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PIGMENTATION AND ENERGY DEPENDENT Sr^{++}-ACCUMULATION OF MOLLUSCAN NEURONS UNDER ANAEROBIC CONDITIONS

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The energetical processes of Molluscan neurons are intriguing first of all because of the ability of certain species to endure prolonged anoxia (see for references: BRAND, 1946; GODDARD and MARTIN, 1966). On the other hand, biochemical measurements failed to reveal an agreement between the amount of carbohydrates consumed and organic acids formed during anoxia (BRAND et al. 1950). In other words, it cannot be assumed that anaerobic glycolysis would be the only possibility to produce energy during prolonged anoxia even of 8—10 days. The nerve cells of certain Molluscs contain numerous cytosomes to which a "metabolic reserve" function has been attributed by NOLTE et al. (1965). They show histochemical and structural changes under anaerobic conditions (BARANYI and SALÁNKI, 1967; ZS.-NAGY and CSUKÁS, 1969; ZS.-NAGY, 1969). The cytosomes correspond to transformed mitochondria, contain a lot of lipochrome pigment, have respiratory enzyme activities and take part in the oxidative energy production of the cell (ZS.-NAGY, 1967, 1969; ZS.-NAGY and KERPEL-FRONIUS, 1970a, 1970b; KERPEL-FRONIUS and ZS.-NAGY, 1971; LÁBOS et al. 1966).

Our present investigations were aimed to clear up whether any relationship exists between the degrees of pigmentation of nerve cells, i.e. the frequent occurrence of cytosomes and anoxibiotic tolerance of different Molluscs. Furthermore, the cytosomal energetical processes were investigated under anaerobiotic conditions by means of an indirect electronmicroscopic histochemical method.

Material and methods

Fresh-water and marine Molluscs have been investigated: the fresh-water *Anodonta cygnea*, the marine *Mytilus galloprovincialis*, *Venerupis decussata*, *Tapes decussatus* and *Pecten Jacobaeus* from Pelecypoda, *Murex trunculus* and *Aplysia limacina* from Gastropoda, and *Octopus vulgaris* from Cephalopoda. The latter was used only in experiments of anoxibiotic tolerance. The fresh-water *Anodonta cygnea* was kept in aquaria supplied with Balaton-water while the marine species were collected in the Gulf of Naples and kept

in marine aquaria for several days before the experiment. Both types of aquaria were well oxygenated.

Investigation of marine animals was carried out at the Stazione Zoologica Napoli in February-April.

For achieving anaerobic conditions the animals were placed into a vessel containing eight times more Balaton- or sea-water than the body weight of the animal, than the surface of the water was covered with a paraffin oil layer of 1—2 cm thickness. Under such circumstances the oxygen content of the water is used up and a total anoxia sets in after some time (BRAND, 1946). The animals were considered as living up to the moment when they ceased to react with movement upon the influence of mechanical stimulation.

For the demonstration of energy production in the nerve cells, the electronmicroscopic histochemical method of KERPEL-FRONIUS and HAJÓS (1970) was employed, the adaptation of which for ganglia of *Anodonta*, and the results of the necessary control investigations are described elsewhere (KERPEL-FRONIUS and ZS.-NAGY, 1971). The ganglia of normal animals as well as those taken from different stages of anoxia were incubated in a buffered solution containing Sr^{++} ions, anorganic phosphate and sodium succinate, for 45 min. In the case of marine animals the suitable osmolarity was achieved by adding sodium chloride to the incubation mixture.

Ganglia of *Anodonta* taken from anoxia of 4—8 days were also investigated in incubation mixtures being devoid of oxygen by perfusion with pure nitrogen. The incubation itself took place hermetically sealed from reoxygenization of the solution. Inhibitory effects of KCN and DNP were tested on all animals according to the description of KERPEL-FRONIUS and ZS.-NAGY (1971). Apart from the histochemical reaction materials have also been processed for normal electron microscopy. Fixation in 1—1.5% OsO_4 buffered with collidine was used for this purpose. The collidine buffer was prepared in sea-water for the ganglia of marine animals, thus the final osmolarity of the fixative was nearly isotonic. The above related histochemical material was also fixed in the same fixative. After a dehydration in ethanol, materials have been embedded into Araldite. Sections were cut on Reichert Omu 2 and LKB Ultrotome III ultramicrotomes and the micrographes taken on Philips EM 200 and TESLA BS 413 A electron microscopes.

Sections prepared for normal electron microscopy were contrasted with uranyl acetate and lead citrate while those processed for histochemical purposes were stained only with the latter.

In order to identify the pigment of marine animals, extracts were prepared from the ganglia and other tissues in ethanol or chloroform and the absorption spectra of them was measured with Beckman spectrophotometer.

Results

1. *The degree of pigmentation and the tolerance of anoxia.*

The tolerance of anoxia is correlated with the lipochrome pigment content of the nervous system and other organs *(Table I)*. The degrees of pigmentation were estimated on the basis of subjective observation and classified as "strong", "moderate" and "weak" categories. One can see from the data that species displaying a strong pigmentation in the nervous system,

TABLE I

Pigmentation and survival capacity in anoxia of different Molluscs

Species	Degree of pigmentation of CNS	Survival times in hours
Pelecypoda		
Anodonta cygnea	Strong	150—200
Mytilus galloprovincialis	Moderate	40—50
Venerupis decussata	Moderate	50—70
Tapes decussatus	Weak	40—50
Pecten Jacobaeus	Unpigmented	2—3
Gastropoda		
Murex trunculus	Strong	100—200
Aplysia limacina	Moderate	10—12
Cephalopoda		
Octopus vulgaris	Unpigmented	2—3

Note: survival times mean the total period after covering the water surface with paraffin oil.

i.e. being rich in cytosomes, can endure anoxia for much longer time than those having less pigments. At the same time, *Pecten* and *Octopus* having unpigmented nervous systems are not able to live in anoxia at all, they die already at a certain level of hypoxia. It should be noted that *Aplysia limacina* behaves to some extent as distinguished from the others. On the basis of the pigmentation of its ganglia one could expect a longer survival time than it showed.

Electronmicroscopic investigation of normal ganglia revealed that the nerve cells of *Anodonta* contain a large number of cytosomes of several micron in diameter *(Fig. 1)* or sometimes reaching even 10—12 μ (Zs.-Nagy, 1968). In the nerve cells of marine species one can find only small cytosomes being less than 1 μ *(Fig. 2)* whereas in those of *Pectan* cytosomes do not occur at all *(Fig. 3)*. It should also be noted that the lipochrome pigment is present in other tissues of *Pecten*. Both the gills and gonads contain a yellow pigment, the ethanol or chloroform extract of which show the absorption spectra characteristic for carotenoids. It should also be mentioned here that in the extracts of pigmented ganglia of marine species investigated, carotenoids were always present.

2. *Energetical processes in anoxia*

Oxidative energy yielding processes can be demonstrated both in mitochondria and certain cytosomes *(Fig. 4)* of nerve cells of normal animals employing the method of Sr^{++}-accumulation. The reaction can totally be abolished by 0.5 mM DNP and to a very large extent, though, not completely by 4 mM KCN. It should be noted that the accumulation of Sr^{++}-phosphate is much more intense in the cytosomes of *Anodonta* than in the small ones

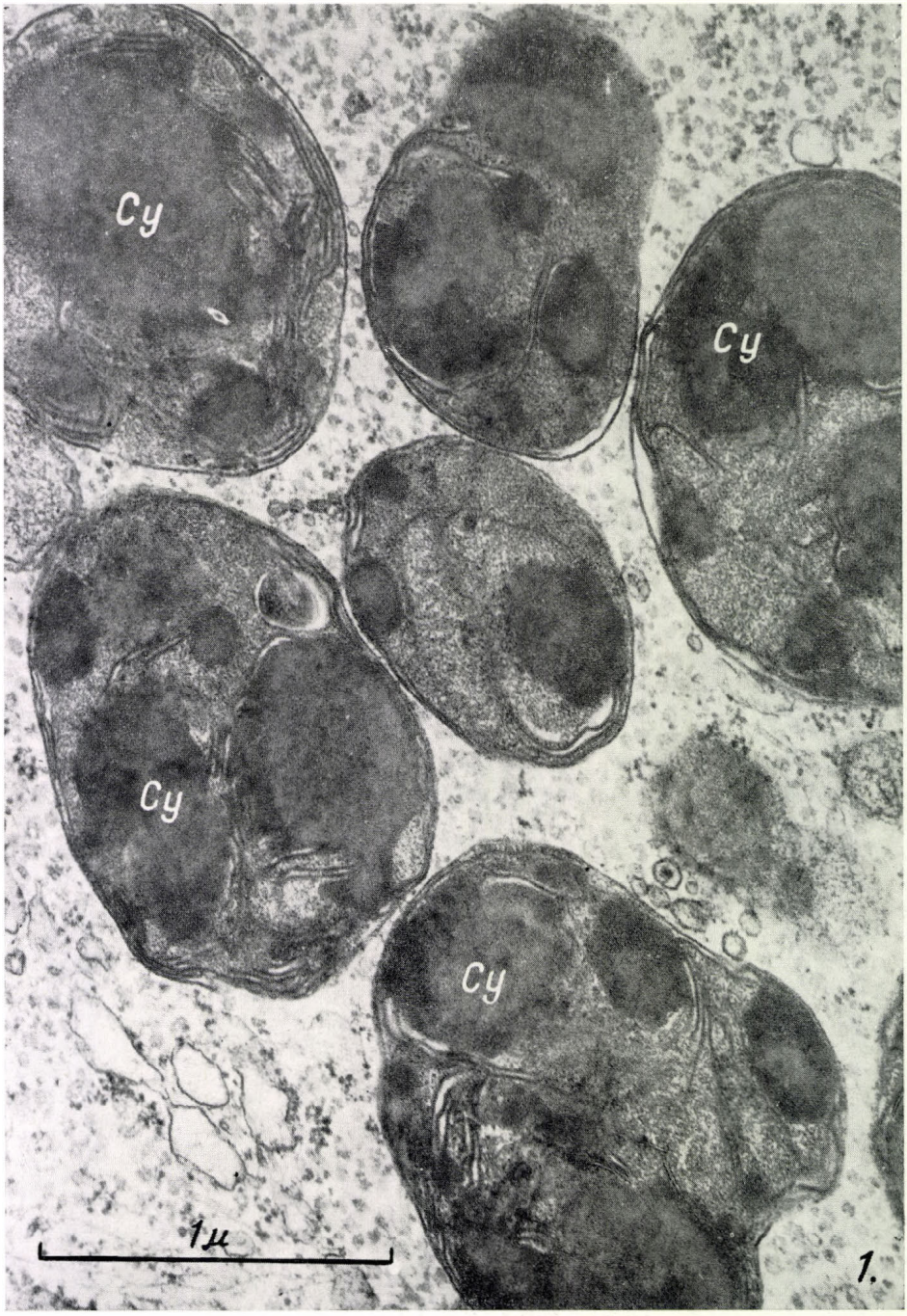

Fig. 1. Detail of a nerve cell of cerebral ganglion taken from normal *Anodonta cygnea*, demonstrating the cytosomes (Cy)

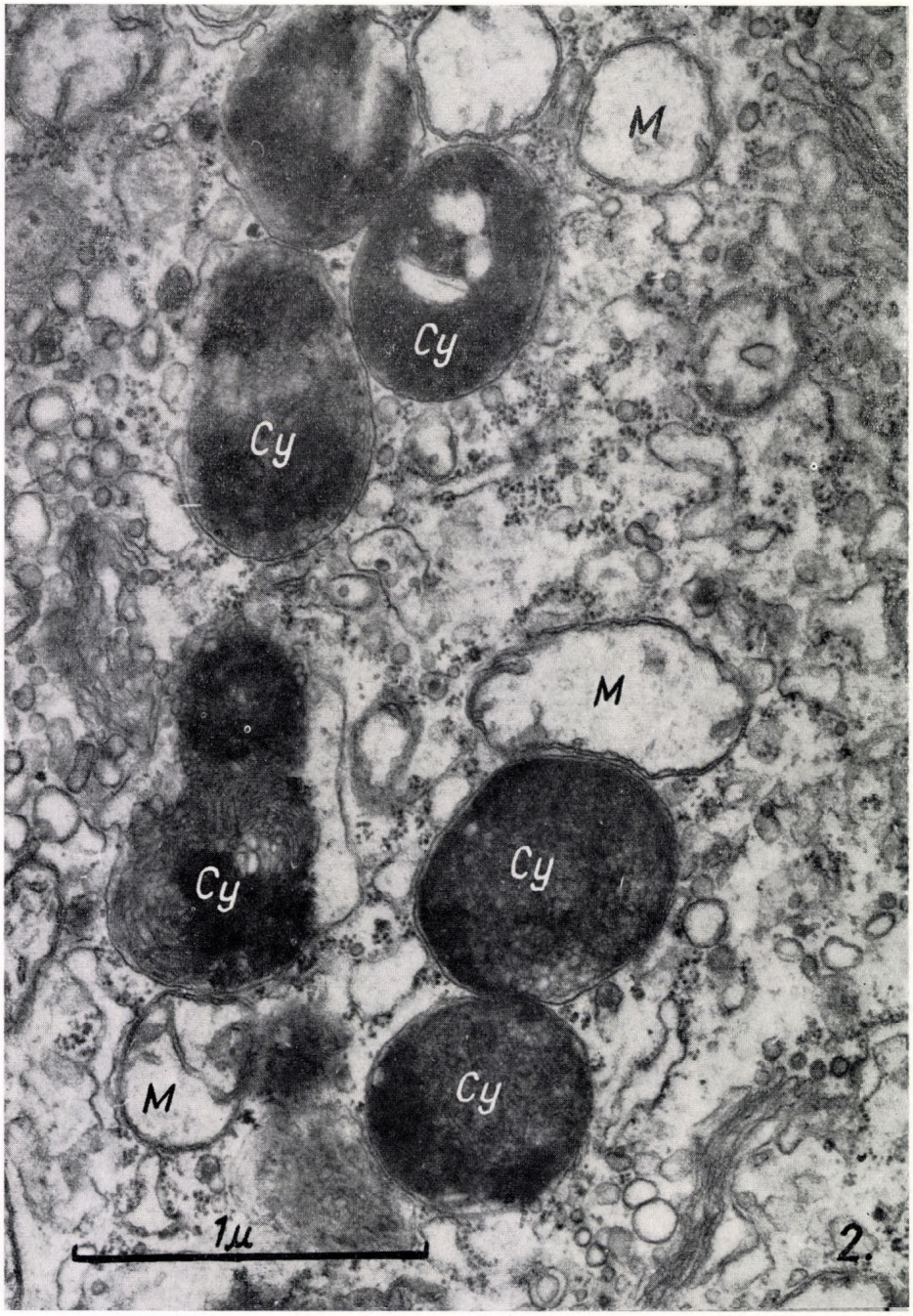

Fig. 2. Detail of the pedal ganglion of a normal *Mytilus galloprovincialis*. Cy - cytosomes; M = mitochondria

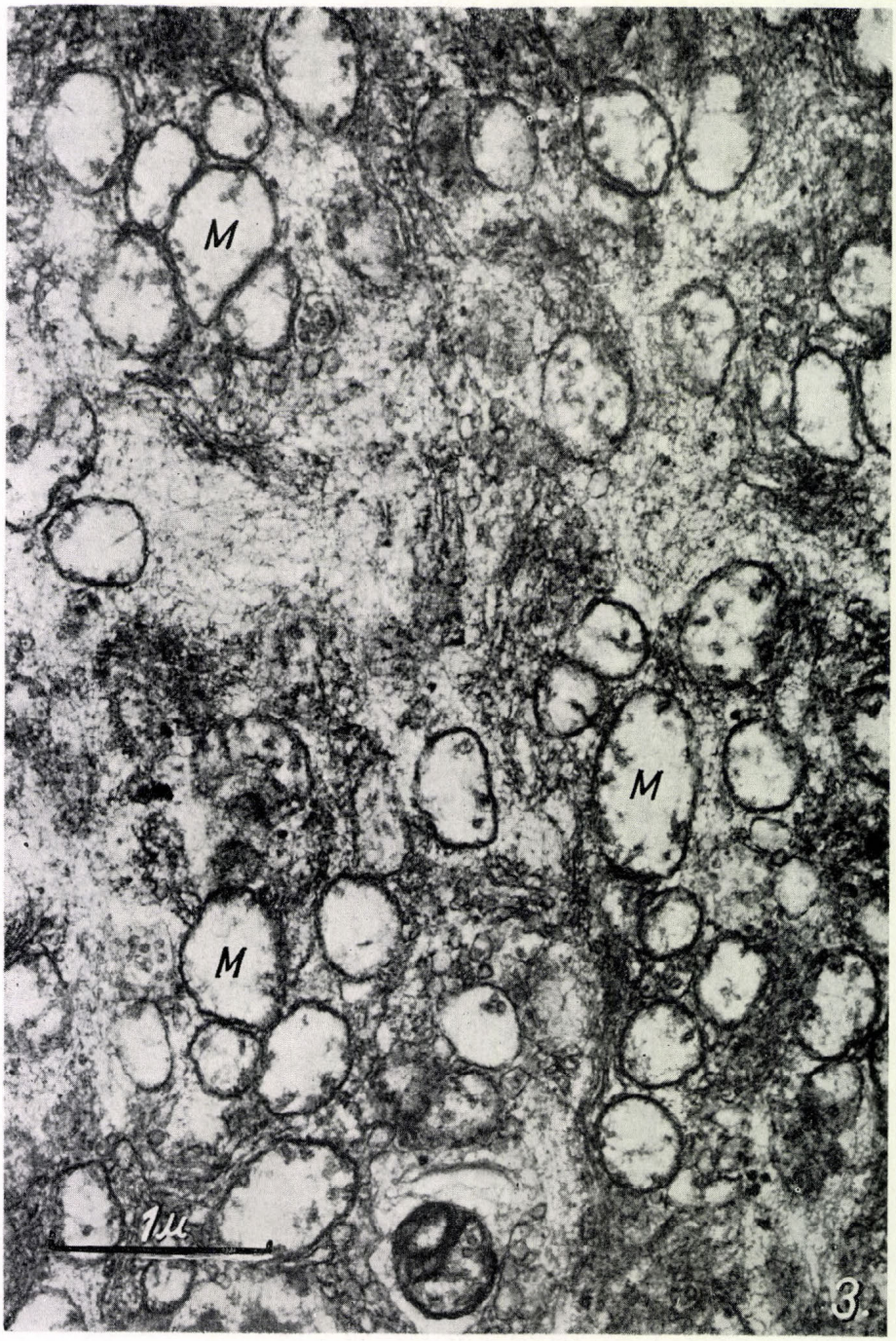

Fig. 3. Detail of the visceral ganglion of normal *Pecten Jacobaeus*. Numerous mitochondria (M) can be seen. No cytosomes

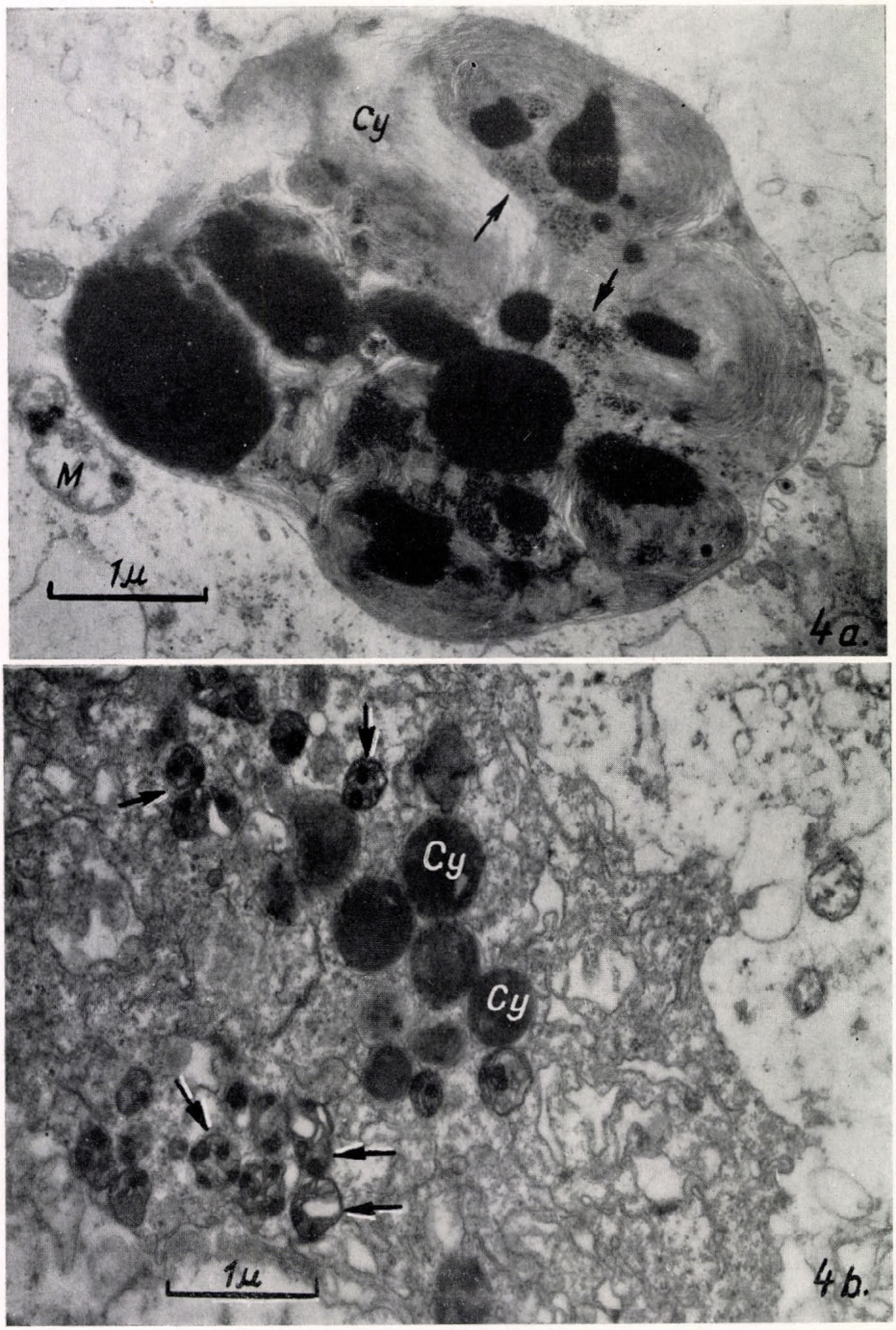

Fig. 4. The result of Sr^{++}-accumulation in normal animals
a) Cerebral ganglion of *Anodonta cygnea*. Cy = active cytosome, the fine granulated Sr^{++}-phosphate deposites are indicated by arrows. M = active mitochondrion
b) Pedal ganglion of *Mytilus galloprovincialis*. Arrows indicate mitochondria of strong activity. Cy = cytosomes displaying only weak activity

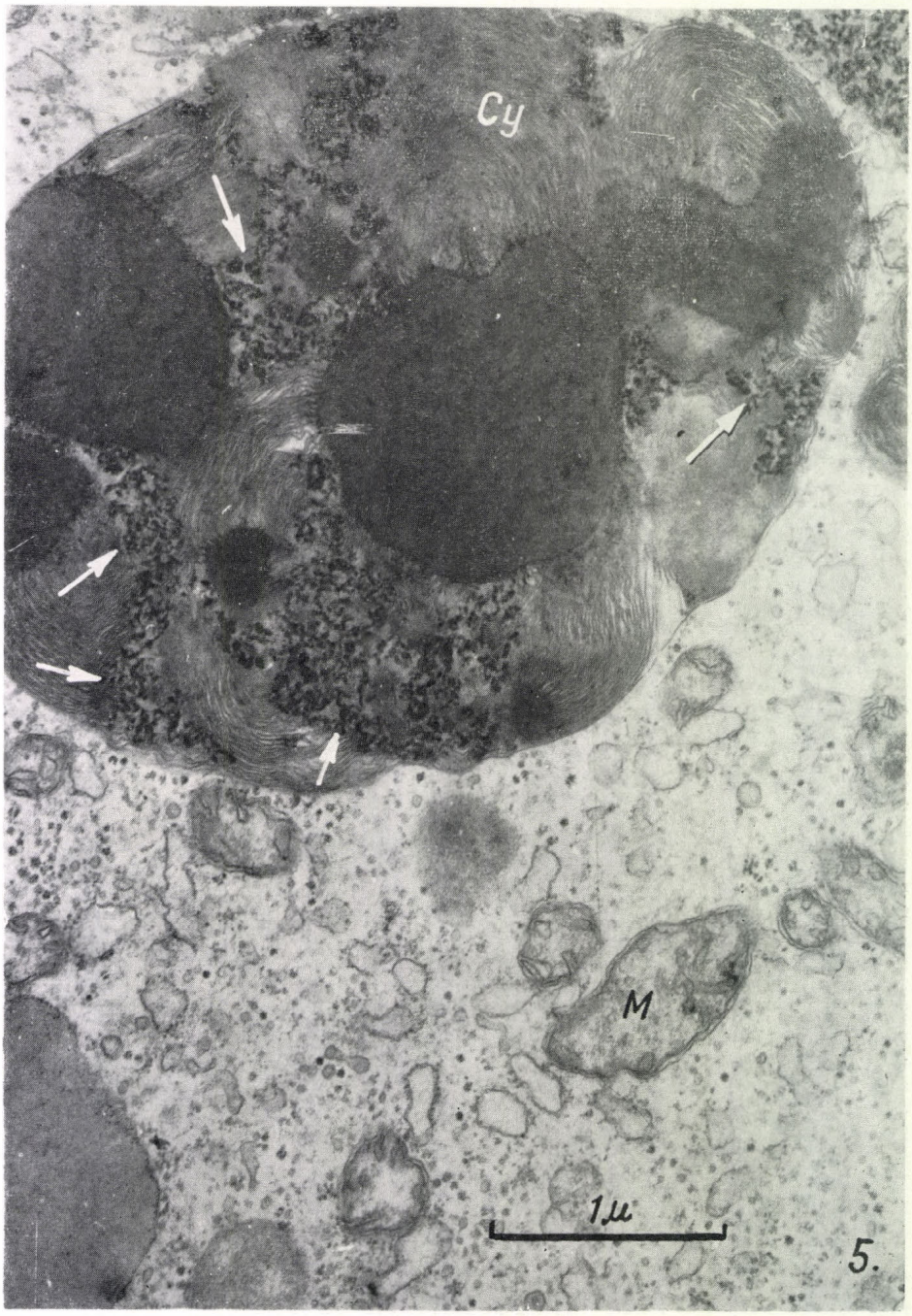

Fig. 5. Pedal ganglion of *Anodonta cygnea* after 5 days of anoxia. Arrows indicate very strong Sr^{++}-accumultation in the cytosome (cy). Incubation was carried out in a mixture devoid of oxygen. M = mitochondrion

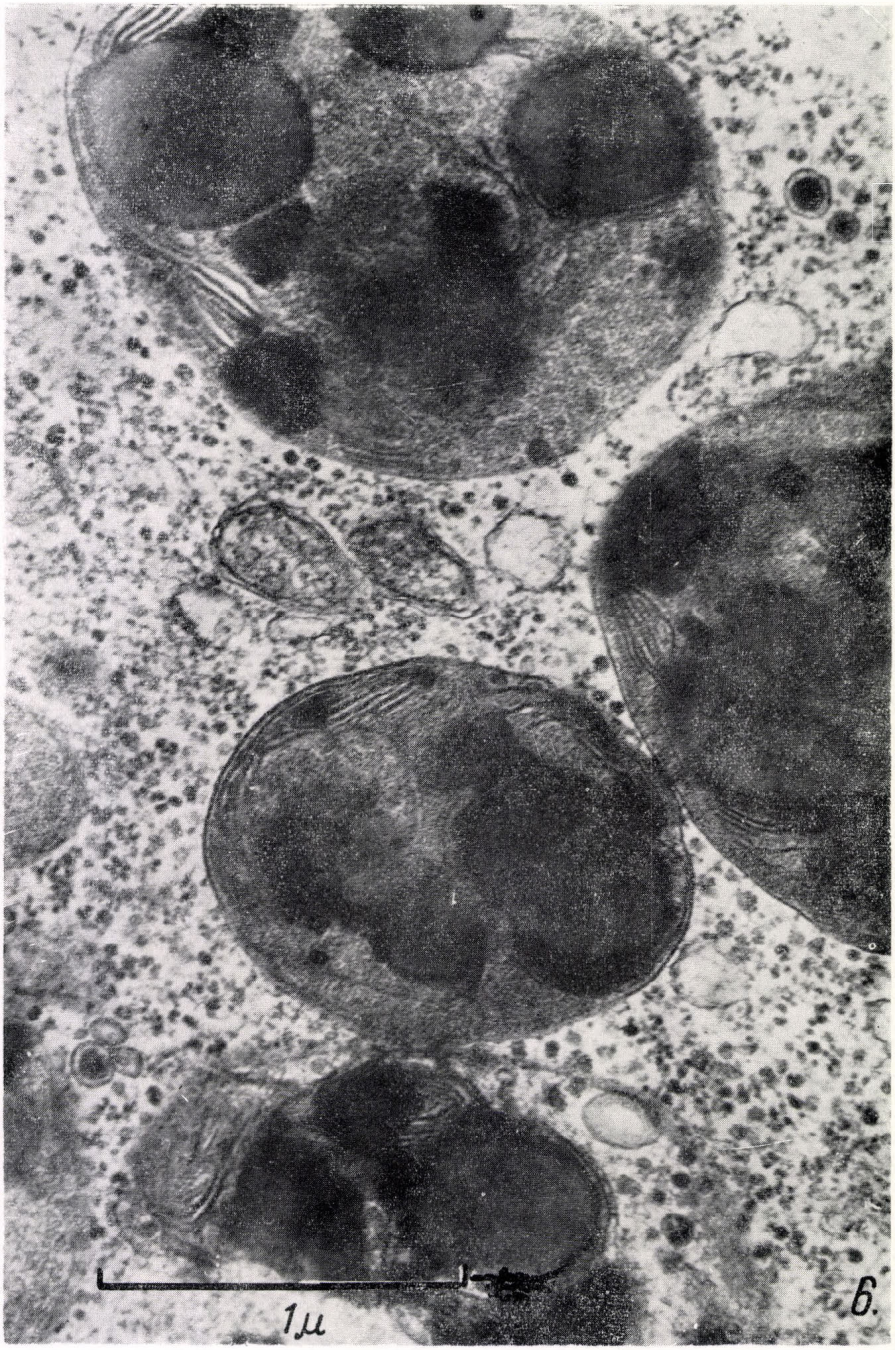

Fig. 6. Cerebral ganglion of *Anodonta cygnea* after 5 days of anoxia. Sr^{++}-accumulation in the presence of 0.5 mM DNP. No Sr^{++}-phosphate deposits in the cytosomes

of marine species. On the other hand, the mitochondria of the latter display a more intense reaction.

The energy production as indicated by the Sr^{++}-accumulation persists for some time even in anoxia, however, only in the cases where the nerve cells contain cytosomes. The Sr^{++}-accumulation was accomplished in the nerve cells of *Anodonta* even after 8 days of anoxia, however, a reversed situation was observed compared to the normal animals: it was weaker in mitochondria and much more intense in cytosomes. The activity of the Sr^{++}-accumulation was not altered by changing the oxygen of incubation mixture for nitrogen *(Fig. 5)*. The Sr^{++}-accumulation observed in anoxia could also completely be inhibited by DNP *(Fig. 6)*. and reacted to KCN in the same way as the normal nerve cells.

The energy production was demonstrated also in the nerve cells of marine animals investigated in anoxia. The individuals investigated parallel could survive only 4—6 hours after the cessation of the Sr^{++}-accumulating mechanism.

Discussion

Our results are in accordance with literary data concenring the anoxibiotic tolerance of Molluscs (see for references: BRAND, 1946; GODDARD and MARTIN, 1966). At the same time, it is also demonstrated by them that there is a correlation between the degree of pigmentation of the nervous system and the anoxibiotic tolerance of the animal, since the nervous system of those well able to endure anoxia is strongly pigmented whereas that of *Pecten* and *Octopus* being destructed already by hypoxia is completely unpigmented and contains no cytosomes at all. A bad anoxibiotic tolerance of *Pecten* has also been observed by SALÁNKI (1966). The ability of the animals for anaerobiosis was considered by BRAND (1946) as an adaptation to ecological circumstances. On the basis of our investigations it seems that the presence of cytosomes represents the morphological basis of this adaptation. This agrees with our observation (ZS.-NAGY, 1969) according to which the cytosomes are formed when the animal is well supplied with oxygen and are structurally altered during the prolonged anoxia.

There are no special results in the literature, answering the question what mechanisms enable the nervous tissue to endure prolonged anoxia. It has been revealed by investigating total animals that in the snails the amount of lactic acid found in the tissues and the environment cannot explain the amount of carbohydrates consumed in anoxia (BRAND et al. 1950) and even the amount of lactic acid produced by different species was strongly variable. This supports the assumption that the glycolysis cannot be the only energy yielding process during the prolonged anoxia so much the more as the lactic acid is excreted from these animals (BRAND, 1946; GODDARD and MARTIN, 1966). Thus the glycolysis being anyway less economical would cause an intense loss of substrates restricting to a great extent the capacity of living of the animals. The same situation would come about by the mechanism described by COLLIP (1920), DUGAL (1939), DOTTERWEICH and ELSSNER (1935), neutralizing the organic acids produced in anaerobic glycolysis by means of calcium carbonate liberated from the calcareous shells. The possible significance of

such processes, cannot be excluded, however, our experiments revealed that the metabolism of isolated ganglia of *Anodonta* under anaerobic conditions is basically not of glycolytic character (Zs.-Nagy, 1971).

The possibility that the energy requirement of the animal is extremely restricted during the anaerobiosis may also arise. It is out of question that the rhythm of activity of bivalves is changed in anoxia (Salánki, 1965; 1966). Although there are no concrete measurements, it is quite clear that it is just the nervous system that needs a certain energetical level even in this stage. It has been indicated also by our present investigations that the energy yielding mechanisms of the nervous system remain intact as long as the animal is alive.

A further possibility would be the supposition of an oxygen-storing system being able to accumulate the suitable amount of oxygen for long periods of time of anoxia. According to our present knowledge *Anodonta cygnea* do not have such systems thus it cannot be the basis of the significant anoxibiotic tolerance. Experimental results also contradict this assumption (Zs.-Nagy, 1971).

The changes of cytosomes observed in anoxia indicate even in themselves that the cytosomes play an important role in this respect (Baranyi and Salánki, 1967; Zs.-Nagy, 1969; Zs.-Nagy and Csukás, 1969). Their participation in energy yielding processes could also be evidenced in present investigations using the Sr^{++} accumulation method of Kerpel-Fronius and Hajós (1970) adapted for the nervous tissue of bivalves (Kerpel-Fronius and Zs.-Nagy, 1971). Since the cytosomal Sr^{++}-accumulation takes place only at intact terminal oxidation and oxidative phosphorylation mechanisms, one has to accept that even the cytosomal energy production is basically of oxidative character.

Under normal circumstances the mitochondrial energy production is preponderant in the neurons and the cytosomes show only little activity. In anaerobiosis the situation is reversed: the Sr^{++} accumulation of mitochondria is significantly less intense, however, it persists whereas the cytosomal activity strongly increases. For explanation of this phenomenon the assumption seems to be obvious that some cytosomal compounds function as electronacceptors being able to substitute the molecular oxygen in anoxia. Experimental evidences have been described in another paper (Zs.-Nagy, 1971) that the lipochrome pigment of cytosomes displays considerably electron-acceptor capacity.

The basic importance of cytosomal energy production in anaerobiosis is also underlined by the fact that its stopping causes very soon the destruction of the animals.

However, the case of *Aplysia* indicates that apart from the presence of cytosomal pigment there can be other factors limiting the anoxibiotic tolerance, too. Considering the high degree of motility of *Aplysia*, it is obvious that it is generally not exposed to prolonged anoxia. One can also suppose that in our experiments their metabolic products could be accumulated in the closed water, being immediately diluted in its natural environment.

Many details of the cytosomal energy production are unknown. We do not known what part of the lipochrome pigment is actually the electron-acceptor. It seems likely that carotenoids form an interposed link in the chain when considering their ability to transport electrons (Pullman and Pullman,

1963), and some unsaturated fatty acids of phospholipids serve as definitive electronacceptor. Anyway the above related working hypothesis may perhaps offer a basis for clearing up the complex relations of the anaerobiosis.

Summary

Investigating *Anodonta cygnea, Mytilus galloprovincialis, Venerupis decussata, Tapes decussatus, Pecten Jacobaeus, Murex trunculus, Aplysia limacina* and *Octopus vulgaris* it has been established that those species are able to endure prolonged anoxia the nervous system and other tissues of which contain large amounts of lipochrome pigment localized in the cytosomes. Using an electronmicroscopic histochemical method it was shown that the energy production of oxidative character persists in mitochondria and mainly in the cytosomes of the nerve cells even during the prolonged anoxia. As far as the mechanism of this process is concerned, it seems likely that the lipochrome pigment substitutes the molecular oxygen as electronacceptor. On the basis of this hypothesis one can explain many problems of the anaerobiosis.

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ÖSSZEFÜGGÉS PUHATESTŰEK IDEGSEJTJEINEK PIGMENTÁCIÓJA ÉS ENERGIAFÜGGŐ Sr^{++}-AKKUMULÁCIÓJA KÖZÖTT ANAEROB ÁLLAPOTBAN

Zs.-Nagy Imre

Összefoglalás

Anodonta cygnea, Mytilus galloprovincialis, Venerupis decussata, Tapes decussatus, Pecten Jacobaeus, Murex trunculus, Aplysia limacina és *Octopus vulgaris* vizsgálata alapján megállapítást nyert, hogy azok a fajok képesek igen hosszú ideig elviselni az oxigénhiányt, amelyek az idegrendszere és más szövetei jelentős mennyiségű lipochrom pigmentet tartalmaznak, amely a citoszónákra lokalizálódik. Elektronmikroszkóposan hisztokémiai módszerrel kimutatható, hogy anoxiás állapotban is fennáll az idegsejtek mitochondriumaiban és főleg citoszómáiban az oxidatív jellegű energiatermelés. A folyamat mechanizmusára nézve az a valószínű, hogy a lipochrom pigment helyettesíti a molekuláris oxigént, mint elektron-akceptort. E hipotézis lehetőséget nyújt az anaerobiózis több kérdésének megválaszolására.