in cytoplasmic Ca^{++} concentration can modify the frequencies of ciliary beat followed by an inhibition as shown by Murakami and

Takahashi and Walker and Satir on ciliary epithelium of freshwater mussels [(18, 27)], and by Girard and Kennedy (8) on rabbit tracheal epithelial explant. The ciliary beat and cell motility of *Dunaliella* a biflagellate unicellular algae have been studied by computer analysis of high-speed movies (25). The two symmetrical anterior flagella have an asymmetric bending pattern similar to the ciliary beat of epithelial cilia, but the two anterior flagella never beat at the same frequency. Drugs which interfere with Ca^{++} homeostasis revealed that initiation of the bend at the base of the flagella, could be Ca^{++} dependent, and could greatly influence the regulation of beat frequency (15). In the present work we have attempted to obtain a better understanding of the role of extra and intracellular Ca^{++} in the regulation of cell motility of *Dunaliella*, and we have localized important intracellular reserves of Ca^{++} which could permit movement and survival of the alga for several weeks in Ca^{++} free-medium.

2 - MATERIALS AND METHODS

2 - 1 : Materials

Dunaliella bioculata (Cambridge collection) was axenically grown in synthetic sea water (SM) - Cell culture were maintained at 22°- C and synchronized to 12 hr light-12 darkness (17) - ^{45}Ca (31-2 mCi - mg^{-1}), was provided from New England Nuclear and all chemicals were from Merck or Sigma.

2 - 2 : Methods

LASER DOPPLER VELOCIMETRY (LDV) : Cell motility was quantified by LDV as described by Betrencourt and Volochine (4) - A beam of monochromatic light was applied to a population of algae moving at an isotropic velocity. The monochromatic light source was a He-Ne-laser ($\lambda = 632.8 \text{ nm}$, maximum output

power : 6 mw) and the scattering angle was 8°- By Doppler effect, the intensity of the scattered light was shifted to frequencies giving a continuous spectrum shape; these frequencies depend on the kinetic parameters that define the motility of the population.

The alga concentration was less than ($3 \cdot 10^6$ cells/ml) (to avoid spurious effects of multiple scattering), the optical path was (2 mm), the temperature approximately 20. C, the incident power of the laser beam less than 10^{-5}W and the incident beam diameter less than (1 mm).

This technic was used to determine the average modal velocity of control algae (i.e. algae grown in SM containing 6.8 mM Ca^{++}).

The value obtained served as the reference for comparison with those obtained with algae in two different culture conditions, either in SM with no Ca^{++} added (SM 0 Ca) or in SM 0 Ca containing (1 mM) EGTA. In both cases, velocities were measured 1h after cells were transferred from SM containing (6.8 mM) Ca^{++} to the test media. In certain assays, ($5 \cdot 10^{-5}\text{M}$) verapamil or ionophore A 23 187, were added to the SM containing (6.8 mM) Ca^{++} . The error of the value of modal velocity is less than 5%, as determined by recording and analyzing the same spectrum ten times.

At the end of the exponential growth phase, the algae were centrifugated and resuspended in SM 0 Ca. After 10 to 15 days of culture in this medium, cells were concentrated to a density of ($7 \cdot 10^6$ cells/ml) and transferred to MS 0 Ca containing (2 μM) ^{45}Ca . At different times, three (1 ml) aliquots were removed, added to (7 ml) of unlabeled SM, promptly filtered through Schleicher and Schuell glass fiber filters (n°6) and washed with (15 ml) of cold medium, the radioactivity retained on the filters was measured on the presence of (3 ml) Econofluor (New England Nuclear).

PREPARATION AND REACTIVATION OF PERMEABILIZED CELLS :

Cells were harvested from growth medium and centrifuged 10 minutes at 2 500 g in reactivation medium composed and adapted (6) containing : (0.1 M) K-glutamate, (0.5) glucose, (20 mM) tris, (2 mM) EGTA, (4 mM) Mg acetate, (1 mM) dithiothreitol, adjusted to pH 7.8 with KOH.

The cells were permeabilized by incubating for 20 to 30 seconds at room temperature into the reactivation medium containing triton X-100, they were centrifuged for (3 min at 365 g). Under these conditions the cells were not affected but above 0.001% triton X-100, they are totally disrupted. The aspect of the permeabilized cells verified by TEM (transmission electron microscopy).

After centrifugation the cells were resuspended in reactivation medium ; (0.2 ml) of the suspension was added to (0.2 ml) of (1 mM) Mg-ATP in a spectrophotometric quartz cuvette.

The swimming behavior of the reactivated cells at room temperature was recorded by LM (light microscopy) (Nikon Optiphot) at 1 400 magnification. The cells were filmed by a video-camera (BST 400 A) 15-20 s after initiation of reactivation. The tracks of permeabilized cells were analyzed frame by frame as described (25).

The motility of the reactivated cells was carried out as described previously (4) by LDV. The modal velocity was measured in reactivation medium containing different Mg-ATP and Ca^{++} concentrations. To test the activity of dynein-ATPase, Vanadate-orthophosphate was used.

INTRACELLULAR LOCALIZATION OF Ca^{++} USING THE PYROANTIMONATE TECHNIC :

Wick and Hepler (28) have reported that when X-ray microanalysis is used after pyroantimonate treatment, Ca^{++} is the only cellular cation associated with the precipitates. Consequently this technic appears to be well

suited to study Ca^{++} distribution in several tissues.

After centrifugation (2500g, 10 min), the alga pellet was fixed according to the technic of Kashiwa and Thiersch (13) for 2h 30 at room temperature, in 10% (v/v) paraformaldehyde, 1% (w/v) pyroantimonate and 5% (w/v) sucrose, adjusted with KOH to pH 7.8. The cells were then washed twice with the same solution, dehydrated with alcohol and embedded in Epon. Thin sections (50 to 60 nm) were cut for ultrastructural studies whereas 100 to 200 nm sections were used for X-ray analysis in a transmission electron microscope (Jeol 100 CXII) equipped with a scanning system (STEM ASID-4-D) and an energy-dispersive X-ray analyser (Link, model AN 1000), acceleration voltage 100 KV, probe surface 30 mm² (Si-Li) crystal with ultra-thin windows.

3. RESULTS

QUANTIFICATION OF ALGAL SPEED UNDER VARIOUS CULTURE CONDITION BY LASER DOPPLER VELOCIMETRY

The velocity of algae was measured at different times for up to 70h. Algae were grown in three different media : SM (synthetic ; medium), SM O Ca (synthetic medium with no Ca^{++} added) fig 2a and SM containing (1 mM) EGTA (table I.). The velocities of algae grown in SM and SM O Ca ranged from 103 to 130 $\mu\text{m.s}^{-1}$, comparable results were obtained with algae grown in SM O Ca containing (1mM) EGT^+ . In the presence of EGTA the pH decreased from 7.4 to 5.5. The low pH is toxic for algae therefore it was necessary to increase the pH to 7.4 with KOH. After 24h the EGTA has an agglutination effect on the algae and increase the number of algae that vibrate in the place, making impossible to measure the velocity.

Modal velocity of algae			
Time (h)	SM	SM O Ca	SM O Ca + EGTA
0	122 ± 7	130 ± 7	141 ± 8
1	127 ± 7	131 ± 7	146 ± 8
2	128 ± 7	128 ± 7	138 ± 7
3	131 ± 7	131 ± 7	137 ± 8
4	125 ± 7	131 ± 7	135 ± 7
20	121 ± 7	118 ± 7	113 ± 6
22	126 ± 7	134 ± 7	122 ± 7
24	114 ± 6	116 ± 6	N - M
25	113 ± 6	122 ± 7	N - M
26	124 ± 7	130 ± 7	N - M
44	101 ± 6	105 ± 6	N - M
45	94 ± 5	108 ± 6	N - M
46	96 ± 5	100 ± 5	N - M
49	110 ± 6	121 ± 7	N - M
50	108 ± 6	115 ± 6	N - M
51	95 ± 5	100 ± 5	N - M
69	94 ± 5	103 ± 6	N - M
70	95 ± 5	103 ± 5	N - M

Table I : Variations of velocities of algae as a function of the extracellular Ca^{++} concentration. Velocities are measured by LDV. The standard deviation on the values of the velocities (v) is 5%. N . M : not measurable.

Ca^{++} UPTAKE

The failure of verapamil or ionophore A 23 187 to alter cell motility could be explained by insensitivity of *Dunaliella* to these drugs.

Therefore we investigated Ca^{++} uptake into the cells. No Ca^{++} uptake could be detected

on normal medium (6.8 mM Ca^{++}) during at least 8h. Indeed cell growth in a SM O Ca was not affected after until 15 days : the cells retained their shape and their movement (see above). Consequently Ca^{++} uptake was examined using cells that had been raised for 15 days in SM O Ca. The rate of Ca^{++} uptake (Fig.1) was constant for about 3 to 4h then decreased. The effect of compounds known to alter Ca^{++} flux (A 23187 or verapamil at $5 \cdot 10^{-5}\text{M}$) showed that very little effect on Ca^{++} flux was obtained (result not shown).

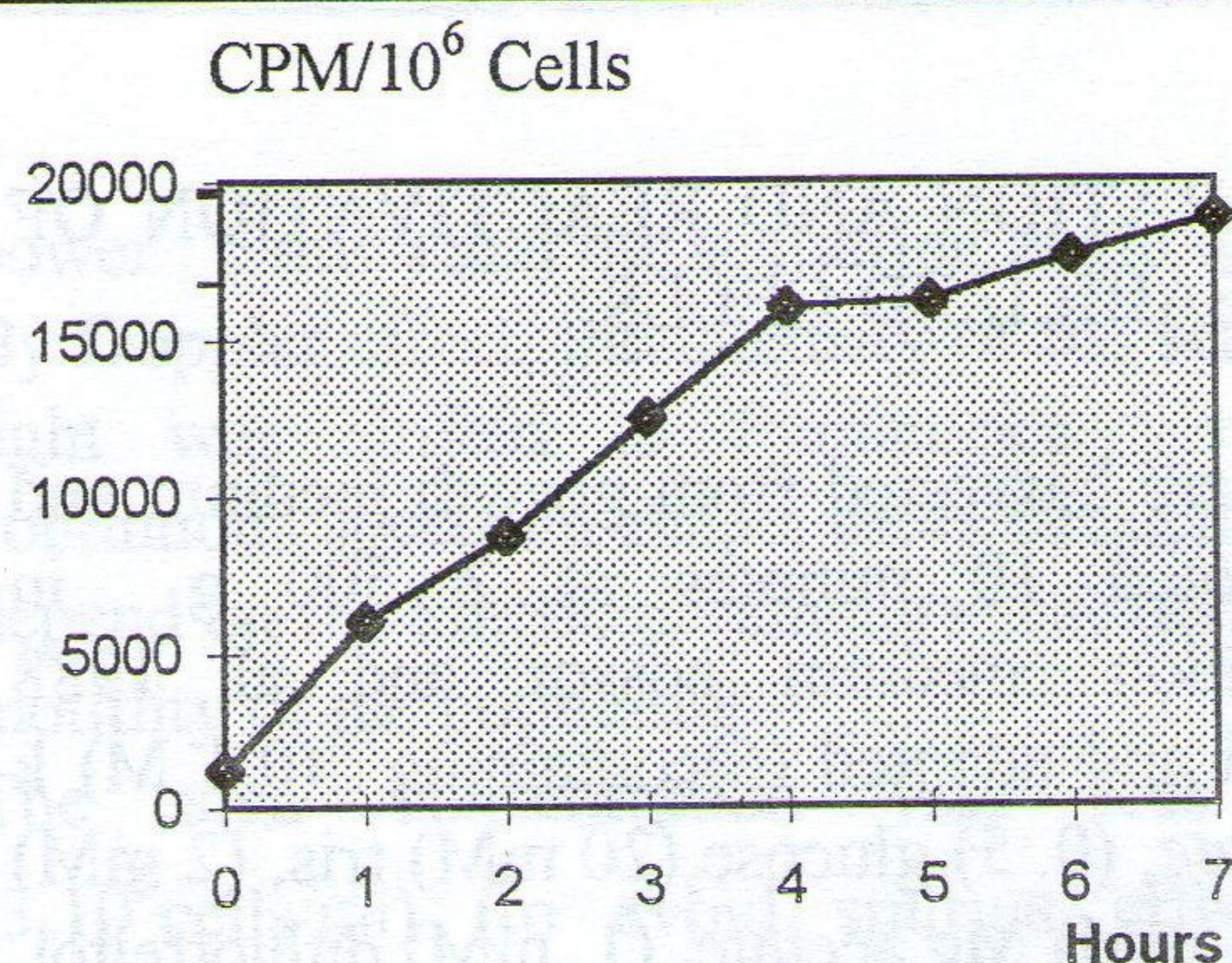


Figure 1 . Time course of ^{45}Ca uptake by *Dunaliella bioculata*. All values are averages of three samples

REACTIVATION OF PERMEABILIZED CELLS

Immediately after permeabilization by triton X-100 the motility of *Dunaliella* was inhibited. However the addition of (1mM) Mg-ATP (the substrat for ciliary dynein) triggered beating the cilia.

Observed by LM (light microscopy) the reactivated cells appeared as cellular bodies which have lost part of their contents. The aspect of these cell fragments was verified at the ultrastructural level using TEM : the flagellar membrane has disappeared but the axonema is intact and is directly in contact with the extracellular medium (Fig.2 c). The two flagellae stay bound together by the striated fibers which connected the two basal bodies. In our preparations, most of the flagellae remained attached to fragments of the cell body (Fig.2 b). In standard conditions that intrinsically contains ($2 \cdot 10^{-8}\text{M}$ free Ca^{++}), The addition of (1 mM) Mg-ATP induced virtually total flagellar reactivation. Two types of movements were observed among the reactivation. Two types of movements were observed among the reactivated cells :

- Spin path movements (Fig.2 d).
- Helicoidal paths as the swimming paths of intacts cells (Fig.2 e,f).

After quantification of flagellar velocity by LDV, the reactivated « fragments » moved with a velocity of 80 to 90 $\mu\text{m.s}^{-1}$ for 30 to 45 min. After 45 min the reactivated « fragments » progressively stopped beating

(the velocity of intact cells was about 110 $\mu\text{m.s}^{-1}$).when vanadate (1 nM) was added in the reactivation medium, movement stopped immediately.

a-Effect of various ATP-Mg concentrations:

The influence of various ATP-Mg concentrations on the reactivated flagella was examined. Below (0.1 mM) and above (5 mM) ATP-Mg, permeabilized cells vibrated but did not move; they agglutinated at high (>5 mM) ATP-Mg concentration and between (0.1 mM) and (5 mM) ATP there was no effect on velocity (table II).

Modal velocity of algae ATP (m M)					
Time (min)	0 - 1	0 - 5	1	2 - 5	5
0	N -M	72 $\pm$ 3,6	93 $\pm$ 4,6	88 $\pm$ 4 ,4	N -M
5	N -M	72 $\pm$ 3,6	80 $\pm$ 4,0	80 $\pm$ 4,0	N -M
10	N -M	60 $\pm$ 3,0	77 $\pm$ 3,8	73 $\pm$ 3,7	N -M
15	N -M	71 $\pm$ 3,6	78 $\pm$ 3,9	57 $\pm$ 2,8	N -M
20	N -M	50 $\pm$ 2,5	54 $\pm$ 2,7	56 $\pm$ 2,8	N -M
30	N -M	58 $\pm$ 2,9	84 $\pm$ 4,2	51 $\pm$ 2,6	N -M
40	N -M	58 $\pm$ 2,9	53 $\pm$ 2,6	56 $\pm$ 2,8	N -M

Table II : Mean values of velocities of demembranated Dunalella as a function of ATP concentration . Velocities are measured by LDV. N.M : Not measurable

b - Effect of various ca++ concentrations :

In standard conditions (1 mM ATP, 2. 10⁻⁸ M free Ca⁺⁺) the speed was maintained at about 80 $\mu\text{m.s}^{-1}$ during the first 20 min of reactivation and then decreased progressively.At (10⁻⁴ M) Ca⁺⁺ even if cells were reactivated, they vibrated without swimming, and it was impossible to quantify this velocity (table III).

Modal velocity of algae Ca++ Free (M)					
Time (min)	2 - 10-8	10-7	1,3 - 10-6	10-5	10-4
0	92 $\pm$ 4,6	79 $\pm$ 3,95	79 $\pm$ 3,95	75 $\pm$ 3,75	N - M
5	77 $\pm$ 3,85	62 $\pm$ 3,1	65 $\pm$ 3,25	76 $\pm$ 3,8	N - M
10	77 $\pm$ 3,85	70 $\pm$ 3,5	55 $\pm$ 2,75	64 $\pm$ 3,2	N - M
15	54 $\pm$ 2,7	71 $\pm$ 3,55	60 $\pm$ 3,0	53 $\pm$ 2,65	N - M
20	64 $\pm$ 3,2	67 $\pm$ 3,35	55 $\pm$ 2,75	47 $\pm$ 2,35	N - M

Table III : Mean values of velocities of demembranated Dunaliella as a function of Ca⁺⁺ concentration . Velocities are measured by LDV . N.M : Not measurable .

At (10⁻³ M) Ca⁺⁺ there was no reactivation. The reactivation ceased, never could a new reactivation process be obtained by further ATP addition.

INTRACELLULAR LOCALIATION

To examine the ultrastructural Ca⁺⁺ localisation in *Dunaliella* the pyroantimonate detection technique was applied.This technique is widely used with cellular material of animal and plant origin (2; 10; 14).

The ultrastructural study of algae grown in SM (6.8 mM Ca⁺⁺) (fig 3a) and in SMOCA (fig.3b) revealed the presence of numerous pyroantimonate precipitates on the outside surface of the plasma membrane and in large vesicles; a few precipitates also appeared on the nucleus, chloroplast and mitochondria .

To obtain direct information on the composition of the precipitates contained in the vesicles, sections were submitted to energy-dispersive x-ray microanalysis. Figure 4 shows the pressence of Ca⁺⁺ in the precipitates at the plasma membrane (Fig. 4 a) or in the vesicles (fig. 4 b).

Attempts to determine directly the cytoplasmic concentration of free Ca⁺⁺by the use of Fura 2 (a fluorescent Ca⁺⁺ chelator); were unsuccessful (not shown) because of the autofluorescence of the chloroplast between 340 and 380 nm, and because of the lack of penetration of Fura 2 through the plasma membrane.

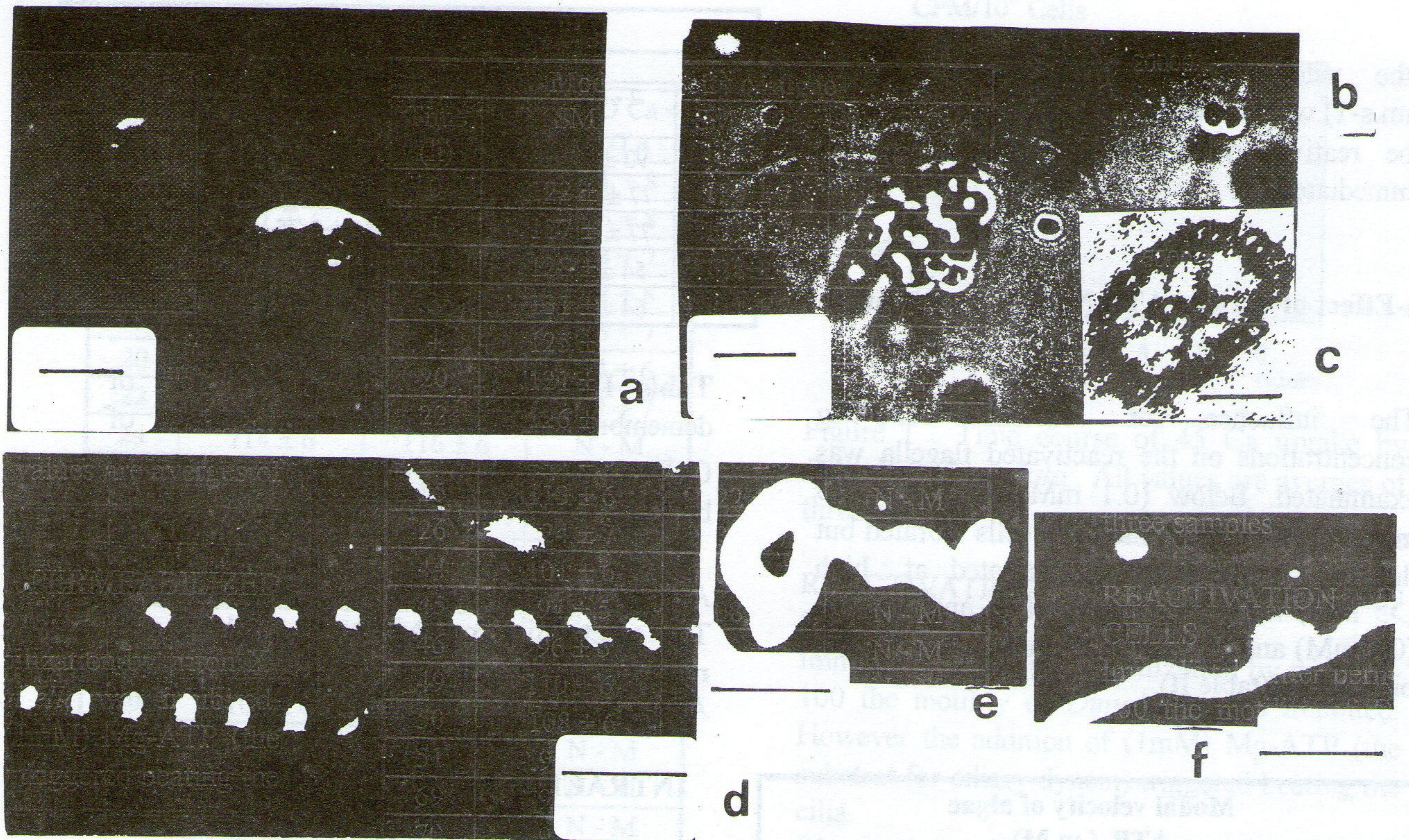
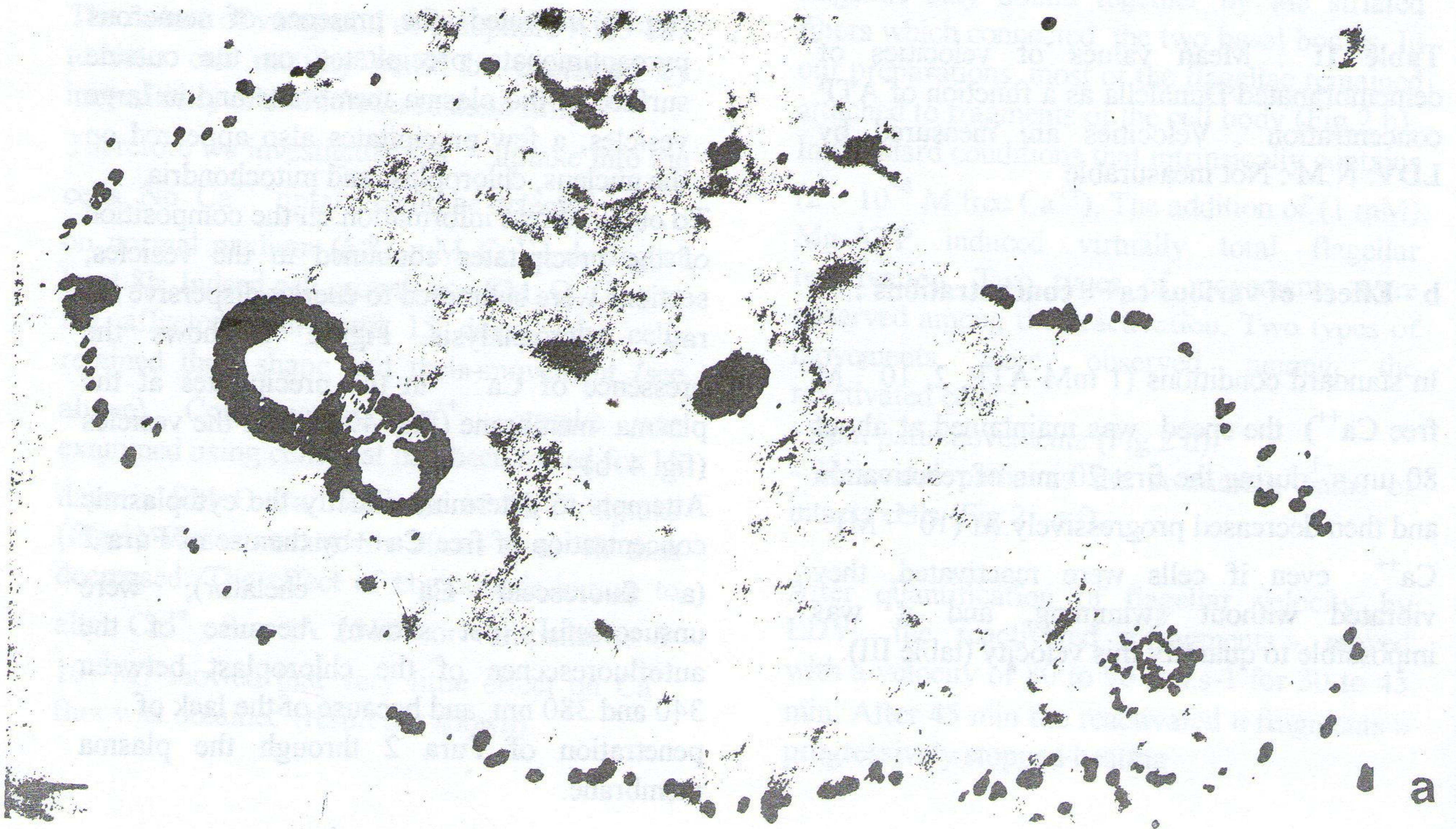
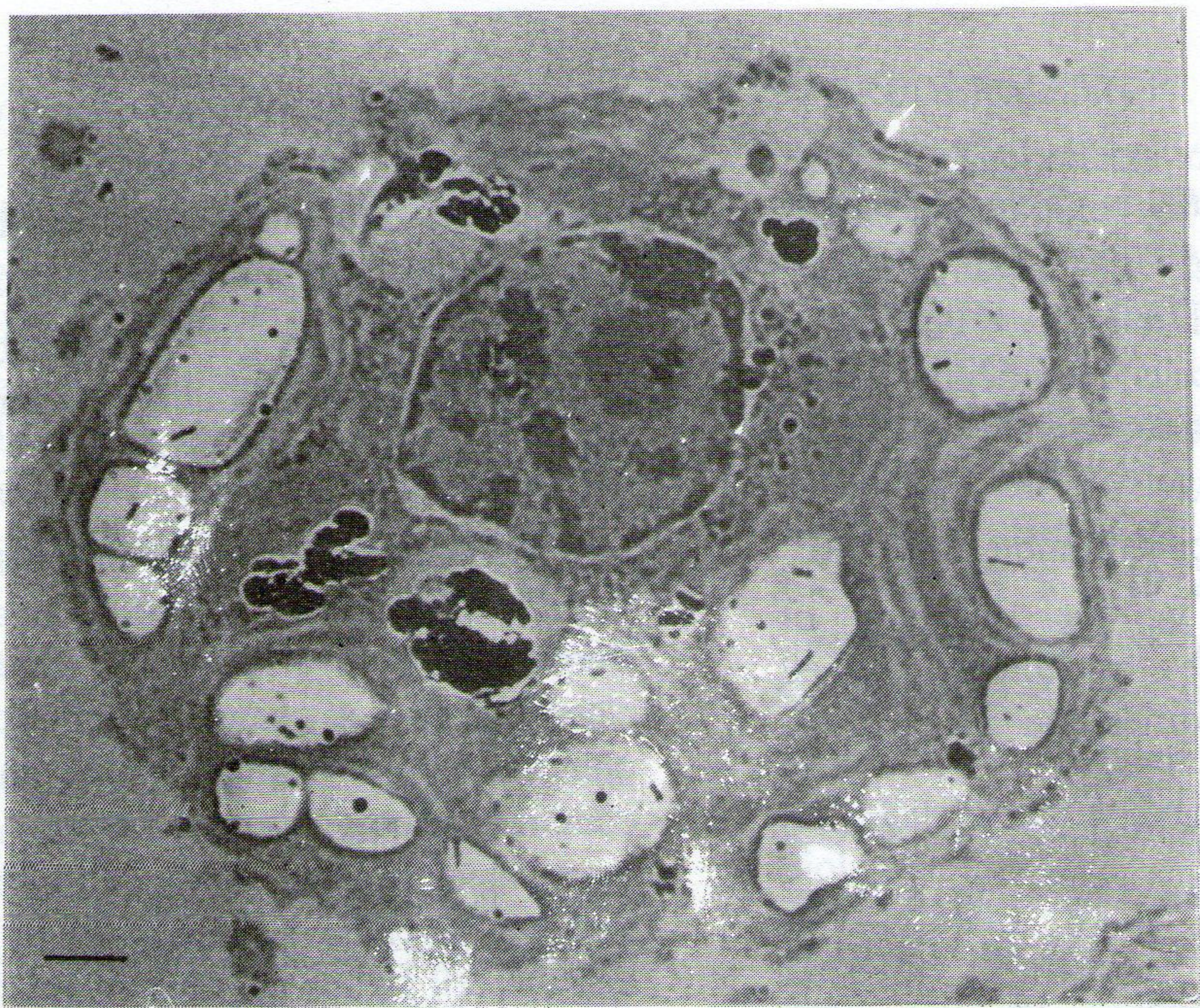


Figure 2 . a - *Dunaliella* control (MS 0 Ca) observed by LM (— 10 μ) .
 b - *Dunaliella* demembrated with triton -100 (LM) (— 10 μ)
 c - Transversal section of demembrated flagellum of *Dunaliella*, observed by LEM .
 (— 0.5 μ) .
 d-e-f - Exemples of trajectories of demembrated *Dunaliella* under standard conditions .
 (— 25 μ)





b

Figure 3 . Ultrastructural localization of intracellular Ca^{++} in *Dunaliella* .

- a - Grown in a SM (6-8 mM Ca^{++}) . Ultralocalized by the pyroantimonate detection technic .
(— 1 μ) .
- b - Grow in a SM 0 Ca (— 1 μ)

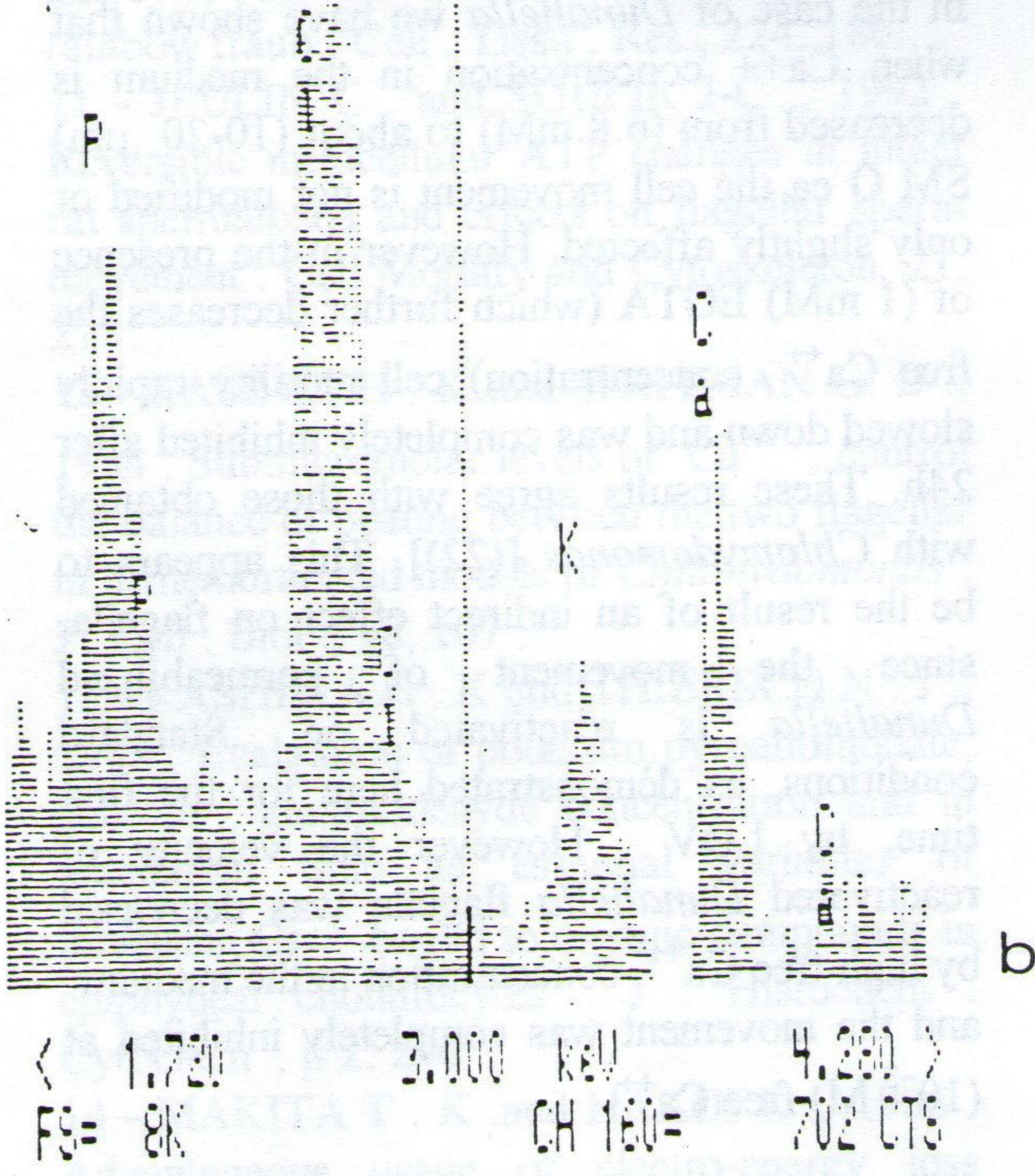
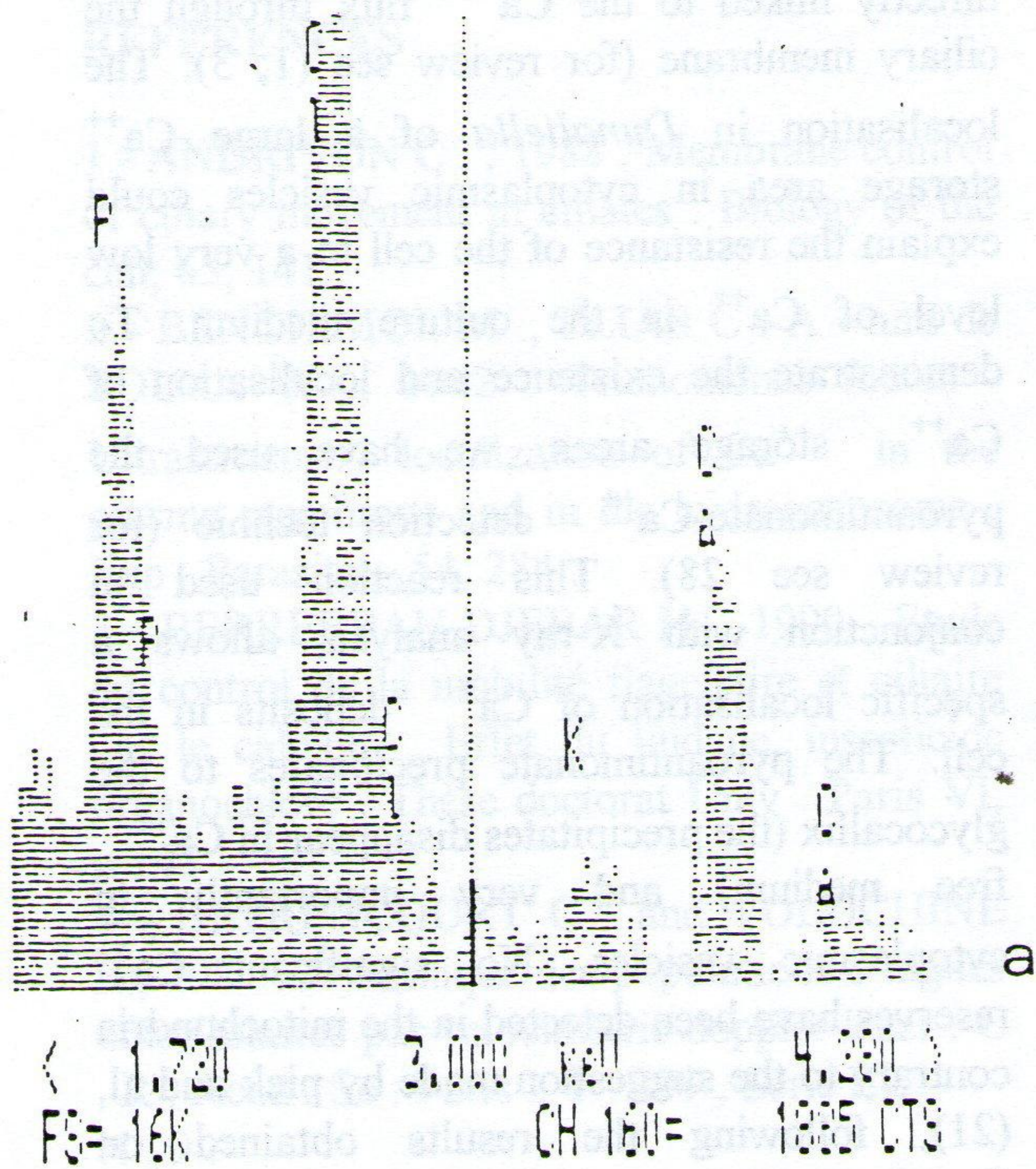


Figure 4 . x-ray energy spectrum from electron dense granules .

- a - Associated with the plasmic membrane in *Dunaliella* grown in (6-8 mM Ca^{++}) medium .
- b - Associated with the vacuoles in *Dunaliella* grown in a SM 0 medium .

4. DISCUSSION

In the literature, there is little agreement concerning the effect of external Ca^{++} concentrations on cell motility. For many ciliated and flagellated cells, modification of the external Ca^{++} concentration strongly affect the beat of cilia or flagella. For example a decrease of the Ca^{++} concentration, from (10^{-5} M) to (10^{-8} M) in the medium of *Chlamydomonas* induces a decrease in cell motility (22), and modifies the photophobic response (24).

The movements of spermatozoa of several species are inhibited by Ca^{++} in a dose-dependent manner (5; 11; 15). In *Oxhyrris marina* the modification of the ionic composition of sea water (decrease or increase of Ca^{++}) induces an abrupt change of direction in cell tracks (6). Other kinds of ciliated cells such as those from the rabbit oviduct (26) or rabbit tracheal epithelium (7) are less sensitive to external Ca^{++} fluctuation.

In the case of *Dunaliella* we have shown that when Ca^{++} concentration in the medium is decreased from (6.8 mM) to about (10-20 μM) SM O ca the cell movement is not modified or only slightly affected. However in the presence of (1 mM) EGTA (which further decreases the free Ca^{++} concentration) cell motility rapidly slowed down and was completely inhibited after 24h. These results agree with those obtained with *Chlamydomonas* [(22)]. This appears to be the result of an indirect effect on flagella, since the movement of permeabilized *Dunaliella* is reactivated on standard conditions, as demonstrated here for the first time, by LDV. However the velocity of reactivated *Dunaliella* flagella was decreased by high free Ca^{++} concentration in the medium and the movement was completely inhibited at (10^{-3} M) free Ca^{++} .

These results are in agreement with those obtained with demembrated *Chlamydomonas* reactivated with various concentrations of Ca^{++} (10^{-6} M to 10^{-3} M), (9); and suggested that Ca^{++} acts via calceins and particularly calmodulin which is present in the axoneme of *Chlamydomonas*.

In a previous study, it was shown that the two flagella of *Dunaliella* never beat at the same frequency. This discrepancy appears more clearly during a change of direction, under phototaxis for example when one flagellum continues to beat whereas the other is inactivated causing the cell to rotate. The inactive flagellum then recovers its movement and the cell swims in the new direction. It was suggested that the discrepancy in frequency between the two flagella could be explained by a selective sensitivity to the intracellular Ca^{++} concentration.

Many authors consider that the regulation of flagellar or ciliary beat by Ca^{++} could be directly linked to the Ca^{++} flux through the ciliary membrane (for review see (1; 3). The localisation in *Dunaliella* of a large Ca^{++} storage area in cytoplasmic vesicles could explain the resistance of the cell to a very low level of Ca^{++} in the culture medium. To demonstrate the existence and localisation of Ca^{++} storage areas, we have used the pyroantimonate- Ca^{++} detection technic (for review see 28). This reaction used in conjunction with X-ray analysis allows a specific localisation of Ca^{++} deposits in the cell. The pyroantimonate precipitates to the glycocalyx (the precipitates disappear in Ca^{++} free medium) and very importantly in cytoplasmic vesicles. No significant Ca^{++} reserves have been detected in the mitochondria contrary to the suggestion made by pick and al. (21), following the results obtained on *Dunaliella Salina* showing that respiratory induced Ca^{++} release. Ca^{++} storage in cytoplasmic vesicles has been also observed (23) in another flagellate unicellular algae

Tetraselmis, where transient release of Ca^{++} from these vesicles and its subsequent sequestration could induce cyclic contraction and extension of large striated flagellar roots. A similar mechanism could regulate the beat frequency in *Dunaliella*. phosphorylated phosphatidylinositols are present in these cells and could be involved in the regulation of the release of Ca^{++} storage vesicles (results not shown).

In conclusion, we suggest that *Dunaliella* growing in the presence of very high Ca^{++} concentrations (6.8 mM) has developed powerful mechanisms to maintain a low cytoplasmic free Ca^{++} concentration; these mechanisms are : storage in vesicles and pumps at the level of the plasma membrane (21). We suppose that these storages of Ca^{++} could interfere in different mechanisms which require an elevation of Ca^{++} concentration such as change of direction or the modification, or inhibition of the ciliary beat as well as the other Ca^{++} regulated functions.

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