ANNAL. BIOL. TIHANY	32	29–36	HUNGARIA 1965

INFLUENCE OF METABOLIC INHIBITORS ON LIGHT SENSITIZED CONTRACTIONS BY ERYTHROSIN IN MUSSEL'S LARVAE (*ANODONTA CYGNEA* L.)

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Received: March 15th. 1965.

It has been established under previous investigations, that the larvae of fresh-water clam (*Anodonta cygnea* L.) display motor activity in the presence of certain xanthene dyes — eosin, erythrosin, rose bengal, phloxine — if exposed to visible light. Light sensitization is more effective if the fluorescence energy yield of the sensitor is smaller (LÁBOS 1965 a). Monovalent electron-donors, as serotonin and chlorpromazine may influence considerably the reaction (LÁBOS 1965 b). It has been demonstrated in the case of chlorpromazine that this agent may react also with rose bengal, erythrosin and in a lesser degree with eosin and may presumably form with them complexes showing at a greater wavelength maximum absorption in the visible spectrum than the stains themselves (LÁBOS 1965 c). The fact that light response is effectively eliminated by cysteine indicates that in the course of light sensitization phenomena analogous to processes occurring in high energy radiation effects are involved (LÁBOS 1965 b).

In this present paper the effect of some substances on contraction sensitized to light by erythrosin is investigated. The inhibitory influence of these agents in the concentrations applied at various stages of the metabolism of the living system is known (HOCHSTER — QUASTEL 1963). In this work it was not intended to perform quantitative studies of kinetic character.

It seems obvious from previous investigations that light reaction is founded also on endogenous energy basis (LÁBOS 1965 b), though the absorbed and in the form of fluorescence nonemitted light energy is in direct connection with the reaction. For this reason our discussion is connected with the energy production of the affected metabolic processes. Of course the metabolic inhibitors may interfere also in more primary physical and chemical processes.

Methods

The experiments were conducted on mature glochidia of *Anodonta cygnea* L. in winter months. 100 glochidia were used for investigating the effect of the inhibitors each. The influence of the dye-solution used as control was also examined on 100 glochidia under equal conditions. The larvae were divided into groups of 25 and were put in twice filtrated Balaton-water. After control examinations the water was syphoned down and the investigated dye solution or the mixture of the dye-solution and the inhibitor was poured on the larvae in exchange. In some cases the animals were preincubated in the inhibitory

agent. Preincubation did not cause essential changes if subsequent to it a mixture of inhibitor and dye-sensitor was applied. Smaller effect was produced if only dye-solution was applied after preincubation. The concentration of control stain solution and the illumination was adjusted that approximately 100% of the larvae should enter into the condition of lasting tonic contraction within 10—20 minutes (25 μg/ml or 28.5 μM erythrosin).

Illumination was supplied by 15 watts tungsten incandescent lamp from 150 mm distance and at an incidence of 50°. The agents used were: KCN, NaF, NaN_3, thiosemicarbazide (TSC), monoiodo acetic acid, hydroxilamine, erythrosin B (525 mμ), 2,4-dinitrophenol (DNP).

Results

1. *The influence of monoiodo acetic acid and NaF*

Light reaction observable in the presence of erythrosin was not influenced by the applied 100 μg/ml (0.54 and 2.4 mM respectively) concentration of monoiodo acetic acid and NaF. In the number of rhythmic concentrations there was no deviation until the end of the reaction neither in the case of con-

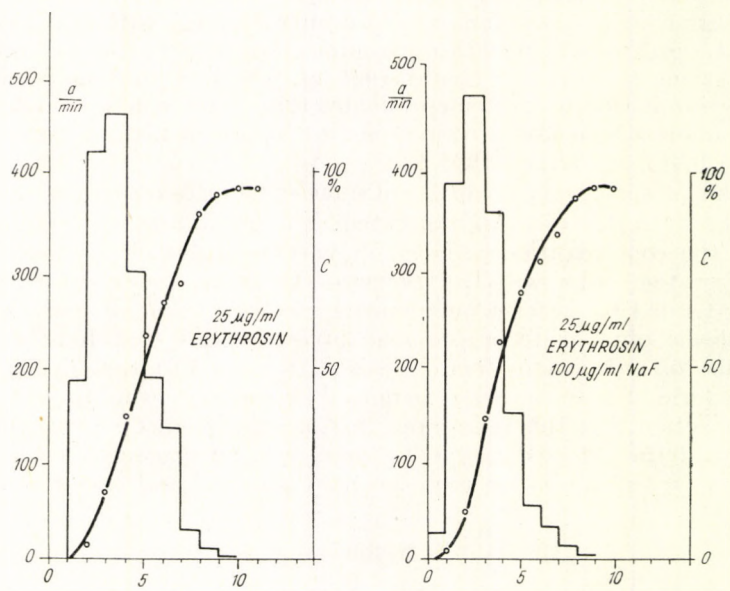

Figure 1. Effect of NaF on contraction sensitized by erythrosin. Abscissa: time in minutes. Ordinate on the left: frequency of rhythmic activity (*a*/min), on the right: the percentage of tonically contracted glochidia (*c*) in case of control and inhibitor. Rhythmic activity and closures are illustrated graphically by columns and lines respectively

1. ábra. A NaF hatása az erythrosinnal fényérzékenyített kontrakcióra. Abszcissza: idő percekben. Ordináta baloldalt: a ritmikus aktivitás frekvenciája (*a*/min), jobboldalt: a tónusos zárásban levő glochidiumok %-os aránya (*c*) kontrol és gátlószeres kísérlet. A ritmikus aktivitást oszlop-, a zárást vonaldiagramm ábrázolja

trol nor in the examinations with the inhibitor. Tonic reaction took place also unchanged. The influence of NaF is illustrated in *figure 1.* There was no inhibition after preincubation.

2. *The influence of hydroxilamine*

In the presence of 1 mM hydroxilamine the light sensitizing influence of erythrosin is tonic. This manifests itself in the decrease in number of rhythmic acts *(Fig. 2)* preceding tonic contraction occurring a little earlier than

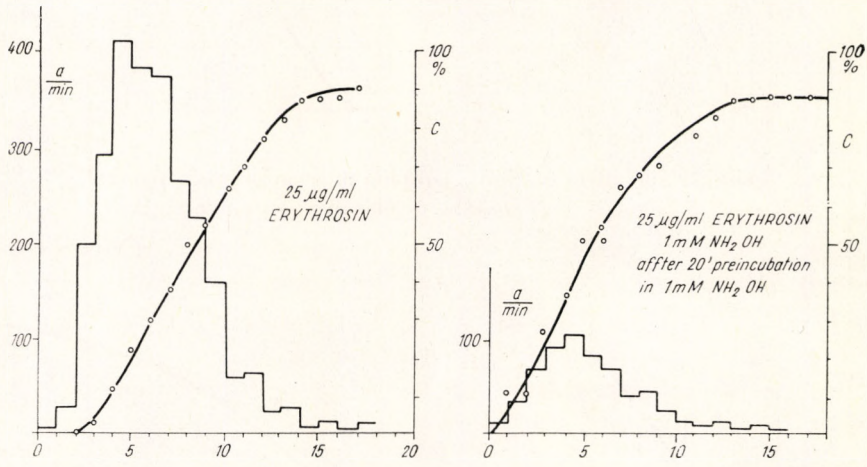

Figure 2. The effect of hydroxilamine. Graphic illustration as in *figure 1.*
2. ábra. A hidroxilamin hatása. Jelölése mint az *1. ábrán*

usual. For example 2500 contractions were produced by 25 µg/ml erythrosin under the given conditions, whereas under the same circumstances the number of contractions was about 500 when applying 1 mM hydroxilamine during a 20 minutes long preincubation and subsequently together with the dye. As a result of this the frequency of rhythmic contractions decreased considerably.

3. *The influence of* NaN_3

Only little changes took place in tonic light reaction on the combined application of 100 µg/ml (1.53 mM) NaN_3 and 25 µg/ml erythrosin. The number of rhythmic contractions, however, decreased considerably. The effect produced by NaN_3 is presented in *figure 3.*

4. *The effect of thiosemicarbazide*

It is visible in *figure 4* that a 20 minutes long incubation in 1 mM thiosemicarbazide and a subsequent application of this solution together with 25 µg/ml erythrosin resulted in a decrease in the rate of tonic closures. The number of rhythmic contractions taking place until the ultimate closure of the larvae is also only 1/5th of that in the control group. In the presence of 1 mM

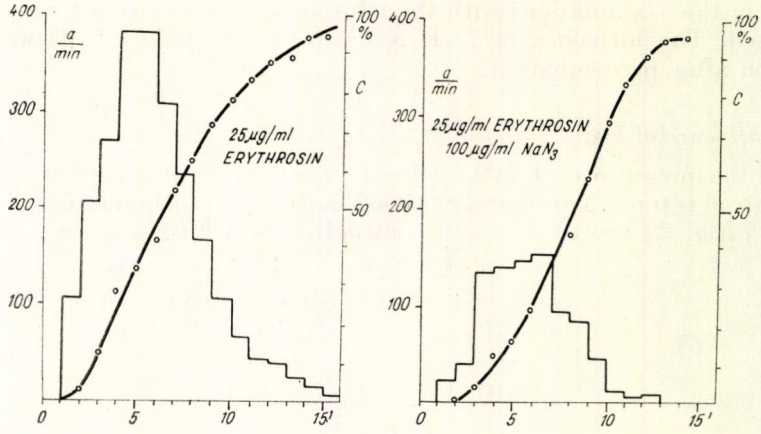

Figure 3. The effect of NaN_3. Graphic illustration as in *figure 1.*
3. ábra. A NaN_3 hatása. Jelölés mint az *1. ábrán*

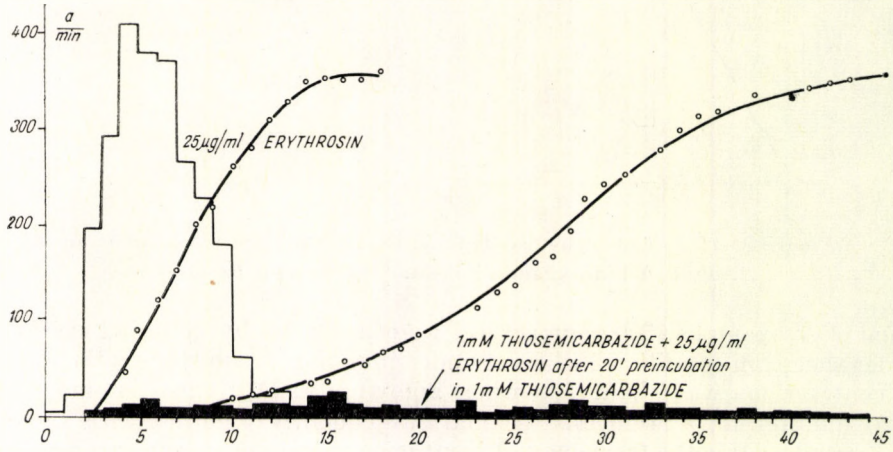

Figure 4. The effect of thiosemicarbazide. Graphic illustration same as in *figure 1.* Control experiment and experiment with the inhibitor is illustrated in the same system of coordinates

4. ábra. A tioszemicarbazid hatása. Jelölése mint az *1. ábrán.* A kontrol és gátlószeres kísérlet ugyanazon koordinátarendszerben

thiosemicarbazide a three times longer period was needed for the tonic contraction of 50 per cent of larvae than in the absence of this agent (8 and 27 minutes respectively). The maximum frequency of rhythmic activity is the most markedly inhibited parameter.

5. *The effect of KCN*

On the combined application of 100 µg/ml KCN (1.51 mM) and 25 µg/ml erythrosin rhythmic activity becomes inhibited and tonic reaction takes place a little faster. The same mixture influences light reaction in a similar way

after a 10 minutes long preincubation in 100 μg/ml KCN with the difference that tonic reaction becomes also inhibited. The inhibitory effects produced by KCN on its application in two different ways is illustrated in *figure 5.*

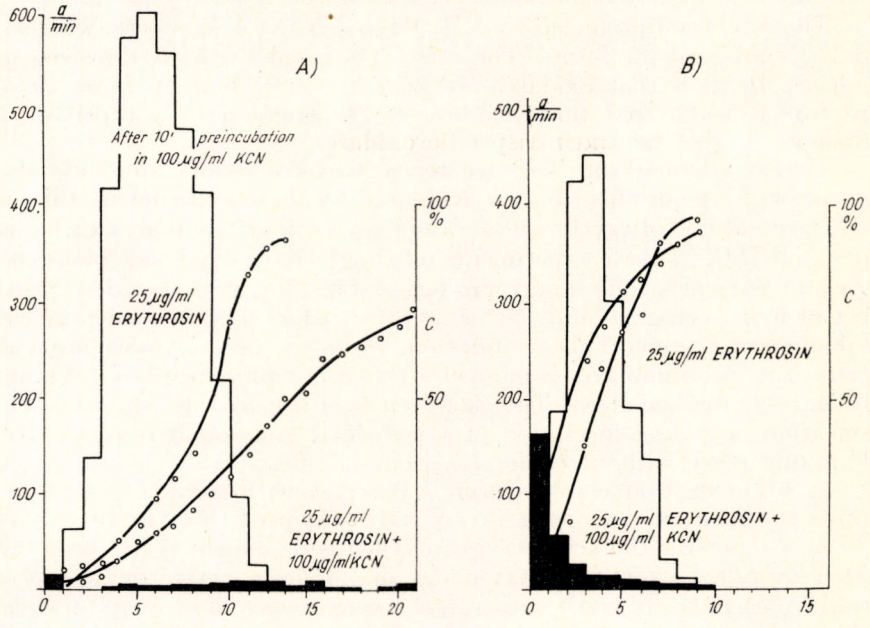

Figure 5. The effect of KCN. Illustration as in *figure 1. A* — the effect of the mixture of dye and inhibitor after preincubation in KCN, *B* — using the mixture of KCN and the dye immediately the effect of KCN itself becomes manifested

5. ábra. A KCN hatása. Jelölés mint az *1. ábrán. A* — előzetes KCN-inkubáció után a festék és gátlószer keverékének hatása, *B* — azonnal a KCN és festék keverékét alkalmazva a KCN saját hatása is érvényesül.

6. The effect of dinitrophenol (2,4-DNP)

DNP is ineffective in 25 μg/ml (0.13 mM) concentration. It does not induce reaction when used by itself in the above concentration. The influence of this substance when in combination with erythrosin was measured after 0—120 minutes long preincubation in DNP. After two hours long preincubation there was a small increase in the tonicity of light reaction. It was not possible to use this agent in higher concentrations because it would have resulted in considerable pH changes and because light responses would be covered up by ion-effects in case if buffers are used.

Discussion

On basis of the accepted point of attack of the metabolic inhibitors investigated definite conclusions may be drawn on the relationship between dye-sensitor and biological energy source. Under the conditions applied NaF and monoiodo acetic acid may paralyse glycolysis dependent processes. Light-

sensitized reaction is considered glycolysis-independent because it is ineffective. 2,4-DNP inhibits oxidative ATP synthesis by separating oxidation and phosphorylation. Because light response is not suspended by 0.1 mM 2,4-DNP after long incubation and in the presence of erythrosin it is suggested that it is independent of oxidative phosphorylation and of its energy production.

The inhibition produced by NH_2OH and NaN_3 is mediocre, whereas TSC and KCN are real inhibitors. The effect produced by KCN deserves special emphasis. In view that oxidases are primarily inhibited by these metabolic inhibitors it is inferred that the process investigated is susceptible to disturbancies in specific and nonspecific oxidases.

Hydroxylamine and TSC are ready to form oxim and semicarbazone together with oxo-groups. As it is evidenced by these experiments the oxygen on the aromatic C of erythrosin does not give colour reaction with hydroxylamine and TSC. In these experiments one might have expected colour changes in case of semicarbazone- and oxim-formation. Thus it is considered that the effect of hydroxylamine and TSC is an effect which is produced primarily on the biological system. This assumption, however, has not absolute validity because TSC as an inhibitor is more effective if it is present, than if it is applied only during preincubation. This fact refers either to the inhibition of dye-permeation or to the formation of a molecular compound between dye and TSC taking place without colour reaction.

It is known that cianide forms a complex with trivalent iron and thus inhibits the reduction of oxidized respiratory ferment (KEILIN 1929). Accordingly in the process the activation of oxygen is damaged. It has been demonstrated by SCHENCK (1948) that under illumination active oxygen is transported by eosin to different substrates. Excited eosin is of long duration and in triplet state (OSTER, ADELMAN 1955) it reacts with oxygen and thereafter with the substrate, and in damped, normal state is released again. Erythrosin is capable of producing the same photosensitizing effect on glochidia as eosin but at a concentration which is of about 2 times smaller order than in the case of eosin (LÁBOS 1965 a). Erythrosin was used in these investigations because it is more effective in lower concentrations. It is assumed that in the presence of oxygen this substance may catalyse similar photooxidation as eosin. Because lightsensitized contraction is susceptible to cyanide but is not inhibited by DNP it is assumable that the energy producing processes in question become inhibited without the participation of cytochromoxidase and as a result of this the photocatalysed contraction of mussels will not be accomplished. Light reaction is thus independent of the control of oxidative phosphorylation but not of terminal oxidation.

It has been demonstrated by OSTER et al. (1959) that only dyes that may enter into photoreduced condition are capable of catalyzing photooxidation. The fact that cytochromeoxidase and the dye-sensitor are both capable, independent of each other of oxygen-activation and substrate-oxidation, and because light reaction is inhibited by cyanide it is inferred that the processes may undisputably be interlinked in lightsensitized contraction. Certain xanthen dyes are able to activate oxygen in the presence of light, the oxygen forming peroxides with the substrate (SCHENCK 1948). It is not out of question that in the present case one of the cytochromes itself is the substrate of the dye.

It is assumed that cyanide does not inhibit dye-sensitized photooxidation in vitro, because this substance blocks catalizators containing heavy

metals and the dye does not contain metals. It is also known that cyanide does not form complexes with cytochromes (KEILIN 1929). Accordingly, the inhibition by cyanide is primarily attributable to the inhibition of cytochrome-oxidase.

The brutto process is illustrable by the following schema:

$$E + C + S + O_2 \xrightarrow{h\nu} E + C + SO_2$$

where E = erythrosin, C = cytochromeoxidase, S = substrate. On basis of data thus far obtained it is difficult to locate the intermolecular pathways of electrons. Cyanide inhibits the transfer of the cytochrome-system or that of the electron of the stain. In this way cytochromeoxidase becomes omitted in oxidized form from the process. These present investigations do not allow of statement as to the number of exchange-electrons, nevertheless the close connection with terminal oxidation refers to the transfer of one electron.

Summary

Light sensitized rhythmic and tonic contractions by erythrosin B in glochidia of freshwater clam *(Anodonta cygnea* L.) was investigated in the presence of various metabolic inhibitors (10^{-4}—10^{-3} M).

On basis of examinations it is established that the process is not sensitive to monoiodo acetic acid, NaF, 2,4-DNP. It is considerably more sensitive to thiosemicarbazide, NH_4OH and NaN_3. It is most sensitive to cyanide.

The findings show that the reaction is independent of glycolysis. Oxidative phosphorylation cannot play an important role either, whereas various oxidases are involved in the reaction.

It may be concluded primarily on basis of the susceptibility to cyanide that light reaction is related to terminal oxidation and in particular to cytochromeoxidation. It is assumable that the photoreduced dye joins the process of terminal oxidation by means of monovalent electron-transfer.

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ANYAGCSEREGÁTLÓ SZEREK HATÁSA ERYTHROSINNAL FÉNYÉRZÉKENYITETT KONTRAKCIÓRA KAGYLÓLÁRVÁKON

Összefoglalás

Lábos Elemér

Édesvízi kagyló *(Anodonta cygnea* L.) glochidiumainak erythrosin B-vel fényérzékenyített ritmikus és tónusos kontrakcióját vizsgáltuk különböző anyagcseregátló szerek (10^{-4} — 10^{-3} M) jelenlétében.

A vizsgálatok alapján megállapítható, hogy a folyamat monojódecetsavra, NaF-ra, 2,4 DNP-ra nem érzékeny. Lényegesen érzékenyebb tioszemikarbazidra, NH_2OH és NaN_3-ra. Kiemelkedően érzékeny cianidra.

Fentiek alapján a reakciót a glikolízistól függetlennek tekintjük. Az oxidatív foszforiláció hasonlóan nem játszhat fontos szerepet, viszont a különböző oxidázok a reakcióban résztvesznek.

Elsősorban a cianidérzékenység alapján mondható, hogy a fényreakció a terminalis oxidációval nevezetesen a citokromoxidázéval kapcsolatos. Feltehető, hogy a fotoredukált festék monovalens elektronátadás révén kapcsolódik be a terminális oxidáció folyamatába.

ВЛИЯНИЕ ИНГИБИТОРОВ ОБМЕНА ВЕЩЕСТВ НА ФОТОСЕНСИБИЛИЗИРОВАННОЕ ЭРИТРОЗИНОМ СОКРАЩЕНИЕ ГЛОХИДИЕВ БЕЗЗУБКИ

Э. Лабош

Была изучена фотосенсибилизированная при помощи эритрозина-В ритмическое и тоническое сокращение глохидиев беззубки (Anodonta cygnea L.) в присутствии разных ингибиторов обмена веществ в концентрации 10^{-4}—10^{-3} М.

На основе полученных данных было установлено, что это влияние не изменяется под влиянием монойодуксусной кислоты, NaF, 2,4—DNP, гораздо лучше реагирует на тиосемикарбазид, NH_2OH и NaN_3 и особенно чувствительно к цианиду.

На основе высшеизложенного реакция считается независимой от гликолиза. Окислительное фосфорилирование в этой реакции тоже не может играть важной роли, но различные оксидазы могут принимать участие в ней.

На основе чувствительности к цианиду можно сказать, что свето-реакция связана с терминальным окислением, а именно с цитокромоксидазой. Можно предположить, что фото-восстановленная краска включается в терминальное окисление при передачи моновалентных электронов.