PRO EXPERIMENTIS

Some remarks concerning staining methods for neurosecretory products in insects[1]

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Summary. Insect neurosecretory cells show great diversity and respond differently to reportedly homologous stains, including Victoria blue, chrome hematoxylin-phloxin and paraldehyde fuchsin. Apart from this diversity, it is shown on a phasmid *Clitumnus* and a bug *Roscius*, that the mode of application of technics have a great influence upon the staining properties of the cells.

It has been recently reported that the pars intercerebralis (p.i.) in insects includes 2 types of A neurosecretory cells (NSC), types A_1 and A_2. The 1st, more easily stained, contains strong acids, while the 2nd, which is less easily stained, contains weak acids[2]. The p.i. also includes Cr NSC, the presence of which can be revealed by azan after Helly fixation[2], as well as other NSC categories such as the B and D NSC of the Heteroptera, and the C NSC of the Orthoptera.

To this great diversity of NSC types corresponds an equally great diversity of functions. As shown by Gersch et al.[3–5], and others, the insects possess several neurohormones which regulate the majority of their physiological processes.

Materials and methods. For this study a heteropteran *Roscius* and a phasmopteran *Clitumnus extradentatus* were used. Following fixation with Bouin or Helly fixatives, various stains (Victoria blue, chrome hematoxylin-phloxin, paraldehyde fuchsin, and azan) were applied using the double or triple staining technique. This technique consists of the placing of adjacent sections on different slides which are then treated with different stains; this allows the determination with certainty of the affinity of a particular cell for several different treatments. Enzymatic destruction has also been carried out, as have histochemical methods such as bromophenol blue and RSR.

Results. Differential reactivity of neurosecretory products depending upon the stain used. It is generally considered that Victoria blue (VB), chrome hematoxylin (CH) and paraldehyde fuchsin (PF) are homologous stains. Nevertheless, in numerous cases, VB colors only the A_1 MNC in the p.i., whereas A_1 and A_2 MNC are stained by CH and PF. PF itself is not entirely homologous with CH. This is not surprising since in addition to its affinity for SS or SH groups, it binds to aldehyde groups freed by oxidation[6].

Some NS products which are not stained by CH are well colored by PF. This is the case, in the LNC (lateral protocerebral NSC) of various species, in some TNC (tritocerebral NSC), and in the glandular cells in the Phasmids corpora cardiaca.

Within a single cell, 2 different types of secretion with different affinities has been recognized. In the A NSC of the p.i. and the ventral nerve cord of Phasmids, secretions appear essentially like voluminous round grains which are colored by HC and PF; we also see fine granules which are disposed in a crescent-like manner, in contact with the Golgi apparatus. These granules, which represent the neurosecretory product at the first phase in its manufacture, are not colored by HC but retain FP. The difference between grains and granules is confirmed by the use of bromophenol blue and RSR. These 2 techniques, employed respectively for the demonstration of proteins and of SS or SH groups, stain the granules but not the grains. In addition, proteolytic enzymes destroy only the granules.

Influence of procedure on results seen. The type of fixative, and the exact modality for the application of stains, have a great influence upon the affinity of NSC. Cr NSC are not detectable except after fixation with Helly. A method such as CHP includes several steps, the duration of which

Color of neurosecretory products as a function of the staining procedure, pars intercerebralis, *Roscius*

a) Chrome hematoxylin-phloxin

Procedure			Color of neurosecretory products				
Oxidation	Chrome hematoxylin	Phloxin	Black	Grey	Pink	Slight pink	Colourless
Slight: 1 min	Slight: 1 min	Moderate: 5 min		A_1	B_1B_2		A_2D Cr
	Strong: 5 min	Strong: 10 min	A_1	A_1	B_1B_2	Cr	D
Moderate: 3 min	Moderate: 3 min	Moderate: 5 min	A_1	A_2D	B_1	B_2	Cr
Strong: 5 min	Moderate: 3 min	Moderate: 5 min	A_1A_2	D		B_1	B_2Cr
	Strong: 5 min	Strong: 10 min	A_1A_2D			B_1B_2	Cr

b) Azan

Procedure		Color of neurosecretory products				
Azocarmin differentiation	Heidenhain blue	Red	Orange red	Orange	Purple or violet	Blue	Colourless
Slight: 5 min	Slight: 1 min	$A_1B_1B_2$Cr	A_2				
	Strong: 30 min	B_1 Cr	A_1	A_2	B_2		
Moderate: 10 min	Slight: 1 min	B_1 Cr	A_1	A_2	B_2		
	Moderate: 5 min	Cr	A_1	A_2	B_1B_2		
	Strong: 30 min	Cr		A_1A_2	B_1B_2		
Strong: 20 min	Moderate: 5 min	Cr		A_1A_2	B_1	B_2	

The durations mentioned are given as illustration. They are not absolute values.

modifies the final result: oxidation, staining with CH, differentiation, phloxinic staining, and the differentiation of this latter.

Thus, for the same tissue, by varying time between 1 and 5 min, we may obtain clearly different results (table). If the duration of oxidation is short (1 min), the affinity of type A secretion for CH is slight, and if the actual staining step is not prolonged certain cells remain unstained (A_2NSC). If oxidation is excessive (5 min), the affinity for phloxin of type B substances will decrease and disappear. An overly pronounced phloxin coloration, after medium or weak oxidation will, on the other hand, yield a uniform color for a large number of cells, some of which are not neurosecretory. Depending upon the mode of application of technique, there is variation in the number of B NSC, and demonstration of 1 or 2 different categories in this population.

For azan, the situation is similar. Results depend upon azocarmin differentiation, the intensity of staining by the orange G-blue aniline mixture, constituting Heidenhain blue, and the differentiation of this stain (see table). The different shades of coloration of A_1 and A_2 NSC, as well as the 2 categories of B NSC, depend upon the exact application of the method.

While a time standard may appear desirable, it will not resolve these problems since, depending upon species, results vary slightly, due either to the permeability of the neurilemma, or the pH of the medium.

Concluding remarks. The complexity of the neurosecretory system of insects, the differences among various species and the variations in applying staining techniques lead, in some cases, to conflicting results. Our experiments on *Clitumnus* and *Roscius* suggest adoption of the following methods for the determination of the various types of neurosecretory cells: use of several techniques including azan, HC and FP in double or triple staining, and application of each technique not according to a single method, but using a range of variants.

1 Dedicated to Professor Dr M. Gersch's on the occasion of his 70th birthday.
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Soybean photosynthesis: simultaneous $^{14}CO_2$ and O_2 estimates

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Summary. A method of estimating the photosynthetic rate of soybean leaves using an oxygen electrode is presented. The procedure is rapid, requires small samples and compares favourably with estimates by other techniques. Light saturation occurs at 1200 $\mu E \cdot m^{-2} \cdot sec^{-1}$. The apparent K_m for HCO_3^- is 3.2 mM at pH 7.6.

This communication demonstrates that net photosynthesis of soybean leaf slices measured in an oxygen electrode is the same as that reported for intact leaves using an infrared gas analyzer. Furthermore, the photosynthetic responses to light intensity and carbon dioxide concentration are consistent with those reported in the literature. The rate of O_2 evolution by leaf slices is compared directly with the rate of carbon fixation, measured with $^{14}CO_2$.

Soybeans (*Glycine max* (L.) Merrill) cv. Wayne were grown in solution culture (pH 5.5) under glasshouse conditions (24–19 °C). All experiments were performed on the 2nd expanded trifoliate leaf. Leaf slices (1 mm wide, 10 mm long) were hand cut, rinsed and kept in 0.5 mM $CaSO_4$ (pH 7.6) in the dark, then illuminated for 1 h at 200 $\mu E \cdot m^{-2} \cdot sec^{-1}$ before use. Oxygen exchange was measured at 25 °C with a Clark-type oxygen electrode. A 20–30 mg subsample of leaf slices from a single leaflet was added to 4 ml 20 mM HEPES (N-2-hydroxyethylpiperarzine-N[1]-2-ethanesulphonic acid), pH 7.6, in the incubation chamber. The solution was partly de-oxygenated with N_2 to 60% of its saturated soluble O_2 content and the CO_2 concentration obtained by adding $KHCO_3$. Dark respiration was measured for 8 min followed by net photosynthesis for 5 min. For $^{14}CO_2$ fixation, 2 μCi $NaH^{14}CO_3$ was injected into the incubation solution (sp. act., 21.8 mCi/mole HCO_3^-). After illumination for 35 sec, the slices were rinsed in fresh incubation solution, blotted dry, killed, decolorized with benzoyl peroxide and counted for radioactivity in a liquid scintillation counter. Controls were performed to account for adsorption or dark fixation.

CO_2 response: Under the prevailing conditions of pH 7.6 and 2000 $\mu E \cdot m^{-2} \cdot sec^{-1}$ light intensity, there was no significant increase in the rate of oxygen evolution by leaf slices from 8 mM to 35 mM HCO_3^-. Thus photosynthesis was saturated by 8 mM HCO_3^- (figure). The concentration which gave half-maximal rates (apparent K_m) was calculated from the regression equation as 3.2 mM. The CO_2 concentration in solution was not significantly depleted as the rate of O_2 evolution by the slices during exposure to light was always linear. The calculated free CO_2 concentration[2] at saturation was 450 μM and the apparent K_m was 180 μM; it is the free CO_2 rather than the HCO_3^- which is the chemical species taken up by leaf slices[3,4].

Light response: Light saturation occurred at 1200 $\mu E \cdot m^{-2} \cdot sec^{-1}$, at 20 mM HCO_3^-, when the photosynthetic rate was expressed in terms of chlorophyll content or fresh weight (figure). Photosynthesis, determined by infrared gas analysis on individual soybean leaves at 25 °C, is light

Net photosynthetic and respiration rates for soybean leaf slices (mean ± SE)

Units	Photosynthesis (n = 9)	Respiration (n = 11)
nmoles $CO_2 \cdot g\ (f.wt)^{-1} sec^{-1}$	77 ± 9*	
nmoles $O_2 \cdot g\ (f.wt)^{-1} sec^{-1}$	67 ± 3**	6.5 ± 0.4**
ng $CO_2 \cdot cm^{-2} sec^{-1}$	49 ± 6*	

* Determined as $^{14}CO_2$ fixation. ** Determined as O_2 evolution (consumption). f.wt, fresh weight.