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Changes in the structure and function of plant cell protoplast due to energy deficit

MARIA PODBIELKOWSKA and EWA KUPIDŁOWSKA

Institute of Botany, Laboratory of General Botany, University of Warsaw

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Abstract

The influence of glycolysis inhibitors and of the respiratory chain inhibitors on the structure and function of protoplast were investigated in *Allium cepa* and *Tradescantia bracteata* meristematic cells. Hypertrophy of rough ER and modification of active transport were found, which was expressed by changes in the permeability of the plasmalemma and tonoplast and by depressed level of active ATP-ase.

It is supposed that the formation of the characteristic rough endoplasmic reticulum (ER) configurations plays a significant role in bioenergetic processes by increasing the surface area active in glycolysis and in the respiratory chain connected with ER membranes.

INTRODUCTION

After long lasting biochemical experiments aimed at a better knowledge of the structure and function of the cell at the molecular level, recently attention is called to the crucial moment of intracellular integration depending on the kinetics of enzymes structurally bound with biological membranes. The latter, differentiate the internal medium of the cell. This makes possible a course of biological cycles which differ in rate, spatial parameters etc. and which are regulated by the varying permeability of the membranes.

In consideration of the role of biological membranes in cell metabolism particular attention should be devoted to energetic metabolism. Most data concerning this problem refer to mitochondrial membranes (Green et al., 1968; Hackenbrock, 1968; Wojtczak, 1971; Duszyński, 1975). The relation between mitochondrial respiration and glycolysis occurring within the cytoplasm has also been investigated (Bryła and

Frąckowiak, 1968). Within the ER, glycolysis enzymes organized in aggregates and distributed along the membranes were observed (Nejfach, 1973). A respiratory chain was also found there differing in enzymatic composition from that of the mitochondria (Nejfach, 1973; Gordon et al., 1975). The numerous experiments carried out in the previous studies with the application of low temperature, oxygenless atmosphere and respiratory inhibitors demonstrated that energy deficit in the cell, notwithstanding the way in which it was elicited, causes characteristic hyperthophy of the rough ER. The hypertrophied membrane systems assume characteristic spherical or parallel forms noticeable both in the electron and in the light microscope. Their formation is associated with an increase in lipid content in the tissues (Podbielkowska and Kacperska-Palacz, 1971; Podbielkowska et al., 1975; Podbielkowska and Borys, 1975). It was supposed that an essential role in the process of formation of the characteristic ER structures is played by the rise of the glycolysis level occurring within the ergastoplasm.

The purpose of the present study was to demonstrate the role of ER membranes in metabolic processes in cells affected by energy deficit. If it was established the corelation between specificity of the membranization process of the cytoplasm and the conditions stimulating glycolysis it would explain the eventual participation of regions of hypertrophic ER in the process of anaerobic respiration. Determination of the content of enzymes taking part in active transport and measurement of plasmalemma and tonoplast permeability will give supplementary information to what extent the energy deficit affects transport through these membranes.

MATERIAL AND METHODS

The experiments performed here were based on the supposition that there exists a relation between ER hypertrophy and the rise of the glycolysis rate. The factors stimulating glycolysis in the experiment were: oxygenless atmosphere (N_2) and the analogously acting sodium azide (NaN_3) which block the last reaction in the electron transport chain. As inhibitors monoiodoacetate (CH_2JCOOH) and sodium fluoride (NaF) were used according to the data of James (1953b).

The experiments were performed on two objects: *Allium cepa* L. and *Tradescantia bracteata* Small roots. *Allium cepa* was chosen as experimental material because glycolysis, particularly its last steps leading to pyruvic acid synthesis, occur here in a different way than in other plants (James, 1953a). Comparison of the changes in the structure of protoplast in cells of *Allium cepa* and *Tradescantia* roots, elicited by glycolysis inhibitors could make it possible to establish which changes may be

interpreted as a reaction of protoplast to inhibition or stimulation of glycolysis and which as a reaction to blocking of nonglycolytic enzymes.

Adventitious roots of *Allium* and *Tradescantia* used in the experiments were cultured in tap water changed every day in shaded vessels of 200 ml capacity. When the roots reached a length of 2—4 cm the onions and *Tradescantia* shoots were transferred to an incubation solution of the given inhibitor, prepared with tap water as well or to a nitrogen chamber (anaerobic conditions).

Such conditions were achieved by passing a stream of nitrogen under 5 atm pressure for 5 min through the exsiccator with the cultured plants. The course of the experiment is illustrated by Table 1.

In all variants of the experiments the reversibility of the changes was checked by transferring the plants with rest part of the roots incubated in the above described solution for postincubation to tap water for 24 h in air atmosphere (normal conditions).

For observation in the light microscope root tips about 0,5 cm long were fixed in chromium-aceto-formalin (CrAF) in a percentual proportion of 0.5-1-20 for 24 h. Then, after being passed through a series of alcohols and xylene they were embedded in paraffin blocks from which microtome sections 3-5 μm thick were prepared. The serial sections were stained with iron haematoxylin after Heidenhain.

The ultrastructure of meristematic cells of *Allium cepa* and *Tradescantia* roots treated for 12 h with NaF (1000 ppm) was investigated in the electron microscope in anaerobic atmosphere. The material was fixed in 2 per cent $KMnO_4$ or glutaraldehyde with postfixation in OsO_4. The sections were contrasted with uranyl acetate after Raynolds (1963).

For checking whether the observed hypertrophy of ER is not a specific reaction of the cell to blocking of one of the steps of the respiratory chain, an additional experiment was performed with the use of KCN and 5-ethyl-5-(1-methyl)-thiobarbituric acid. The course of the experiment is shown in Table 2.

Moreover, orientational cytochemical investigations were carried out on living material, concerning the action of the respiratory inhibitors applied on enzymes involved in energetic metabolism in the cell (ATP-ase, succinic dehydrogenase). As experimental object served inner (upper) epidermis cells from *Allium cepa* scales treated for 20-30 min with solutions of the particular inhibitors in a 20 ppm concentration. The content of active enzyme finding its expression in the intensity of the colour reaction was orientationally assayed by comparison with control material. For detection of succinate dehydrogenase neotetrazolium staining according to Seligman and Rutenburg (1951 — quoted after Zawistowski, 1965) was applied.

ATP-ase was revealed by incubating the sections for 25-30 min at room temperature (37°C) in the following mixture:

Table 1

Object	Glycolysis time stimulator (h)		Glycolysis inhibitor		Time (h)	Inhibitor and stimulator		Time (h)	
	Atmosphere N_2	NaN_3	NaF		CH_2JCOOH	Incub.	Postincub.	Incub.	Postincub.
			50 ppm	1000 ppm		$NaF+N_2$	N_2	$NaF+NaN_3$	NaN_3
Allium	3	3							
	6	6	6	6	6	6	12	6	24
	12	12	12	12	12	12	12	12	12
	24	24	24	24	24				
								$CH_2JCOOH+N_2$	N_2
Tradescantia	6		6	6	6	6	12	6	6
	12	—	12	12	12	12	12	9	12
	24		24	24	24			12	12

1 ml ATP solution (sodium salt)
1.4 ml distilled water
0.6 ml TRIS buffer (0.2 M solution, pH 6.0)
0.5 ml $Pb(NO_3)_2$ — 1 per cent
1 ml 0.015 M $MgSO_4$.

After washing in water the sections were treated with 1 per cent NH_4S solution for 15-60 sec.

Table 2

Time (h)	KCN				5-ethyl-5-(methyl) -thiobarbitur acid	
	concentration ppm					
6	50	100	500	1000	1000	2000
12	50	100	500	1000	1000	2000
24	50	100	500	1000	1000	2000

In order to establish whether there is a relation between the degree of permeability of the plasmalemma and tonoplast and the action of the respiratory inhibitors applied, an experiment was performed with measurement of the time of deplasmolysis. For this purpose fragments of the inner (uper) epidermis of *Allium cepa* scales were icubated for 3 min in 2,4-DNP, NaN_3, NaF, KCN, and malonic acid of 20 ppm concentration and subjected to plasmolysis in 30 per cent sucrose solution. The deplasmolysis time was measured in 0.6 M ethyl glycol solution which penetrates into the cell rather slowly.

The time was measured from the moment when the tissue was placed in the solution of the deplasmolyser to complete deplasmolysis of all cells visible within the field of vision (ca. 10 cells).

RESULTS

In the light microscope changes in the protoplast structure of meristematic cells from *Allium cepa* and *Tradescantia* roots under the influence of stimulating factors and glycolysis inhibitors were observed, and particularly the morphology and degree of membranization of the cytoplasm and the number and morphology of the mitochondria. Changes in the protoplast structure were evaluated by comparison with control material. The results are shown in Table 3.

Observations in the light microscope of changes in the protoplast structure under the influence of glycolysis inhibitors and stimulators allowed to establish the following regularities:

1. Factors stimulating glycolysis (N_2 atmosphere, NaN_3) cause the formation of characteristic membraneous structures within the cytoplasm (Plate I, Fig. 3).

Table 3

Course of experiment showing shanges in protoplast structure under the influence of stimulating agents and glycolysis inhibitors

Objects	Stimulator		Inhibitor			Stimulator + inhibitor			
	Atmosphere N_2	NaN_3	NaF		CH_2 JCOOH	$NaF+N_2$ Incubation	N_2 Postincubation	$NaF+NaN_3$ Incubation	NaN_3 Postincubation
			50 ppm	1000 ppm					
Allium	Intensity of cytoplasm membranization process and number of mitochondria increases proportionally to incubation time from 0-24 h. After 24 h decrease of number of mitochondria, slight vacuolization of cytoplasm	Protoplast membranization increasing proportionally to time of inhibitor action	No membranization of cytoplasm. No changes in structure of protoplast components found	Cytoplasm membranization noticeable after 12h. After 24h or more protoplast disorganization, swelling of mitochondria and vacuolization of cytoplasm, disapperance of memb. structures begin	Sporadically membraneous structures in dermatogen	Typically formed membr. structures. Very numerous polymorphic mitochondria	Cytoplasm membranization process started in incugation continues in postincubation. Number of mitochondria and morphology typical	Cytoplasm membranization process dependent on time of exposure to inhibitors. Typically formed memb. structures rather numerous	Number of membraneous structures depends on time of incubation in $NaF+NaN_3$. Membranization continues in postincubation. Number of mitochondria gradually decreases
Tradescantia	Formation of membraneous structures proportional to time of exposure N_2 atmosphere	as above	No membr. structures noted	Tipical membr. structures not noted. Single spherical structures found within dermatogen	Few membr. structures spherical mitochondria prevail	Very numerous polymorphic mitochondria. Few membr. structures mostly in peripheral parts of roots (dermatogen, outer periblem)	Typical numerous membr. structures in all root layers. Numerous polymorphic mitochondria	$CH_2JCOOH+N_2$ Membr. structures within cytoplasm not numerous	Postincubation N_2 Cytoplasm membranization process started in incubation continues in postincubation

2. Glycolysis inhibitors, particularly NaF produce membranization of the cytoplasm only in concentrations as high as 1000 ppm and when applied for a longer time (12-24 h). This phenomenon is somewhat less pronounced in the cells of *Tradescantia* than in the meristematic cells of *Allium cepa* (Plate I, Fig. 1). At lower concentrations of this inhibitor (50-500 ppm) membraneous structures were not observed within the cytoplasm.

3. After exposure to the glycolysis inhibitor, postincubation in an agent stimulating glycolysis (anaerobic atmosphere, NaN_3) causes an increase in the number of ergastoplasmic structures.

4. Application of the glycolysis inhibitor simultaneously with the stimulating agent (NaF 1000 ppm in oxygenless atmosphere) reduces the number of ergastoplasmic structures (Plate I, Fig. 4).

5. Respiration inhibitors cause a decrease in the number of mitochondria and their gradual shortening and swelling (Plate II, Fig. 4).

6. The observed changes in protoplast structure are completely reversible during 24-h postincubation under air atmosphere in tap water (normal conditions).

The observations noted in the electron microscope allowed a more accurate analysis of the effect exerted by one of the glycolysis inhibitors, sodium fluoride (NaF) in a 1000 ppm concentration in an anaerobic atmosphere stimulating the process of glycolysis.

The membraneous structures of spherical and parallel configuration observed in the light microscope were indentified in the electron microscope as cisterns of the highly hypertrophied ER (Plate II, Figs 5, 6). In material fixed with glutaraldehyde with postfixation in OsO_4 ER was found to be rough.

In the areas of the cytoplasm delimitated by membrane complexes or in the immediate neighbourhood of the membraneous structures numerous mitochondria and dictiosomes are present (Plates IV and V, Figs 9 and 12), what seems indicating metabolic activity in these regions.

It was observed that within the ER cisterns, frequently on their endings, characteristic vesicles form which seem to be the first step in the formation of large vacuoles with a sharply visible surrounding membrane (Plates III and IV, Figs 7, 8 and 10).

Electronograms representing meristematic cells of *Tradescantia* show essential differences as compared with those of *Allium cepa* cells. These differences mainly concern the number of ergastoplasmic structures. Spherical ER cistern systems in *Tradescantia* cells were only sporadically noted (Plate V, Fig. 11), more frequent were parallel systems but with a small number of membranes too. Beside normal mitochondria, less numerous swollen ones with a reduced number of cristae were found (Plate V, Fig. 13).

For further elucidation of the mechanism of ER hypertrophy a supple-

mentary experiment was carried out with the use of KCN and thiobarbituric acid as respiratory chain inhibitors. Both these substances were found to stimulate the process of cytoplasm membranization (Plate I, Fig. 2). These inhibitors also induce changes in the morphology of mitochondria, causing their shortening and swelling.

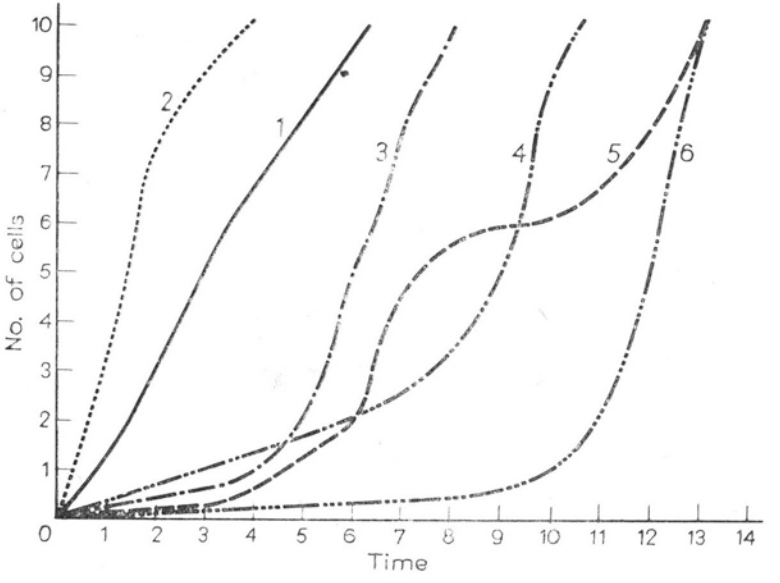

Diagram 1. Time of deplasmolysis dependence on action of respiratory inhibitors
1 — control; 2 — DNP; 3 — NaN_3; 4 — NaF; 5 — KCN; 6 — malonic acid

For demonstrating the influence of the inhibitors used on the contents of certain enzymes associated with energetic processes in the cell, cytochemical reactions were run in vivo. The content of succinic dehydrogenase was determined in epidermal cells of *Allium cepa* subjected previously to the action of inhibitors for 20-30 min. The colour reaction consisting in reduction of neotetrazol by succinate dehydrogenase demonstrated the presence of active enzyme, within the mitochondria. The enzyme content was orientationaly evaluated by comparing the colour intensity in experimental and control material.

It results from the observations performed that respiratory inhibitors reduce the amount of active succinate dehydrogenase. Most pronounced is here the influence of sodium fluoride (NaF) which is the specific inhibitor of this enzyme. The effect of 2,4-DNP is the weakest.

The content of active ATP-ase (assayed orientationally in reference to the control) was also evaluated in epidermal cells of *Allium cepa* scales. The trend of the changes is shown in Table 4 in which the inhibitors are ordered according to their influnence on ATP-ase content.

Table 4
Influence of respiratory inhibitors on the level of ATP-ase

100%	Cytoplasm	Mitochondria
	control	control
	NaN_3, 2,4-DNP	$CH_2JCOONa$
	KCN	
	NaF	2,4-DNP, NaN_3
0%	$CH_2JCOONa$	NaF, KCN

Rather significant differences in the effects of the particular inhibitors on ATP-ase content in the plasmalemma and mitochondria were noted. This may be explained by the different susceptibility of mitochondrial and cytoplasmic ATP-ases. Mitochondrial ATP-ase is inactivated by azides and 2,4-DNP, whereas ATP-ase localized in non mitochondrial (cytoplasmic) membranes is not directly affected by the inhibitors (D o w b e n, 1973).

The metabolic processes associated with energy production are directly related with active transport and membrane permeability. Therefore the next experimental step was to measure the permeability of the plasmalemma and tonoplast on the basis of the plasmolytic method. Diagram 1 shows the mean number of plasmolysed epidermal cells of *Allium cepa* scales in each minute as seen in the field of vision. (Measurements were repeated three times for each experimental combination). A distinct dependence of the membrane permeability on the action of the inhibitor results from these data. All the inhibitors applied with the exception of 2,4-DNP reduced the permeability of cytoplasmic membranes. Under the action of 2,4-DNP the permeability of the plasmalemma and tonoplast increased on the contrary, as compared with the control.

DISCUSSION

It results from the here described experiments that the changes in the configuration of ER membranes may be induced by the raised rate of glycolysis occurring within the ergastoplasm. Glycolysis is stimulated by anaerobiosis, 2,4-DNP and inhibitors of the respiratory chain as a response to the decrease in mitochondrial ATP. It is induced by kinazine released by the mitochondria into the cytoplasm (N e j f a c h, 1973). Biochemical investigations demonstrated that ADP is the inductor of kinazine synthesis and ATP its repressor. Thus it may be concluded that the factor stimulating glycolysis is intensively synthesized under conditions of energy deficit. In the light of these data a raised rate of glycolysis seems quite

probable in energetic processes of cells cultured under conditions of ATP deficiency. The results of our experiments seem to indicate that stimulation of glycolysis by application of anaerobiosis or inhibitors of oxygen respiration (NaN_3) stimulates the development of membranes in regions of the cytoplasm in which processes supplying energy take place. Hypertrophy of the ER membranes thus would aim at increasing the surface active in these processes.

The investigations of Nejfach (1973) show that release by the mitochondria of a factor stimulating glycolysis is simultaneous with their swelling. The increase in the volume of mitochondria observed in the present study under the influence of anaerobiosis and 2,4-DNP would confirm the findings of the above named author. The glycolysis inhibitors used by us (NaF, CH_2JCOOH) in spite of their low specificity (they act as well on other enzymes not associated with glycolysis) did not induce formation of the typical ER structures. This effect could be most distinctly observed in the meristematic cells of *Tradescantia* roots in which the glycolysis cycle runs along the normal pathway. In the roots of onion in which synthesis of pyruvic acid occurs on a different pathway (James 1953a) NaF played above all the role of inhibitor of the respiratory chain, therefore the initiated process of cytoplasm membranization under the influence of this inhibitor was weak. The correctness of this supposition is also supported by the results of enzymatic reactions. The considerable fall of the succinic dehydrogenase level under the action of respiratory inhibitors (NaF) indicates small participation of the respiratory chin in the energy-supplying processes.

It has been observed that glycolysis stimulation is not the only factor inducing cytoplasm reorganization. There is a distinct relation between cytoplasm membranization and disturbances in the reactions of the respiratory chain. The specific inhibitors used by us such as KCN and thiobarbituric acid produced typical ER hypertrophy. This agrees with the observations of Gordon et al. (1975) suggesting the presence of a respiratory chain within ER membranes. Electron transport inhibition in the respiratory chain by the inhibitors used in our experiments might have caused an increase of the ER membranes surface area on which this process occurs as compensation of the energy deficit. The factors disorganizing cell respiration used by us, beside acting directly on the mitochondrial and cytoplasmic electron transport chain are not without influence on active intracellular transport. The result of the enzymatic reactions run by us confirms this conclusion. The fall of the ATP-ase level after exposure to the action of our inhibitors is quite considerable. The observed changes in plasmalemma and tonoplast permeability seem to indicate that, under conditions of energy deficit, disturbances in transport also occur in other plasmatic membranes.

Vacuole formation from ER observed by us agrees with the findings

of Berjak (1972) and Parish (1975). The vacuoles described by Parish formed from ER contained active peroxidase. This fact supports our suggestion as to the role of ER in bioenergetic processes.

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Author's address:
Dr. Maria Podbielkowska and Ewa Kupidłowska
Institute of Botany, Laboratory of General Botany,
University of Warsaw,
Warsaw, Poland

Zmiany w strukturze i funkcji protoplastu komórki roślinnej wywołane deficytem energii

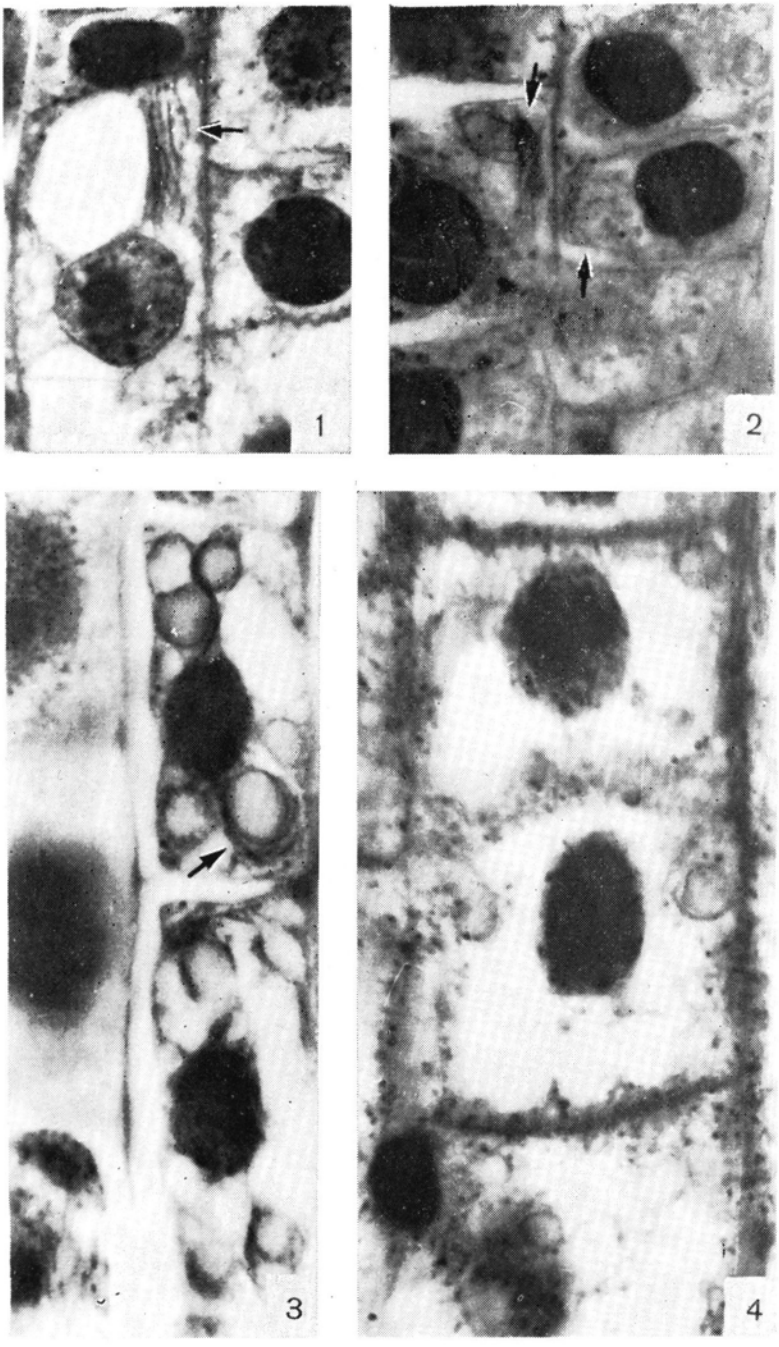
Streszczenie

Przeprowadzone doświadczenia miały na celu wykazanie pozytywnej korelacji między deficytem energii, indukowanym przez inhibitory oddychania tlenowego (NaN_3, KCN, atm. N_2) i beztlenowego (NaF, CH_2JCOOH) a specyficzną reorganizacją protoplastu wyrażającą się hipertrofią ER oraz zmianą w strukturze mitochondriów. Za szczególnie istotne uznano powiązanie rozwoju membran ER ze stymulacją procesu glikolizy. Punktem wyjścia dla tej hipotezy był fakt lokalizacji enzymów glikolitycznych w przestrzeniach międzybłonowych membran cytoplazmatycznych (ER) (Nejfach 1973). Wyniki przeprowadzonego przez nas doświadczenia pozwalają sądzić, że istnieje pozytywna korelacja między powstawaniem charakterystycznych konfiguracji ER a wzrostem poziomu glikolizy. Okazało się jednak, że stymulacja glikozy nie jest jedynym procesem indukującym tę reorganizację cytoplazmy. Istnieje także wyraźny związek membranizacji cytoplazmy ze zmianami w reakcjach łańcucha oddechowego, które wywoływano przez zastosowanie specyficznych inhibitorów oddychania tlenowego (NaN_3). Oceniając rolę tych niezwykle specyficznych zmian w strukturze cytoplazmy, wysunięto przypuszczenie, że jest to wyraz adaptacji komórki przebywającej w warunkach okresowego niedoboru ATP. Poziom ATP w komórce warunkuje także aktywny transport przez błony. Wykazana w doświadczeniu zmiana przepuszczalności plazmalemmy i tonoplastu w wyniku działania inhibitorów oddechowych potwierdziła ten fakt i stała się podstawą do wnioskowania o roli zreorganizowanej ergastoplazmy jako terenu o dużej aktywności, przy czym rozrost błon ER mógłby mieć dwojakie znaczenie: 1) stworzenie większej powierzchni metabolicznie czynnej w procesach dostarczających energię (glikoliza, łańcuch oddechowy związany z ER), 2) regulację aktywnego transportu.

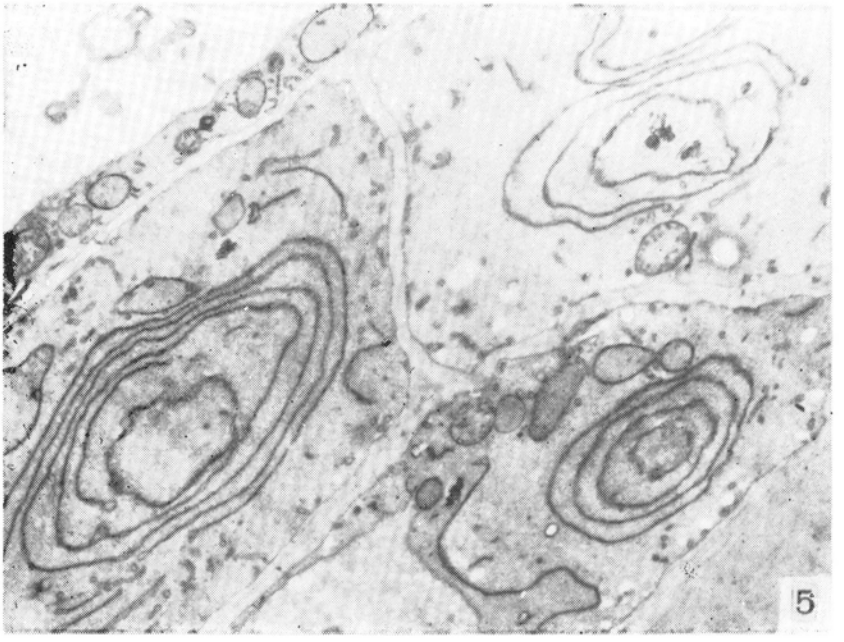
Plate I

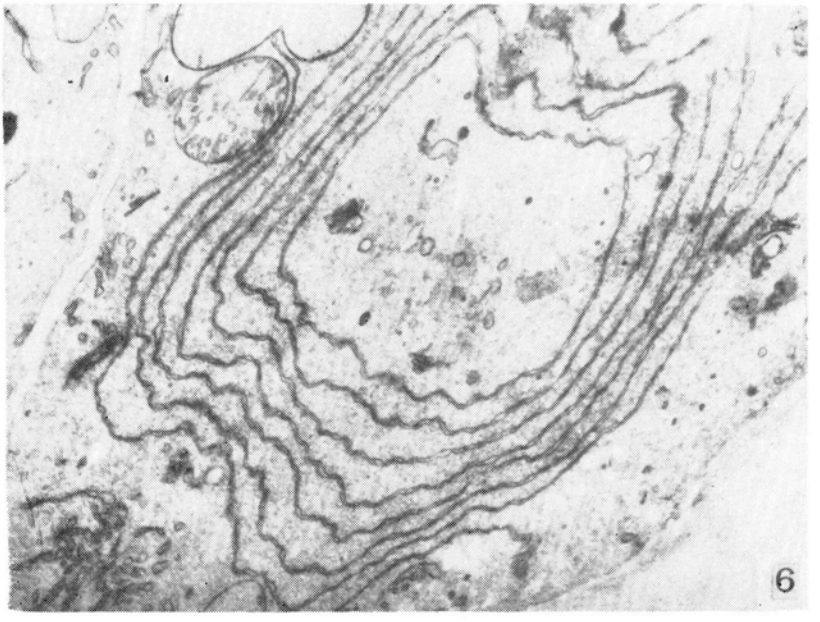
Fig. 1. treated with 1000 ppm NaF for 12 h; membraneous structures visible in parallel arrangement
Fig. 2. treated with 100 ppm KCN for 12 h, membranous structures in parallel and spherical configuration visible
Fig. 3. growing in anaerobic atmosphere for 12 h; membranous structutures in spherical configuration visible
Fig. 4. treated with 1000 ppm NaF in oxygenless atmosphere for 12 h; membraneous structures in spherical configuration and numerous mitochondria visible

Plate I

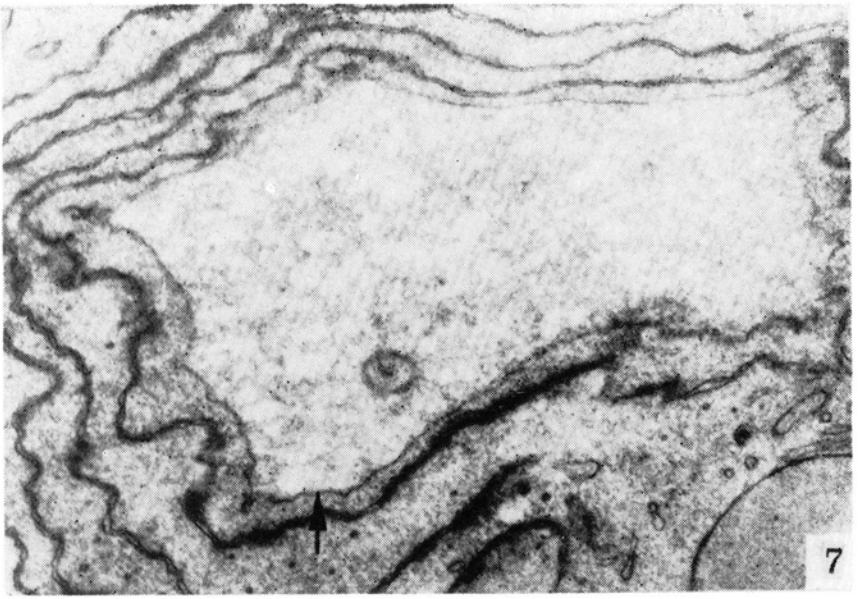


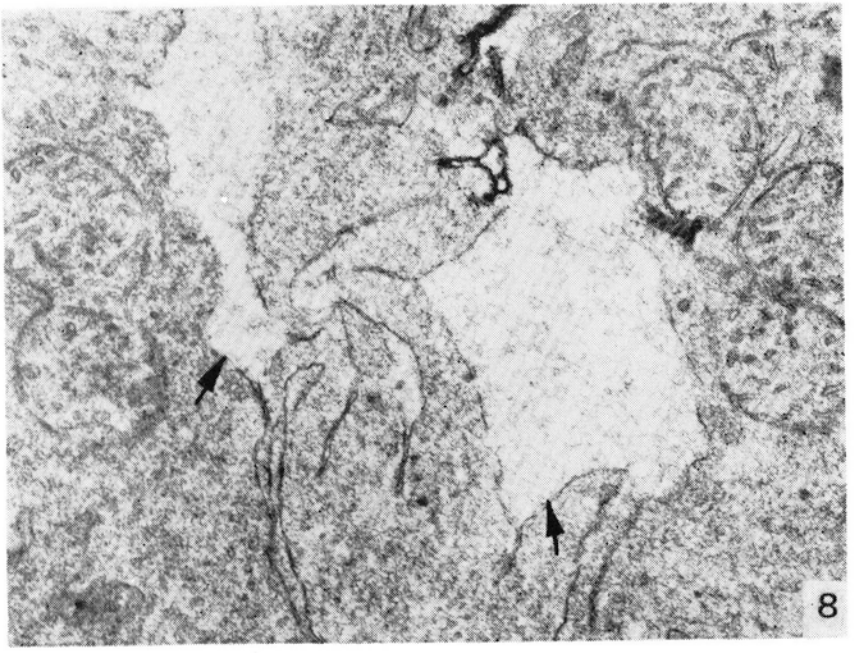
Meristematic cells from *Allium cepa* root tips fixed in CrAF; iron hematoxylin after Heidenhain; × ca 2500





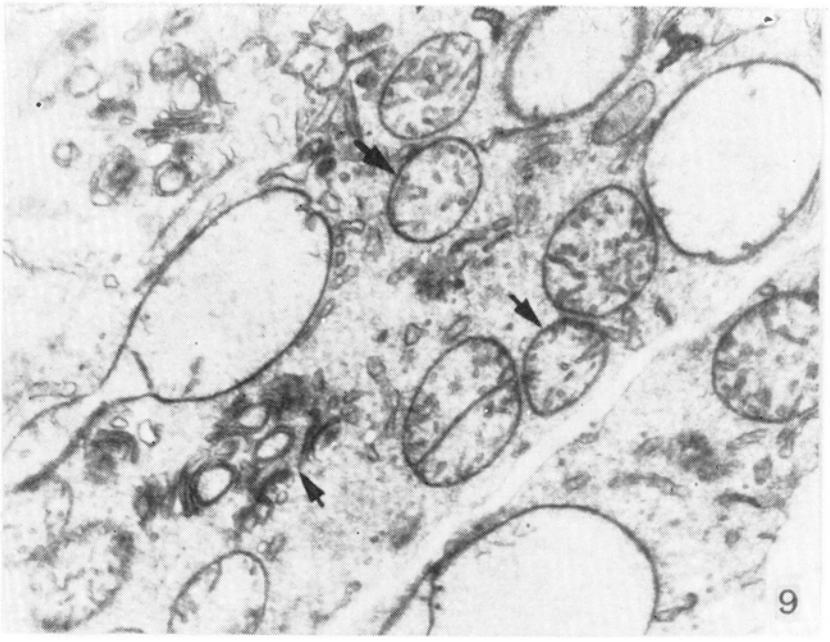
Figs 5, 6. Meristematic cells from *Allium cepa* root tips treated with NaF in anaerobic atmosphere for 12 h; fixed in 2% $KMnO_4$; fig. 5 × 14 000; fig. 6 × ca 18 000, ER in spherical configuration

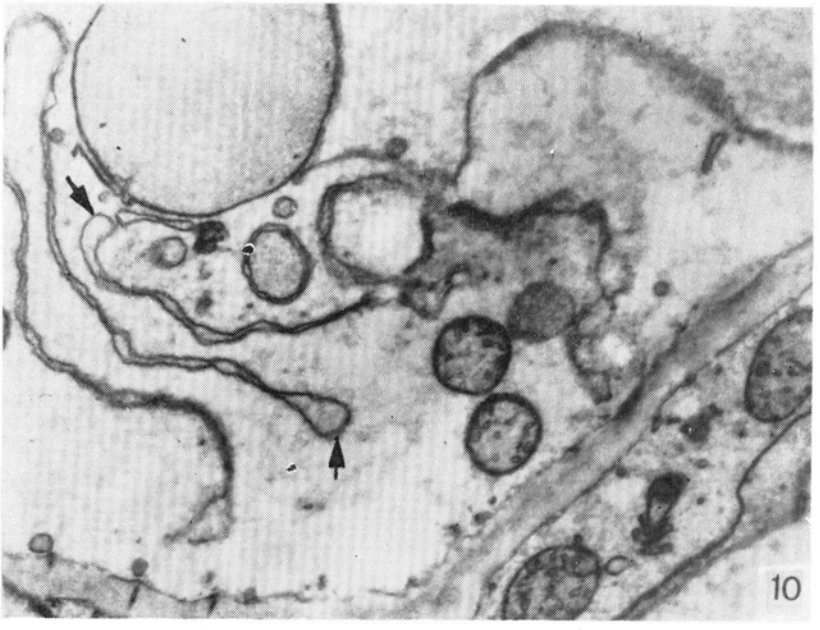




Meristematic cells from root tips of *Allium cepa* treated with 1000 ppm NaF in anaerobic atmosphere for 12 h; fixed in 2 per cent $KMnO_4$; × ca 20 000

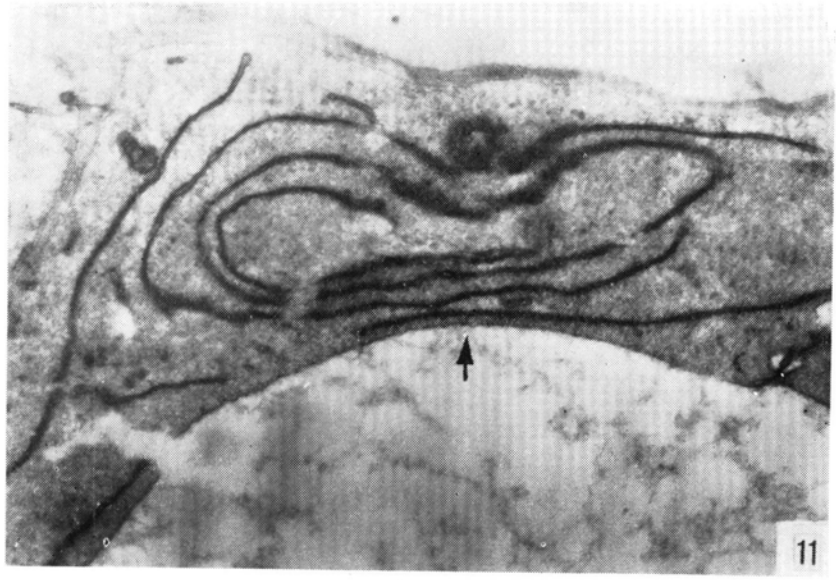
Figs 7, 8. Vacuoles formed of ER membrane (originating from ER)





Meristematic cells from *Allium cepa* root tips treated with 1000 ppm NaF in anaerobic atmosphere for 12 h; fixed in 2 per cent $KMnO_4$, × ca 20 0000

Fig. 9. group of numerous mitochondria and dictiosoms
Fig. 10. vesicle-like distensions at ends of ER cisterns



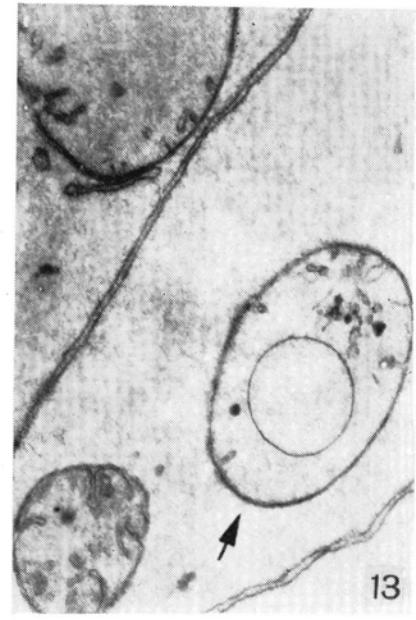


Fig. 11. Meristematic cells of *Tradescantia* root tips treated with 1000 ppm NaF for 12 h in anaerobic atmosphere; fixed in 2 per cent $KMnO_4$; × ca 18 000. Membrane systems in spherical configuration visible

Figs 12, 13. Meristematic cells of *Allium cepa* root tips treated with 1000 ppm NaF for 12 h in anaerobic atmosphere; fixed in 2 per cent $KMnO_4$; × ca 20 000

Fig. 12. Agglomeration of numerous Golgi structures visible

Fig. 13. Degeneration of inner mitochondrial membrane visible