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VOLTAGE-CLAMP AND SINGLE CHANNEL ANALYSIS OF Pb^{2+}-INDUCED CURRENT IN ISOLATED SNAIL NEURONS*

J. GYŐRI, O.N. OSIPENKO[1] and T. KISS

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(Accepted: 1991-08-30)

Pb-activated outward current was investigated in intracellularly perfused, isolated snail neurons. Pb-ions induced non-inactivating but reversible current (I_{Pb}). The I_{Pb} showed concentration dependence. The I-V curve was linear with negative slope and the I_{Pb} proved to be Na-dependent.

Keywords: Snail neuron - heavy metal - lead - intracellular perfusion - lead-activated conductance

Heavy metals permanently accumulate in living organisms and may produce neurological disorders in man and animals. It is difficult to explain mechanisms of their neurotoxic action, since these metals are capable of interacting with many biological ligands on the membrane surfaces and produce a variety of physiological processes. Toxic heavy metals combining with ligands present in all proteins can influence the permeability properties of the membrane. Beside their action on voltage dependent Ca-channels and transmitter release, heavy metals (Pb^{2+}, Cu^{2+}, Hg^{2+}, Ag^{+}) activate a steady-state current in different vertebrate and invertebrate neurons /1-5/. Pb^{2+} induced two-types of currents in snail (Helix pomatia L.) neurons, an inward and an outward one /4/. The present study provides further description of the Pb^{2+}-induced outward current (I_{Pb}) in dialysed

*Presented during the ISIN Symposium "Neurobiology of Invertebrates", June 23-28, 1991, Tihany, Hungary

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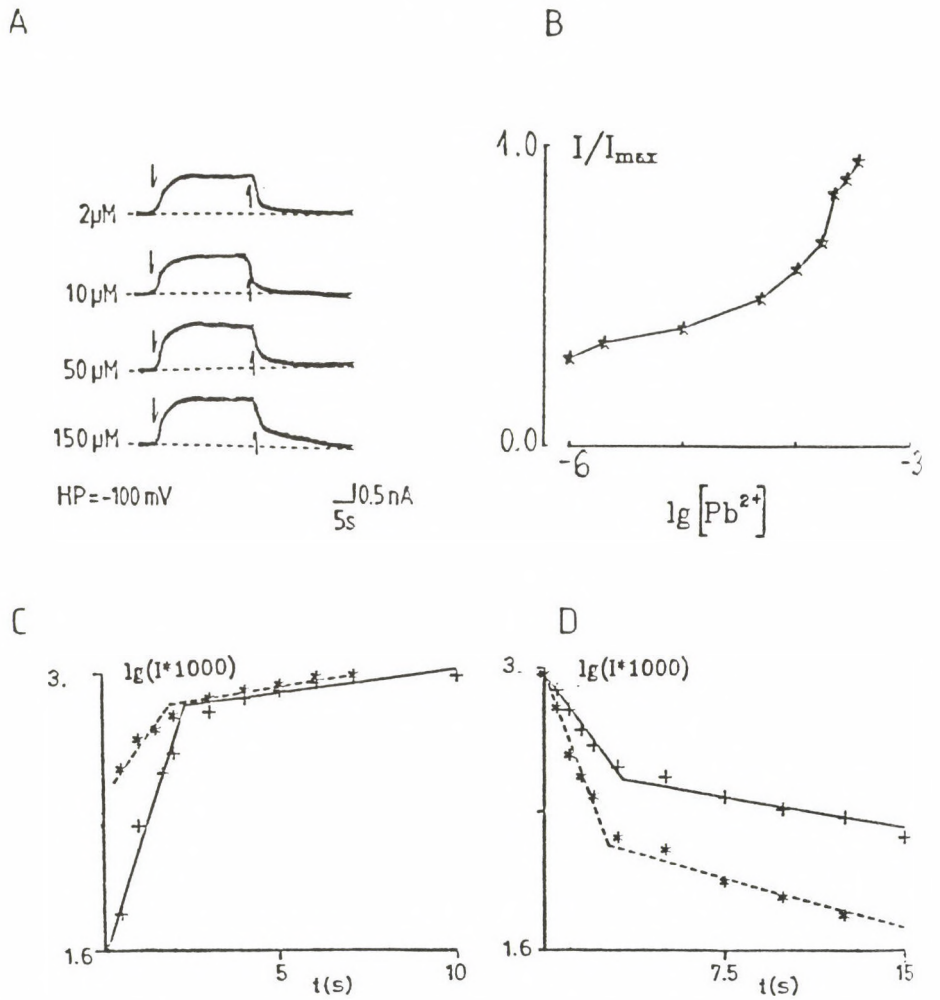

Fig. 1. Concentration dependence of Pb-activated currents (Kd = 63 μM, **A-B**, arrows indicated the application of Pb^{2+}) and the activation (**C**) and relaxation (**D**) kinetics of currents at two Pb^{2+} concentrations (stars: 2 μM, crosses: 150 μM). The activation (τ_{fast} = 1.0 s, τ_{slow} = 19 s at $/Pb^{2+}/_o$ = 2 μM; τ_{fast} = 1.2 s, τ_{slow} = 21 s at $/Pb^{2+}/_o$ = 150 μM) and the inactivation (τ_{fast} = 0.7 s, τ_{slow} = 10.5 s at $/Pb^{2+}/_o$ = 2 μM; τ_{fast} = 1.1 s, τ_{slow} = = 15.4 s at $/Pb^{2+}/_o$ = 150 μM) phases, as shown on the semilogarithmic plots consisted of two components at both Pb^{2+} concentrations

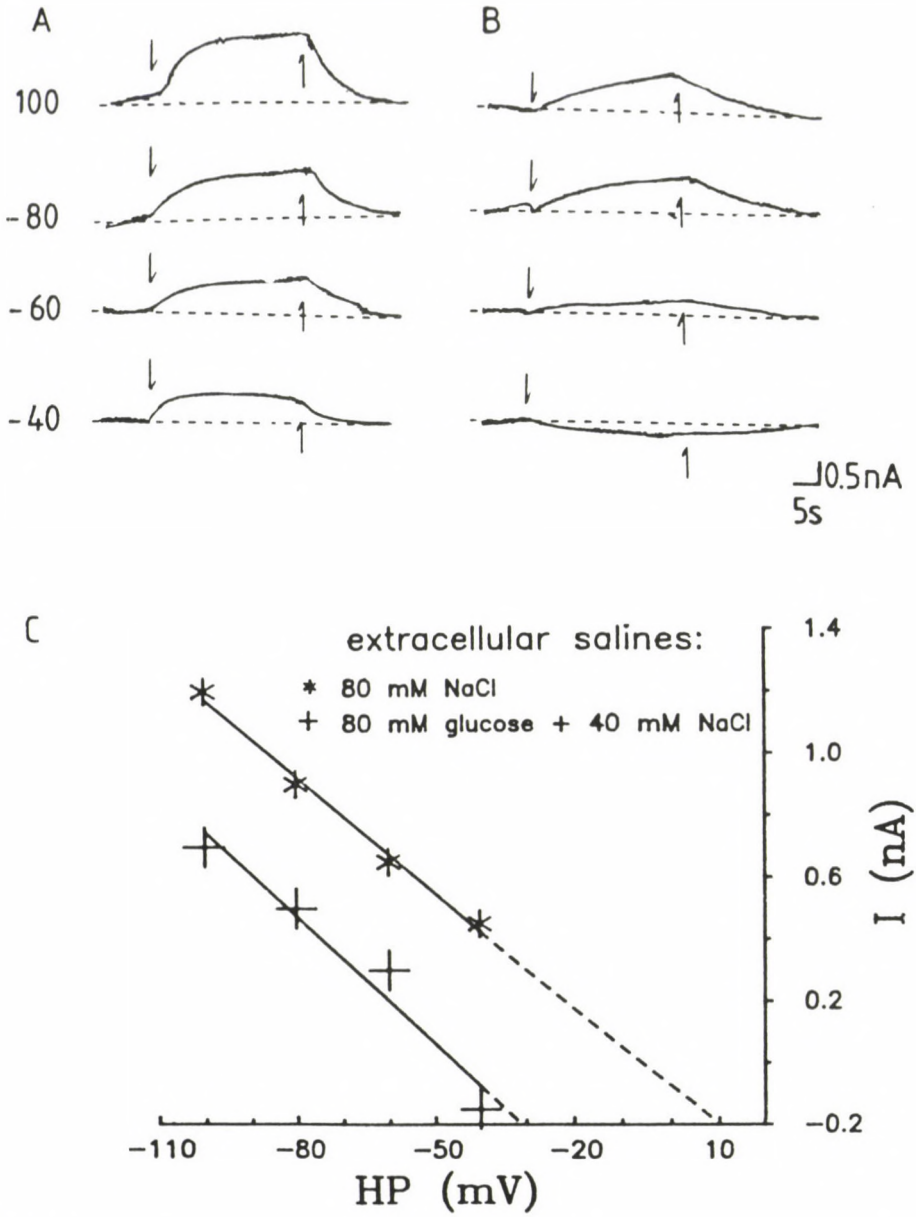

Fig. 2. Pb^{2+}-activated currents at different holding potentials (**A**) and the current-voltage relationships in control saline (**C**). Removing 50% of NaCl from the extracellular saline (**B**) the I-V characteristic was shifted into the hyperpolarizatin direction by 40 mV

voltage clamped Helix pomatia L. neurons. To examine the Pb^{2+}-induced currents we used the concentration-clamp technique /6/. For single channel investigations cell-attached patch-clamp technique has been used /7/. The extracellular solution was containing (mM): NaCl 80, MgCl 5, KCl 4, CaCl: 10, glucose 10, Tris base 5, pH adjusted to 7.8 with HCl. The intracellular saline contained 130 mM Tris-Aspartate and 20 mM KCl, pH = 7.4. Extracellularly applied Pb-ions induced a non-inactivating and reversible I_{Pb} in intracellularly perfused voltage-clamped snail neurons. The amplitude of this current increasd in the range of 0.1-250 μM Pb^{2+} (K_d = 63 μM, HP = 100 mV, Fig. 1A-B). The activation (Fig. 1C) and the relaxation phases (Fig. 1D) of the I_{Pb} were both characterized by two exponentials. It is believed that the two exponentials reflect two steps of activation and relaxation. The time constants (τ) were concentration-dependent. The average activation constant was: 1.95 + 0.8 s (fast component) and 22.7 + + 5.1 s (slow component) at -100 mV HP and $/Pb^{2+}/_o$ = 100 μM. The mean value of the relaxation constants were 1.96 + 0.5 s (fast component) and 31.0 + 6.6 s(slow component). This suggest, that Pb-ions may have effect on the fixed surface charge of the membrane. The current voltage relationship was linear between the potential range of -100 and -40 mV with negative slope conductance. The reversal potential for the Pb^{2+}-activated-current was determined by extrapolation of the I-V curve which gave a value of -18.0 mV (Fig. 2A,C). Increasing the extracellular Cl^--concentration we found that E_{rev} of the I-V curve deviated from the control in both direction insignificantly. Decreasing the extracellular Na^+-concentration shifted the I-V relationship to the left along voltage axis (Fig. 2B). Patch-clamp data showed that the steady-state Na-channel has a conductance of 14 pS and both closed and open time distributions display single exponential characters.

The negative slope of the I-V curve and the decrease conductivity during Pb^{2+} application suggest that I_{Pb} is a result of the blocking of the resting Na-conductance. Data obtained from single-channel measurements also supported this conclusion.

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Acta Biologica Hungarica 44(1), pp. 9-13 (1993)

PATCH-CLAMP STUDIES OF THE OXYTOCIN-INDUCED EFFECTS IN HELIX POMATIA L. NEURONS*

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(Accepted: 1991-08-30)

Effect of extracellularly applied oxytocin was investigated on snail neurons in cell attached single channel recording configuration. OXT activated Cl-dependent inward and outward current with amplitude of 1.4 and 1.6 pA, respectively. OXT have affected the ACh-activated current decreasing or increasing the mean interburst interval of single channel activity.

Keywords: Snail neuron - oxytocin - acetylcholine - patch-clamp - Cl-conductance

In voltage-clamped bursting molluscan neurons depolarization caused by vasopressin or oxytocin (OXT) was due to an increase of Na-dependent slow inward current which is a reason of the negative slope resistance region in the current-voltage relationship /1-4/.

Recently it was found, that on Helix neurons the OXT induced conductance changes were Cl^- and K^+ dependent /5/. Three types of OXT-induced currents were described: a) a Cl^--dependent inward current (OXT_{in}) associated with membrane conductance increase, b) a Cl^--dependent outward current (OXT_{out}) associated with membrane conductance decrease and c) a K^+-dependent outward current associated with increased membrane conductance. It is proposed that the contradiction between data was due to the use of different substitutes for NaCl. Namely, replacement of NaCl by TRIS-HCl could

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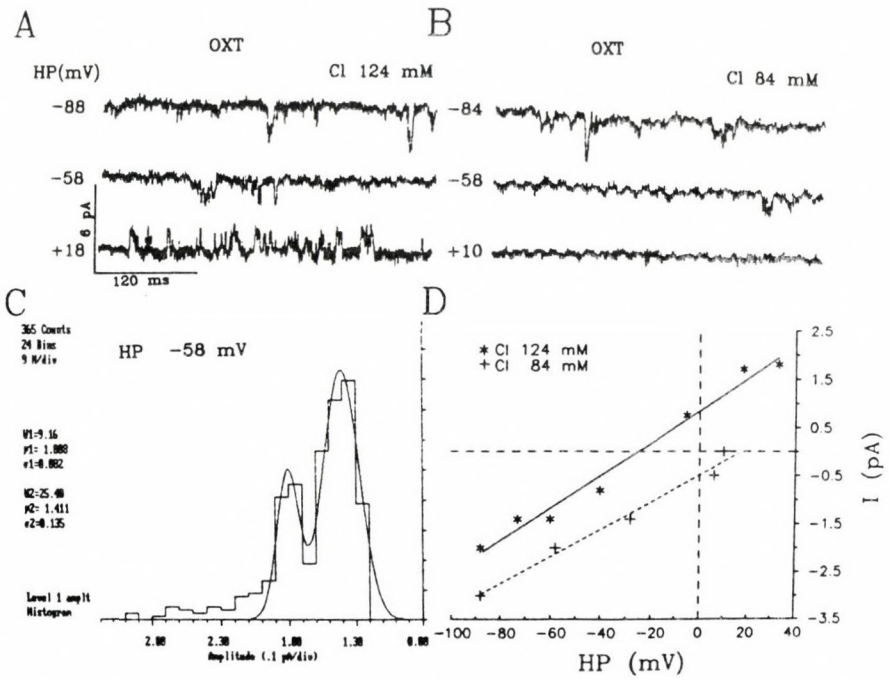

Fig. 1. OXT-activated openings in cell-attached recording mode. Cell was bathed in control solution with following composition (in mM) NaCl 80, KCl 4, $CaCl_2$ 10, $MgCl_2$ 5, glucose 10, Tris 5, pH=7.4 adjusted at two different $/Cl^-/_o$ (**A,B**) see the shift of the reversal. **C**-amplitude histogram of OXT-activated single channel currents. Fit with two Gaussians may reflect different single-channel conductances (see A, too). **D** - I-V curve of the currents seen on A and B - single channel conductance 32 pS. Pipette contained control saline + OXT in 5-10 μM

inhibit OXT-activated conductances whereas sucrose or glucose not /5/. We have now re-analyzed the mechanism of OXT-activated currents using single channel recording technique in cell-attached patch configuration /6/.

The experiment was carried out on isolated non-identified neurones of the suboesophageal ganglia of Helix pomatia L. The isolation procedure was described elsewhere /7/. OXT (Gedeon Richter, 5-10 μM) and/or acetylcholine chloride (Sigma, ACh, 10 μM) were either bath-applied or added to the pipette control saline /7/.

Figure 1A shows single channel currents activated by OXT (OXT_{in}) recorded at three different potentials. The mean amplitude at -58 mV is 1.41 $\pm$ 0.14 mV and the current is inwardly directed. The amplitude histogram could be fitted by two Gaussian distributions with second peak at 1.88 $\pm$ 0.08 mV (Fig. 1C). This may indicate a second open state of the presence of two populations of OXT activated channels. The I-V relation-

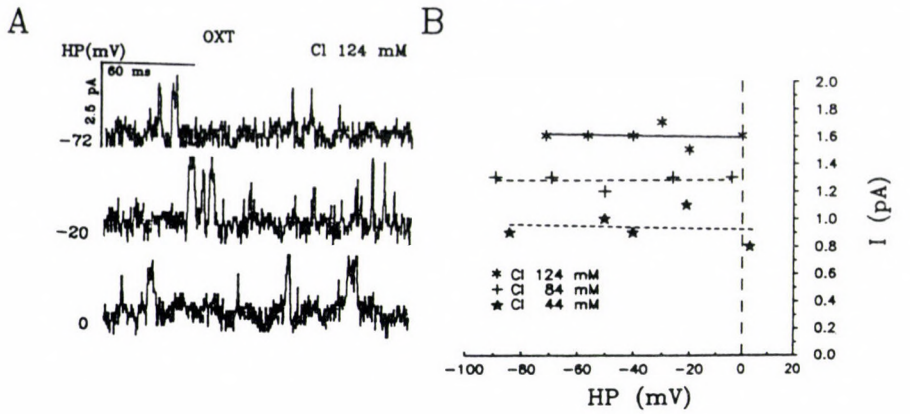

Fig. 2. OXT-induced outward currents recorded in cell attached configuration (**A**). No reversal and no potential-dependence (**B**) was observed, however the Cl^--dependence was clearly shown

ship (Fig. 1D) of the larger component was linear between -100 and +40 mV, with single channel conductance of 32 pS. Decreasing the extracellular Cl-concentration up to 84 mM (NaCl was replaced with sucrose) (Fig. 1B) shifted the reversal potential from -30 mV to +25 according to the Nernst equation increasing the single channel current amplitude at the second component (1.12 $\pm$ 0.11 nA and 2.04 $\pm$ 0.07 nA at -58 mV S.D.). Open time distribution could be well fitted by one exponential (τ_o) at -58 mV. The mean open time showed U-shaped potential dependence. The closed time histogram is a sum of two exponentials with τ_{c1} = 2.25 ms and τ_{c2} = 143.2 ms. The τ_{c1} was voltage-independent while τ_{c2} showed similar voltage-dependence as τ_o.

The OXT_{out} had single channel amplitude 1.6 nA and showed no voltage dependence, however it was sensitive to the extracellular Cl-concentration (Fig. 2A, B). The open and closed time distributions were fitted usually with two time constants. Similar potential-independent cAMP-activated current was described in Helix neurons /8/. These observations may support earlier findings that OXT exerts its effect through adenylate cyclase cascade /4, 9-11/.

As has been shown earlier OXT could evoke both a decrease /9, 10/ and an increase /10/ of the amplitude of the ACh-current in intact Helix neurons. In most cases Cl^--dependent outward ACh-current with E_{rev} near -60 mV can be seen ACh-activated single channel currents normally appeared in bursts, and characterized by the presence of multiple conductance

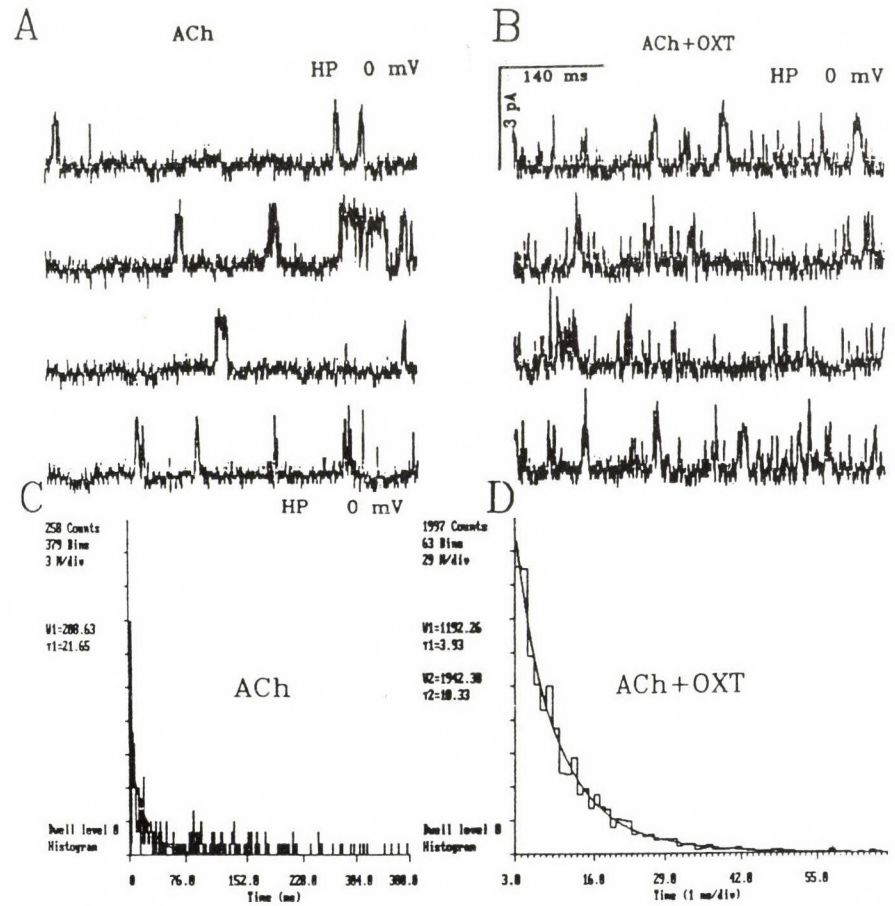

Fig. 3. ACh-activated single channel events in control saline and after OXT (5 μM) treatment (**A, B**). Single channel currents tend to appear in bursts. The interburst interval histograms revealed that in the presence of OXT the distribution could be fitted by two exponentials (**D**) and that the time constants became shorter compared with control (**C**)

levels (Fig. 3A). In the presence of OXT in the pipette or in the experimental chamber (5-10 μM), the mean interburst interval decreased about 50% by OXT treatment (Fig. 3C, D), leaving τ_0 unchanged. In some experiments we also observed an inhibitory OXT effect on ACh-current which consisted of an elimination of τ_{Cl} and a decrease in the rate of the burst generation (not shown).

Results presented above support our earlier finding /5/ that OXT affects on the Cl^--conductance of the neuronal membrane, which in turn leads to the generation of both inward (Fig .1) and outward (Fig. 2) currents. Furthermore OXT both enhanced the ACh-activated outward current decreasing

the interburst duration, leaving the single channel current amplitude and mean open time unchanged (Fig. 3), or inhibited the ACh-current blocking the burst generation. This could be the reason of an increase and a decrease /9, 10/ of the ACh-current by OXT.

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CELL-SPECIFIC EFFECTS OF LEAD ON CULTURED NEURONS OF THE FRESHWATER SNAIL PLANORBARIUS CORNEUS*

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(Accepted: 1991-08-30)

Cultures of isolated neuronal populations from the central ganglia of the gastropod mollusc Planorbarius corneus were used for testing the effects of inorganic lead. The examined parameters were cell survival, neurite outgrowth and cytoskeletal morphology. In large heterogeneous neuronal populations as obtained from a whole cerebral or pedal ganglion, the different sensitivity to lead is reflected mainly on the cell survival. The neurons belonging to the homogeneous E cluster population are more sensitive; in fact a higher percentage of them do not survive in the presence of lead. Moreover, in this neuronal cell type the neurite outgrowth is dramatically affected by lead only when the neurons are cultured on conditioned substrate. Possibly, membrane mechanisms activated for the neurite outgrowth represent a target for inorganic lead. The few neurites sprouted in presence of lead do not evidence changes in the cytoskeletal components.

Keywords: Molluscan neuronal cultures - lead - cell survival - neurite outgrowth - cytoskeleton - Planorbarius corneus (Mollusca)

Lead in both organic and inorganic forms constitutes an important environmental pollutant, and many studies indicate that this heavy metal can dramatically influence neuronal function and cause a variety of behavioral changes /5, 6, 9/. In previous studies we examined the effects that lead can induce on cellular organization and neurochemical correlates in some molluscs /1-4/. Neuronal cultures offer an advantageous tool to

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investigate at the cellular level modifications induced by neurotoxic substances. The aim of this study was to examine the effects of inorganic lead on survival and neurite outgrowth of cultured neurons from selected ganglia of the pond snail Planorbarius corneus.

Two heterogeneous populations of dissociated cells from selected ganglia of juvenile P. corneus, viz. right cerebral and right pedal ganglion, and a homogeneous population from an asymmetrical cluster (E cluster) of the left cerebral ganglion /8/ were cultured. The method has been described elsewhere. Briefly, the isolated neurons were seeded in dishes coated with a poly-L-lysine substrate and containing a modified L15 medium. Neurite outgrowth from heterogeneous cell populations requires a substrate pre-conditioned with factor(s) released from whole ganglia. In this study the medium was supplemented with $Pb(NO_3)_2$ at the same moment of the cell seeding or 18 h later. We tested different concentrations of lead on the E cluster cell cultures and found that lead concentrations ≥ 10 µM were clearly effective on neurite outgrowth (Fig. 1).

The 10 µM lead concentration was then chosen as the experimental dose concentration for subsequent tests including population statistics and immunocytochemical stainings. Atomic absorption spectroscopy indicated that with this initial concentration in our polylysine-coated dishes the lead remaining in solution for the duration of the experiment is about 7 µM.

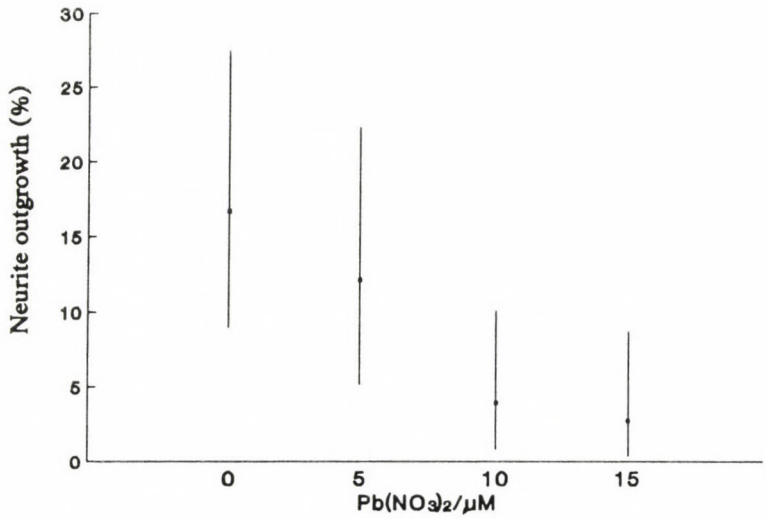

Fig. 1. Dose-response curve of neurite outgrowth of E cluster cells treated with $Pb(NO_3)_2$. Bars indicate confidence level at 95%

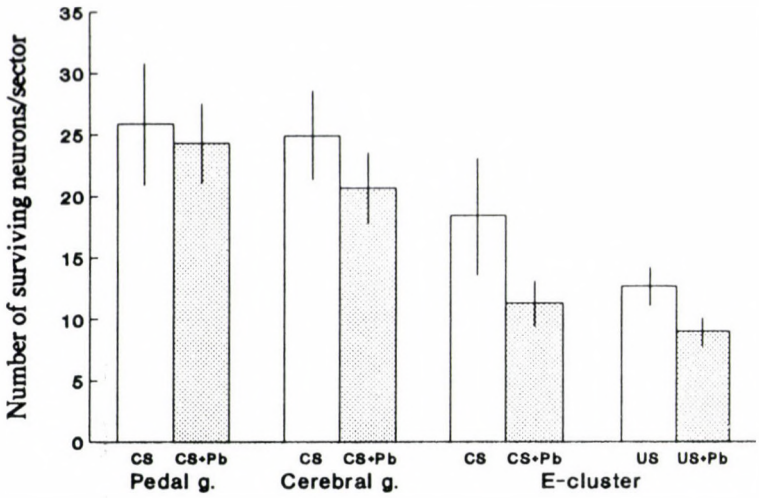

Fig. 2. Lead effect on survival of ganglion cells from two heterogeneous populations (pedal and cerebral ganglia) and a homogeneous population (E cluster) in either unconditioned (US) or conditioned (CS) substrate (mean number of cells scored in randomly chosen sectors)

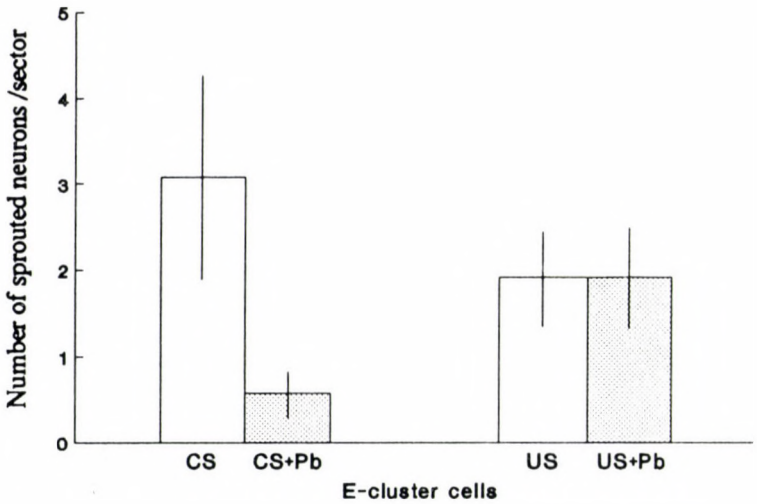

Fig. 3. Lead effect on neurite outgrowth from E cluster cells in either unconditioned (US) or conditioned (CS) substrate (mean number of cells with neurite outgrowth scored in randomly chosen sectors)

Neurons were counted both for survival and neurite outgrowth 40 h after culturing. For each dish, the count was made on 4 equidistant sectors of the same size, delimited by an optical mask (open/whole surface ratio = 1:8.7). A neuron was scored positive for neurite growth if it sprouts at

least one process that is one cell diameter or more in length. In order to study cytoskeletal proteins we performed immunocytochemical fluorescent stainings utilizing monoclonal antisera against β-tubulin and α-actin (Boehringer, Mannheim).

The E cluster cell population, unlike the heterogeneous populations, is able to sprout new neurites at almost the same percentage on both conditioned (CS) and unconditioned (US) substrates /8/. Addition at the time of seeding of 10 μM lead to the conditioned substrate reduces by about 10% the survival of the cerebral ganglion population and by about 40% the survival of the E cluster population while it does not reduce the number of surviving neurons in the pedal ganglion population (Fig. 2). Furthermore the percentage of sprouting neurons among surviving E cluster cells was reduced by about 80% (Fig. 3).

No effect on the number of cerebral and pedal neurons with neurite outgrowth was observed. On the unconditioned substrate the survival of the E cluster neurons was reduced by about 30% (Fig. 2), but neurite outgrowth was unaffected (Fig. 3). Addition of 10 μM lead, 18 h after cell seeding, to the E cluster cell cultures on conditioned substrate does not significantly modify the survival and the number of cells that sprouted neurites.

The immunocytochemical stainings with β-tubulin and α-actin do not evidence changes of the cytoskeletal morphology of neurons that sprouted neurites from all neuronal populations exposed to lead.

We suppose that neurons with different sensitivities to lead are present in the C.N.S. of P. corneus. In large heterogeneous neuronal populations as obtained from a whole cerebral or pedal ganglion, the different sensitivity to lead is revealed by cell survival. The influence of lead on the E cluster is cell-specific. These neurons are more sensitive, and in fact a higher percentage of them do not survive in the presence of inorganic lead. Moreover, in this cell type neurite outgrowth is dramatically affected by lead only when the neurons are cultured on conditioned substrate. Furthermore the addition of lead after the neurons already sprouted new neurites does not seem to cause any changes. We hypothesize that in these cells, membrane mechanisms activated by unknown factor(s) present in the conditioned substrate and responsible for the neurite initiation represent a target for inorganic lead.

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ACETYLCHOLINE LEVEL IN THE BRAIN AND OTHER ORGANS OF THE BIVALVE ANODONTA CYGNEA L. AND ITS MODIFICATION BY HEAVY METALS*

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(Accepted: 1991-08-30)

Acetylcholine was detected and measured in the ganglia (60-80 nmol/g), in the heart (10-15 nmol/g) and in the adductor muscles (4-5 nmol/g) of the bivalve Anodonta cygnea L. using gas chromatographic determination.

Treatment of the animals with low concentration of heavy metals, which cause change in the behaviour, resulted in decrease of the brain acetylcholine level. Within 7 days treatment Cu^{2+} caused 80 per cent, Cd^{2+} and Pb^{2+} 30 per cent reduction with varying types of recovery after wash.

Keywords: Acetylcholine - Anodonta cygnea L. - mussels - Cd^{2+} - Cu^{2+} - Pb^{2+}

The presence and physiological function of acetylcholine (ACh) have been described long ago in various organs of bivalves, namely its role in heart regulation /10/, in evoking contraction of the anterior byssus retractor of Mytilus /9/ and of the adductor muscles of Anodonta /7/, in inhibition of ciliary activity /2/. The detection of cholinesterase activity in the brain /8/ and the effect of ACh application to the ganglia /4/ of Anodonta referred to the central neurotransmitter role of this substance. Until now, however, ACh has not been revealed and measured directly either in the ganglia or in other organs of mussels.

Our aim was first of all to obtain quantitative data on the ACh concentration of various organs of the freshwater mussel by using chemical

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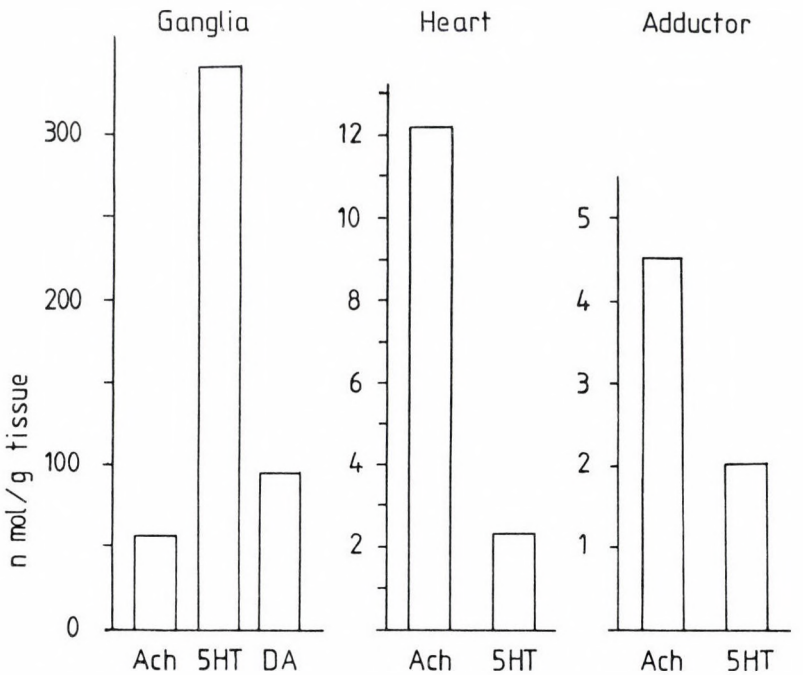

Fig. 1. Transmitter content of different tissues of Anodonta

detection. Further on, since the level of two other well known neurotransmitters (serotonin and dopamine) changes significantly under the effect of some heavy metals /6/ evoking behaviour modulation in the animal /5/ we also studied the effect of Cd^{2+}, Cu^{2+} and Pb^{2+} treatment on the ACh level of the ganglia.

ACh measurements were performed on organs of the mussel Anodonta cygnea L. by gas chromatographic determination. From untreated animals the ganglia, heart and adductor muscles, while after heavy metal treatment only the central nervous system was used. $CdCl_2$, $CuCl_2$ and $PbCl_2$ were applied in concentration of 5 mg/l, 0.2 mg/l and 1 mg/l respectively by perfusion into the water where a group of animals was kept. The duration of the treatment lasted seven days, followed by wash out. ACh concentration was measured with regular intervals, each time from two animals.

After rapid dissection tissues were weighed and homogenized in 1.0 ml of freshly distilled ice-cold acetonitrile containing a known amount of butyrylcholine as internal standard. Homogenates were kept on ice for 30 min, then centrifuged at 20 000 g for 15 min. The supernatants were decanted and dried in a vacuum-centrifuge. Choline esters in the samples were demethylated with sodium benzenethiolate /3/ and measured with a Perkin-

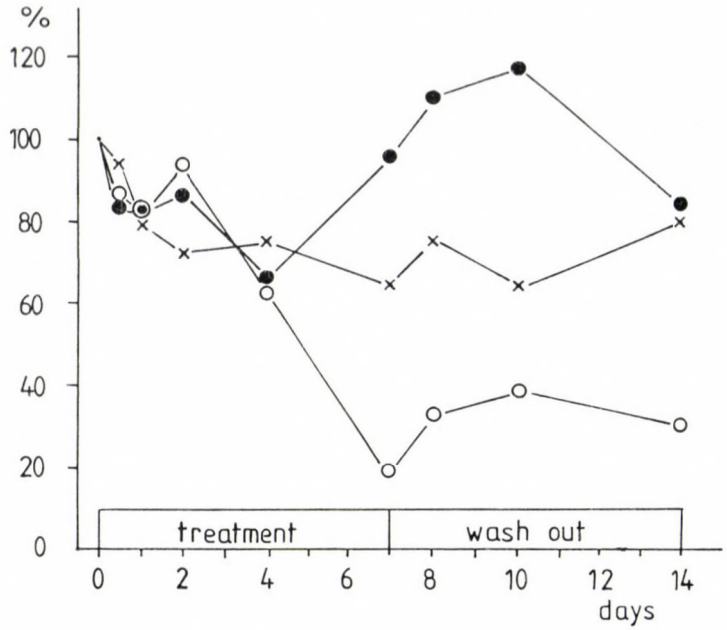

Fig. 2. Effect of heavy metals on the ganglionic ACh level. Values are expressed as per cent of control. Each value is the mean of five experiments. SEM is less than 15 per cent for each point.

● - Cd^{2+}, o - Cu^{2+}, x - Pb^{2+}

Elmer Sigma 1 BGC system, using a nitrogen-sensitive detector /1/. Tissue ACh contents were expressed as nmol/g wet tissue weight.

In untreated animals highest concentration of ACh was found in the ganglia (60-80 nmol/g), followed by the heart (10-15 nmol/g) and by the adductor muscles (4-5 nmol/g) providing an additional proof about the neurotransmitter role of ACh in mussels. We compared these data to the concentration of serotonin (5HT) in each organ and to dopamine (DA) concentration in the ganglia (Fig. 1). In the ganglia, 5HT concentration was five times higher than ACh concentration, while in the heart and adductors the ratio was reversed. In the heart ACh concentration was six times higher than 5HT concentration.

Under the effect of heavy metals there was a considerable reduction in the Ach concentration of the ganglia (Fig. 2). Within seven days treatment Cu^{2+} caused 80 per cent, while Cd^{2+} and Pb^{2+} only about 30 per cent decrease in the ACh concentration. Under cadmium treatment the decrease was transient and restoration started already in the presence of the metal,

but following copper and lead application only partial restoration of ACh level was observed even after seven days wash out.

Important to note, that changes of the filtering behaviour of mussels occurring under the effect of these heavy metals did not correlate with changes in the ACh concentration, since Cd^{2+} and Cu^{2+} caused massive inhibition of activity, while the effect of Pb^{2+} was not significant in the concentrations applied in the present experiments /5/. It is suggested that the involvement of ACh in the central regulation of periodic activity is not significant, but may be important in the regulation of the circulation and of fast adductor contractions assuring water pumping during activity.

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SENSITIVITIES OF ACHATINA GIANT NEURONES TO PEPTIDES ISOLATED FROM MOLLUSCA*

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Sensitivities of the Achatina giant neurones to the peptides isolated from Mollusca were examined by their local application of the pneumatic pressure ejection under current clamp. The peptides tested and their effects were as follows: Ser^2-MIP (inhibitory). CARP (inhibitory), oxytocin (excitatory), SPC_B (excitatory), α-BCP (no effect). ELH (no effect). APGW-amide (inhibitory) and FMRFamide (inhibitory). Membrane conductance (g) of a neurone, d-RPeAN, measured under voltage clamp, was unexpectedly decreased by SCP_B during inward current (I_{in}) caused by the peptide.

Keywords: Peptides - mapping - giant neurones - Mollusca - snail

The effects of the eight peptides isolated from Mollusca (Table 1), applied locally by the pneumatic pressure ejection, on the Achatina giant neurones were previously examined /7, 8/. The present paper aimed to summarize the results of these reports. Another neuroactive peptide having a D-phenylalanine residue, achatin-I, has recently been isolated from the Achatina ganglia /5/. Its pharmacological features will be reviewed in another paper /15/.

Identifiable giant neurones of an African giant snail (Achatina fulica Ferussac) were used in the present study /14/. The abbreviated names of these neurones tested are listed in Table 1. The experimental ways and

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electrical arrangements adopted were described in another paper /15/. The experiments were performed mainly under current clamp. The peptides examined in this study were as follows: Ser^2-Mytilus inhibitory peptide (Ser^2-MIP) /3/, catch-relaxing peptide (CARP) /4/ and APGW-amide /6/ (synthesized by Dr. Hiroyuki Minakata of Suntory Institute for Bioogranic Research, Japan), oxytocin /13/ (from Peptide Institute, Japan), small cadioactive peptides$_B$ (SCP_B) /9/, α-bag cell peptide /12/, egg-laying hormone (ELH) /2/ (Peninsula Laboratories, USA), and FMRFamide /11/ (synthesized by Dr. Eisuke Munekata of Tsukuba University, Japan). Each peptide was dissolved in snail's physiological solution /16/, and applied mainly by the pneumatic pressure ejection (2 kg/cm^2, 400 ms and 10^{-3}M ($3x10^{-3}$ M for ELH) with 0.5% Fast Green locally to the neurone to be tested.

The sensitivities of neurones to the peptides mentioned, tested under current clamp, are summarized in Table 1.

Table 1

Effects of molluscan peptides, applied by pressure ejection (2 kg/cm^2, 400 ms, 10^{-3}M ($3x10^{-3}$ M for ELH), on the identifiable giant neurones of Achatina fulica Férussac /7/

No. Neurone	Ser-MIP	CARP	Oxytocin	SCP_B	αBCP	ELH	$APGW_{NH2}$	$FMRF_{NH2}$
I. Suboesophageal ganglia								
1. PON	(-)	(-)	E	(-)	(-)	(-)	(-)	(-)
2. TAN	I	(-)	(-)	(-)	(-)	(-)	I	I
3. TAN-3	I	(-)	(-)	(-)	(-)	(-)	I	I
4. RAPN	I	I	(-)	(-)	(-)	(-)	I	SI
5. BAPN	(-)	(-)	(-)	E	(-)	(-)	I	I
6. d-RPLN	I	(-)	SE	(-)	(-)	(-)	I	(-)
7. INN	I	(-)	(-)	(-)	(-)	(-)	I	I
8. VIN	(-)	(-)	E	(-)	(-)	(-)	(-)	(-)
9. d-VLN	I	(-)	(-)	(-)	(-)	(-)	I	(-)
10. d-LPeLN	I	(-)	(-)	(-)	(-)	(-)	I	I
11. LPeNLN	I	(-)	(-)	(-)	(-)	(-)	(-)	I
12. d-RPeAN	I	(-)	(-)	E	(-)	(-)	(-)	I
II. Cerebral ganglia								
13. d-LCDN	I	(-)	(-)	(-)	(-)	(-)	(-)	I
14. d-RCDN	I	(-)	(-)	(-)	(-)	(-)	(-)	I
15. v-LCDN	I	I	(-)	E	(-)	SI	I	I
16. v-RCDN	I	I	(-)	E	(-)	SI	I	I

E - excitatory effects, SE - slightly excitatory effects, I - inhibitory effects, SI - slightly inhibitory effects, (-) no effect

Among these, SCP_B produced an inward current (I_{in}) of a neurone, d-RPeAN, unexpectedly with a decrease in the membrane conductance (g) under voltage clamp. This is concordant to the effect of this peptide on an Aplysia neurone /10/ and a Hermissenda crassicornis neurone /1/.

Each of the classic neurotransmitter candidate of Achatina giant neurones, such as dopamine, 5-hydroxy-tryptamine, GABA, β-hydroxy-L-glutamic acid, histamine and acetylcholine, showed the effects of the multiple ionic mechanisms /14/. However, each of the neuroactive peptides mentioned showed one of the effects either excitatory or inhibitory on these neurones. This suggests that the combination of receptors and ionic channels activated by these peptides is less multiple than that of the case of the classic neurotransmitters.

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FREE-RADICAL DAMAGE OF IDENTIFIED NEURONES IN LYMNAEA STAGNALIS*

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(Accepted: 1991-08-30)

We used one of the porphyrin compounds to investigate the selective sensitivity of the different neurones in network to free radical (FR) damage. In general, interneurones are much more resistant to FR damage as compared with other components of the network.

Keywords: Free radicals - photodynamic damage - identified neurons - Lymnaea stagnalis - chlorin e6

Free-radical (FR) reactions are involved in development of many well known pathologies in the nervous system. Moreover, an activation of FR reactions, lipid peroxidation in cellular membranes underlie universal mechanisms of cellular damage. Nervertheless, there are significant differences in properties of particular neuronal clusters both in vertebrate and invertebrate nervous systems. Hence, it is possible to predict that different neuronal groups and individual neurones have different sensitivity and resistance to nonspecific FR damage. To investigate the selective sensitivity of the different neurones in a network to FR damage, a new experimental model has been developed. Central nervous system of freshwater pulmonate snail, Lymnaea stagnalis may be an excellent preparation in this respect. A large number of individual neurones, its synaptic connections and be-

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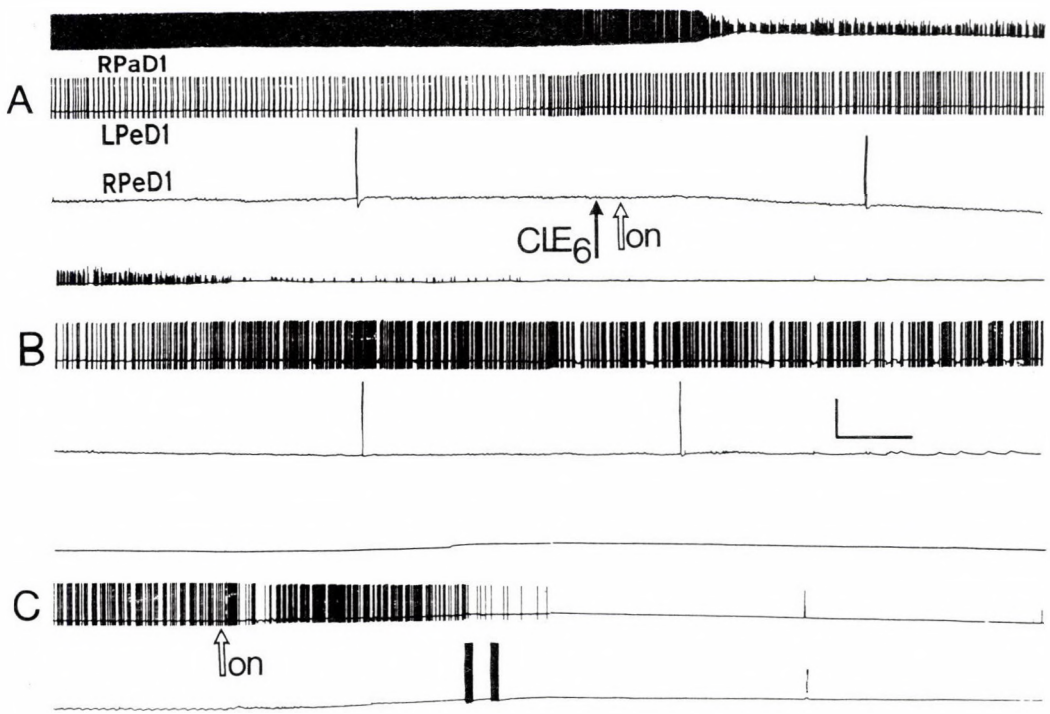

Fig. 1. Effects of the chlorin e6 ($2x10^{-7}$ M) (dark arrow) on three identified neurones in Lymnaea stagnalis related to different functional groups of cells. RPaD1 is putative motoneurone; LPeD1 is putative serotonin-containing modulatory neurone; RPeD1 - dopamine-containing interneurone. Switching on the light is indicated by open arrows. **A**, **B**, **C** are simultaneous recording from three cells. **A** and **B** are continuous recordings. **C** shows partial restoration background activity after 10 min in darkness. Spikes in RPeD1 were induced by electrical stimulation to check the excitability of the neurone. Note the hyperpolarization of the RPeD1 during first minutes of illumination.
Scale: 80 mV, 2 min

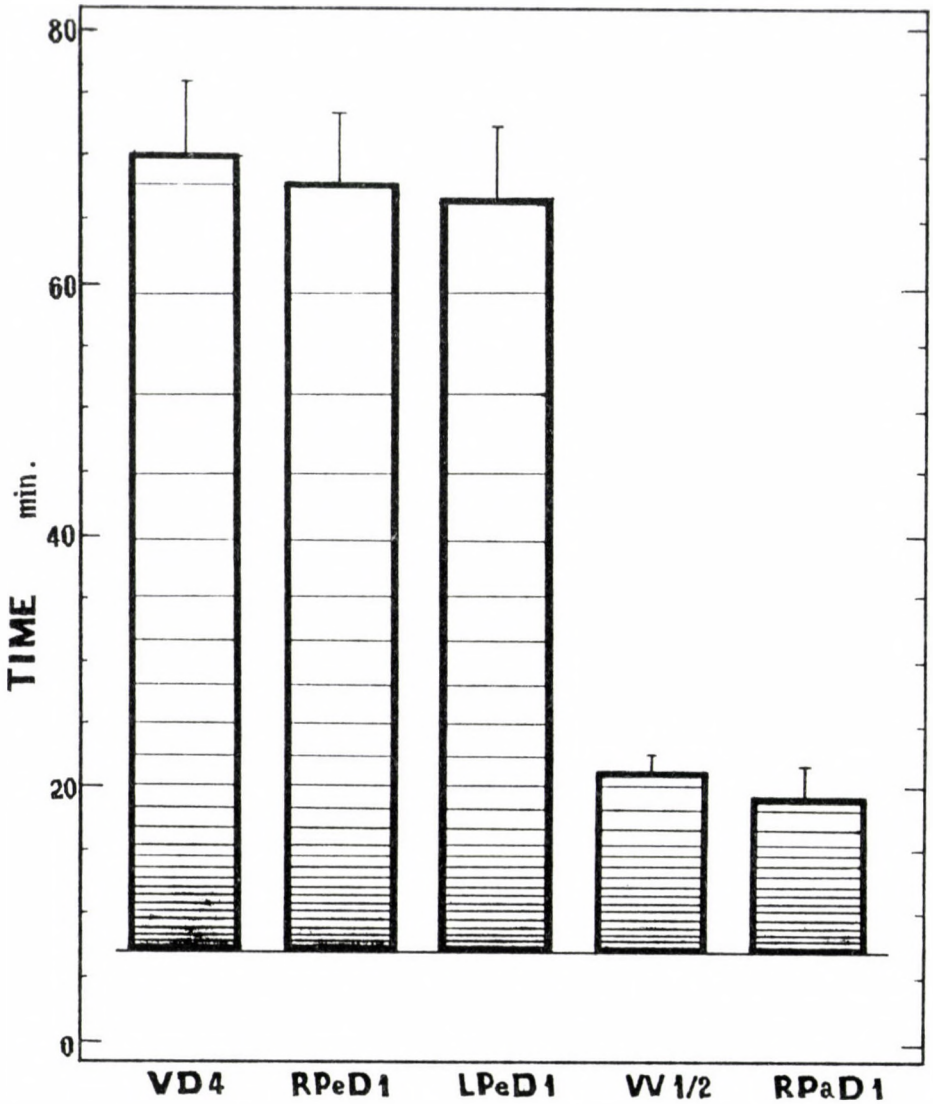

Fig. 2. Time of death (in minutes) for several identified neurones of Lymnaea stagnalis under illumination (light power density 1.76 mW/sm^2) in presence of 5x10^{-7} M chlorin e6. Vertical lines are standard deviations ($p < 0.05$, $n = 7$-12)

havioural outputs have been identified (see /5/ and Winlow et al. in this volume for references).

Experiments were carried out on isolated brains Lymnaea stagnalis using conventional microelectrode techniques. Protease treatment (5-10 min incubation in 0.2% pronase solution) have been used before microelectrode recording.We selected cells (see map in Moroz, Winlow, this volume for lo-

calization of the individual neurones investigated here) independent of functional properties and transmitter specificity (according 1, 4). We used one of the porphyrin compounds, chlorin e6 /2, 3/ to induce photodynamic damage and FR reactions in the central nervous system.

Administration of chlorin e6 (Chl e6) (10^{-9} - 10^{-6} M) in the bathing solution has no detectable effects on neurones during several hours in darkness. However, Chl e6 causes serious neuronal damage following illumination (Fig. 1). Under these conditions singlet oxygen production and induction of FR reactions have been shown by biochemical methods. The threshold concentration was about 10^{-8} M (light power density 1.76 mW/sm^2). The time-course of FR reactions in neurones (as shown by the TBA-testing) and their electrophysiological responses were in good accordance.

There were ne any major differences in the responses between several of the identifiable neurones investigated here. A slow gradual depolarization occurred simultaneously with a decrease in both membrane resistance and action potential amplitude. Nevertheless, the sensitivity of the different neurones and the time-course of damage were very variable and characteristic of particular cell types (Fig. 2). In general, interneurones are much more resistant to FR damage as compared with other components of the network. For example, damage and death of the RPeD1, LPeD1, VD4(=VW1) occurred after 1.5-3 h, while those of the pedal motoneurones (A-groups) or RPaD1, VV1/2 occurred within 15-30 min.

Our initial investigations indicate that the positions of the individual neurone in network is a more important characteristic of the cell (with respect of FR damage) than its transmitter specificity.

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EFFECTS OF GENERAL ANAESTHETICS ON CULTURED LYMNAEA NEURONES*

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In order to elucidate the mode of action of general anaesthetics we are using neurones of Lymnaea stagnalis as a model system. Neurones exhibit mainly two types of responses to anaesthetics delivered at clinical concentrations, i.e., either gradually going into quiescence or exhibiting paroxysmal depolarizing shifts (PDS). In order to determine whether these differences are due to intrinsic membrane properties or because of synaptic effects, cultured neurones are being used so that cells can be studied in isolation from any synaptic effects. Cells in culture retain their basic electrophysiological characteristics and behave in a similar manner to the applied anaesthetics as do whole brain preparations. Demonstration of PDS and quiescence in cultured neurones shows that these phenomena are due to membrane effects and not due to synaptic inputs. The effects of anaesthetics observed seem to be consistent with the suggestion that anaesthetics may influence the inward calcium current or other calcium-dependent currents.

Keywords: Cell culture - general anaesthetics - action potential - snail - halothane

In the whole brain there are important differences in the responses of individual Lymnaea neurones to applied anaesthetics, irrespective of the anaesthetic used /7/: cells either become gradually quiescent or exhibit paroxysmal depolarizing shirts (PDS) and oscillatory behaviour. Only isolated neurones can tell us whether these effects are due to synaptic

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inputs or reflect intrinsic differences between the cells. Here we demonstrate that cultured Lymnaea neurones retain their basic electro-physiological features in culture. Previous reports indicate an increase in the duration of action potential in cultured Helisoma neurones /2/ and an increase in the resting potential and spike-amplitude in at least one category of cultured leech neurones /3/.

Identified neurones of Lymnaea were isolated aseptically from the brain and cultured in poly-L-lysine coated Falcon No. 3001 dishes. Cells could either be grown as spheres or with neuritic extensions depending on the protocol used /5/. Recordings were made from cultured neurones using standard intracellular electrophysiological techniques and anaesthetics were delivered according to the methods of Girdlestone et al. /4/.

Lymnaea neurones can be classified as having type 1 or type 2 action potentials according to the respective absence or presence of a pseudo-plateau during repolarization /1/. Preliminary results indicate that many cultured Lymnaea neurones maintain their characteristic action potential type, in cells grown either as spheres or with neurite extensions (Table 1). The resting membrane potential ranges from -40 to -55 mV which is not very different from that in vivo. As is apparent from Table 2, there appears to be a tendency towards an increase in the duration of action potential with a concomitant decrease in the spike amplitude in some cell types in culture but this is not significant.

Table 1

Neuron types in culture

Neurone types	Type of action potential	Number of cells studied in culture	Number of cells retaining spike shape in culture
M group	II	16	14
RPD1	II	8	7
VV1/2	II	4	4
RPeD1	II	8	5
Total number of cells studied		36	30 (83.33%)

Table 1 demonstrates that the majority of neurones studied maintain their normal action potential shapes in culture

Recordings of cultured neurones in 1.0% (v/v) halothane are similar to those from the intact brain. There is a diminution of the action potential amplitude, plateau and after hyperpolarization, all of which are calcium dependent phenomena (Fig. 1). Some of the isolated cultured neurones also exhibit the ability to generate PDS-like events (n=5) in the presence of halothane. Thus these PDS-like events are due to the intrinsic membrane properties of cells themselves, not because of synaptic inputs from other sources. An increase in intracellular calcium may initiate bursts, and could lead indirectly (via a calcium-dependent potassium conductance) to burst termination /6/. Increases in intracellular calcium ion concentration due to a release from the sarcoplasmic reticulum in chemically skinned rat myocardium appear to be caused by halothane when applied in lower doses /8/.

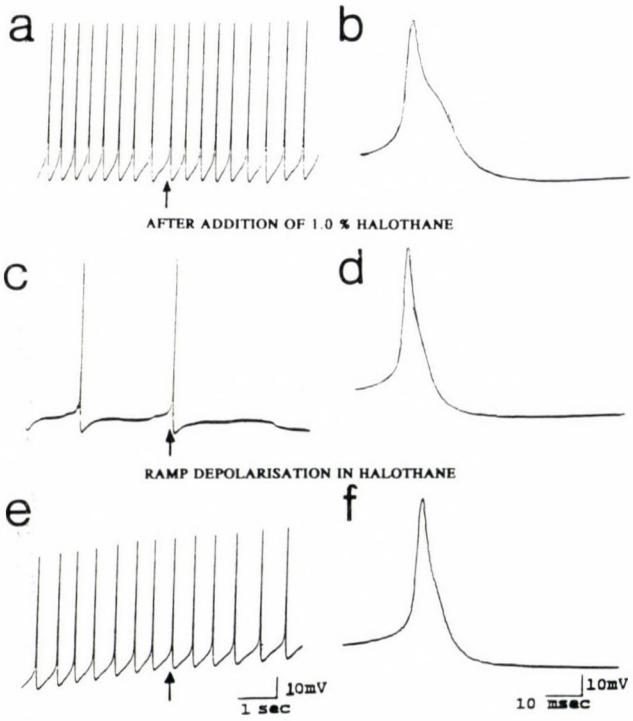

Fig. 1. Effect of 1.0% halothane on a cultured RPeD1 cell.(**a**) Cell firing spontaneously and one of the spikes (**b**) expanded to show the shape with a prominent pseudoplateau. (**c**) After addition of halothane the frequency of firing decreases till the cell becomes quiet. (**d**) One of the spikes on expanded time scale to show the loss of pseudoplateau. (**e**) Even after ramp depolarization to increase the firing frequency the spike shape (**f**) does not recover to normal, i.e., type 2. Thus the calcium dependent plateau is lost in the presence of halothane

Table 2

Comparison of action potential half-width and spike-amplitude of neurones in culture and in tact brains

	Action potential half-width (msec)		Spike amplitude (mV)	
Neurone type	Cultured	Intact	Cultured	Intact
M group	15.64 ± 8.74 (n=16)	21.27 ± 8.08 (n=7)	80.23 ± 8.64 (n=16)	78.42 ± 8.31 (n=7)
RPD1	16.60 ± 5.29 (n=8)	11.85 ± 5.90 (n=5)	76.82 ±11.93 (n=8)	90.61 ± 9.20 (n=5)
RPeD1	13.07 ± 3.37 (n=9)	7.4 ± 4.2 (n=10)	76.70 ±10.97 (n=9)	91.39 ± 7.15 (n=10)

Table 2 compares action potential half-width (i.e., width of action potential at half the amplitude) and action potential amplitude (i.e., the distance between the highest and the lowest points in the action potential) in neurones in culture and in neurones in intact brains. There are no significant differences in these two parameters at 5% level (Student's t-test)

Thus, cultured Lymnaea neurones maintain their basic electrophysiological characteristics and their responses to general anaesthetics are similar to those in the intact brain.

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THE STRUCTURAL MULTIPLICITY OF MYTILUS INHIBITORY PEPTIDES ISOLATED FROM PULMONATE MOLLUSCS*

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Twelve species and 10 species of mytilus-inhibitory-peptide analogues were isolated from the ganglia of Helix pomatia and Achatina fulica, respectively.

Keywords: ABRM - Mytilus inhibitory peptide - Mytilus - Helix - Achatina

Hirata et al. were the first to isolate two Mytilus inhibitory peptides (MIPs) from the pedal ganglia of the bivalve mollusc, Mytilus edulis /2/. Their structures are GAPMFVamide and GSPMFVamide. The MIPs showed a potent inhibitory effect on contractions of the anterior byssus retractor muscle (ABRM) of Mytilus /3/. Recently, Fujisawa et al. isolated five MIP-family peptides from the ABRMs themselves /1/.

The MIPs show inhibitory effects not only on the ABRM but also on many other molluscan tissues /3, 6, 7/. Therefore, it is suspeceted that the MIP-family are widely distributed in molluscs. In the present study, we isolated various species of MIPs from the ganglia of two pulmonate molluscs, Helix pomatia and Achatina fulica.

MIPs in Helix. The cerebral and suboesophageal ganglia of Helix pomatia were excised from 750 specimens. Acetone extracts of the ganglia were forced through C-18 cartridges. The retained material was eluted with

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methanol. The eluate was concentrated and applied to a C-18 reversed-phase column and eluted with a 120 min linear gradient of 0-60% acetonitrile in 0.1% TFA (pH 2.2). The biological activities of the fractions were assayed on phasic contraction of the ABRM in response to repetitive electrical stimulation. Three peaks with inhibitory activity were found. The fractions of each of the active peaks were collected, concentrated and applied to a cation-exchange column. The column was eluted with a 60 min linear gradient of 0-0.6 M NaCl in 10 mM phosphate buffer (pH 6.9). We obtained three inhibitory peaks from each of the foregoing three peaks. The fractions in each of the nine active peaks were then applied to another kind of C-18 reversed-phase column. The active materials were further purified by using reversed-phase columns. These procedures have been described previously /4/. All of the purified substances were subjected to amino acid analysis and amino acid sequence analysis. Thus, we could propose 12 species structures of MIP analogues.

MIPs in Achatina. Acetone extracts of the cerebral and suboesophageal ganglia excised from 940 specimens of Achatina fulica were loaded on C-18 cartridges.The retained material was eluted with methanol and subjected to HPLC purification. The procedures of the purification have been described previously /5/. They were basically the same as in the case of Helix. The purified substances were subjected to amino acid analysis, amino acid sequence analysis and liquid secondary ion mass spectrometric analysis. Thus, we could propose 10 species structures of MIP analogues.

All of the proposed structures of the MIPs of Helix and Achatina, in addition to those of Mytilus /1/, are shown in Table 1.

The MIPs isolated from the ABRMs have -PXFVamide at their C-terminal portion. It has been suggested that -PXFVamide is an important structure for the MIPs to exert their inhibitory effect /1/. This structural property was also observed in almost all of the MIP analogues isolated from Helix and Achatina. Only one out of the 22 MIPs of the pulmonates was found to have -PXFIamide.

The pentadecapeptides isolated from Achatina consist of two MIP-like hexapeptides linked by -GRR-. The structures of dibasic amino acid residues preceded by a glycine residue (e.g., -GRR-) are well known as processing sites in precursors of neuropeptides with an amidated C-terminus. In general, precursor proteins are inactive on target tissues for final products. However, the pentadecapeptides showed a potent inhibitory activity on phasic contraction of the ABRM and spontaneous contractions of

Table 1

Structures of MIP analogues isolated from three species of molluscs

Mytilus edulis (ABRMs)	G S P M F V	amide
	G A P M F V	amide
	D S P L F V	amide
	Y A P R F V	amide
	A S H I P R F V	amide
Helix pomatia (ganglia)	G A P A F V	amide
	A A P R F V	amide
	G A P M F V	amide
	G A P L F V	amide
	G S P Y F V	amide
	G A P Y F V	amide
	R A P Y F V	amide
	S V P I F V	amide
	G V P Y F V	amide
	G P P M F I	amide
	A A P F F V	amide
	R A P F F V	amide
Achatina fulica (ganglia)	A A P K F V	amide
	G A P K F V	amide
	G A P V F V	amide
	G A P Y F V	amide
	A A P Y F V	amide
	G P P M F V	amide
	G A P F F V	amide
	D A P K F V G R R D P P Y F V	amide
	A A P K F V G R R G S P Y F V	amide
	A A P K F V G R R G A P Y F V	amide

See also /1, 4, 5/

the crop of Achatina /5/. The pentadecapeptides might be released without being cleaved at least under specific conditions. Furthermore, it can be supposed that one molecule of the pentadecapeptides released from nerve terminals is able to activate simultaneously two MIP-receptor sites of the target tissues. Further examinations of their mode of action are required.

From the viewpoint of molecular evolution, it is interesting that such a wide structural diversity of analogue peptides is observed in one phylum.

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FMRFamide-RELATED PEPTIDES ISOLATED FROM THE PROSOBRANCH MOLLUSC FUSINUS FERRUGINEUS*

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In addition to FMRFamide and FLRFamide, four FMRFamide-related peptides were isolated from the ganglia of a prosobranch mollusc, Fusinus ferrugineus. Their primary structures were ALTNDHELRFamide, LSSFVRIamide, GSLFRFamide and SSLFRFamide.

Keywords: FaRPs - Fusinus - FMRFamide - FLRFamide - radula protractor

It has been shown that FMRFamide-related peptides (FaRPs) are widely distributed in molluscs /1, 5/. In the present experiments, we searched for FaRPs in the ganglia of the prosobranch mollusc Fusinus ferrugineus. We found six FaRPs including FMRFamide and FLRFamide. The actions of these FaRPs on some molluscan muscles were characterized.

Animals. The animals used in the experiments are Fusinus ferrugineus, Meretrix lusoria and Mytilus edulis.

Purification. The cerebral, suboesophageal and buccal ganglia of 1.100 specimens of Fusinus were excised. The acetone extracts of the ganglia were applied to C-18 cartridges. The retained material was eluted with methanol. The eluate was gel-filtrated with a column of Sephadex G-15. The fractions which showed bioactivities on twitch contractions of the radula retractor muscle of Fusinus were pooled, evaporated and applied to

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a HPLC system with a reversed-phase or a cation-exchange column. After three to five steps of the HPLC separation, we purified 13 peptides.

Structure determination. The purified peptides were subjected to amino acid sequence analysis, amino acid analysis and FAB-MS analysis, and thus probable structures of the peptides were determined. Peptides having the probable structures were synthesized or purchased, and their behavior on HPLC and bioactivities on the radula retractor were compared with those of the native peptides to confirm their structures.

Biological actions. The actions of the peptides related to FMRFamide were examined on the following molluscan muscles: the radula retractor of Fusinus, the heart of Meretrix and the ABRM of Mytilus. The methods of stimulation of the muscles and recording tension from them were described previously /3/.

Salines. The physiological saline for the Mytilus and Meretrix muscles was artificial seawater (ASW) /3/. For the Fusinus muscle, a low-Mg^{2+} ASW (20 $MgCl_2$ ASW) was used. This saline was made by replacing a part of $MgCl_2$ in the normal ASW with osmotically equivalent NaCl.

Results of the chemical analyses showed that six out of the 13 purified peptides were FaRPs. The primary structures of the FaRPs are shown in Table 1.

Table 1

Structures of FMRFamide-related peptides isolated from the ganglia of Fusinus

Peptide 1	H-Ala-Leu-Thr-Asn-Asp-His-Phe-Leu-Arg-Phe-NH_2
Peptide 2	H-Phe-Met-Arg-Phe-NH_2
Peptide 3	H-Phe-Leu-Arg-Phe-NH_2
Peptide 4	H-Leu-Ser-Ser-Phe-Val-Arg-Ile-NH_2
Peptide 5	H-Gly-Ser-Leu-Phe-Arg-Phe-NH_2
Peptide 6	H-Ser-Ser-Leu-Phe-Arg-Phe-NH_2

The decapeptide ALTNDHFLRFamide, as well as FMRFamide and FLRFamide, showed excitatory actions on the radula retractor (Fig. 1A). The actions of the decapeptide on the other molluscan muscles were also excitatory, being similar to those of FMRFamide and FLRFamide. Fujisawa et al. /2/ isolated a FMRFamide-related decapeptide from the ABRM /2/. The proposed sequence of the Mytilus decapeptide is highly homologous with that of the Fusinus decapeptide. Thus, it can be suspected that there exists a decapeptide subfamily of FaRPs in molluscs.

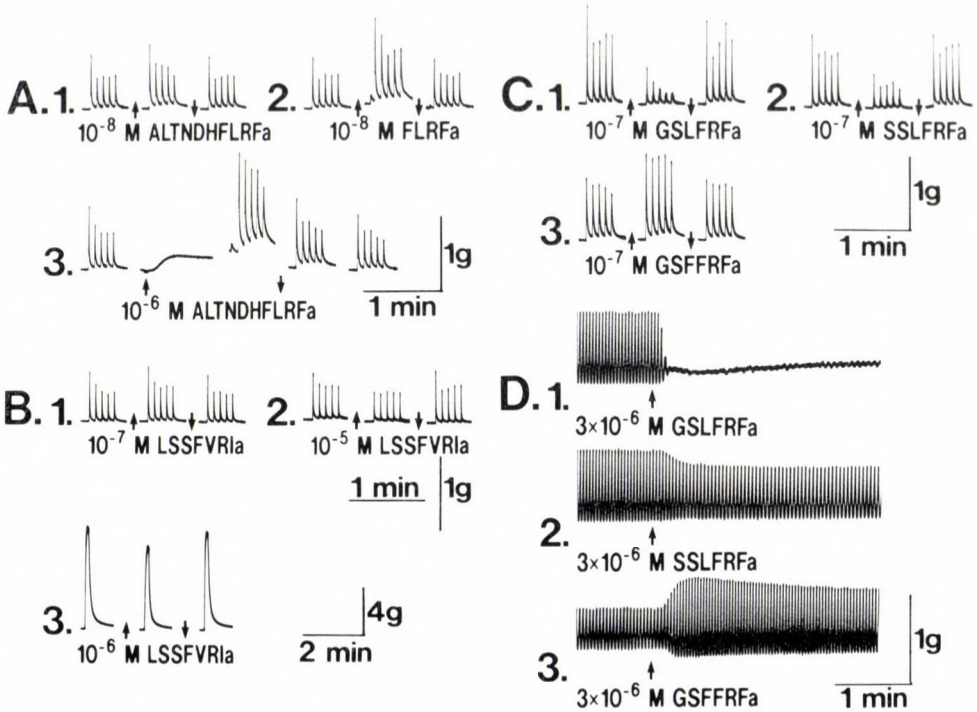

Fig. 1. Effects of FMRFamide-related peptides of Fusinus on contractions of some molluscan muscles. **A**: effects of ALTNDHFLRFamide and FLRFamide on twitch contractions of the radula retractor of Fusinus. **B**: effects of LSSFVRIamide on twitch contractions of the radula retractor of Fusinus /1, 2/ and on phasic contraction of the ABRM of Mytilus /3/. **C**: effects of GSLFRF amide, SSLFRFamide and GSFFRFamide on twitch contractions of the radula retractor of Fusinus. **D**: effects of GSLFRFamide, SSLFRFamide and GSFFRFamide on the heart beat of Meretrix. The twitch contractions of Fusinus were evoked by applying train electrical pulses of stimulation (22V, 1 ms, 0.2 Hz, 5 pulses). The phasic contraction of Mytilus was evoked by applying repetitive electrical pulses of stimulation (15 V, 3 ms, 10 Hz for 5 s)

The heptapeptide LSSFVRIamide showed a weak twitch-potentiating action on the radula retractor at lower doses (Fig. $1B_1$), while at higher doses it showed twitch-inhibiting action (Fig. $1B_2$). In the ABRM, the peptide inhibited phasic contraction in a dose-dependent manner (Fig. $1B_3$). That is, the action on the ABRM is opposite to those of FMRFamide and FLRFamide. The C-terminal tetrapeptide fragment structure of LSSFVRIamide is closely related to those of the peptides QFYRIamide and EFLRIamide which have been shown to be encoded by a gene in Lymnaea with FMRFamide and FLRFamide /4/.

The hexapeptides GSLFRFamide and SSLFRFamide showed an inhibitory action on twitch contractions of the radula retractor (Fig. $1C_{1,2}$) and on spontaneous contractions of the heart of Meretrix (Fig. $1D_{1,2}$). These actions are also opposite to those of FMRFamide and FLRFamide. The analogue peptide GSFFRFamide showed excitatory actions on the muscles of Fusinus and Meretrix (Fig. $1C_3$, D_3). It is of great interest that the substitution of Phe for Leu^3 of GSLFRFamide changes its inhibitory action into excitatory one.

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STRESS-INDUCED RELEASE OF OCTOPAMINE IN THE AMERICAN COCKROACH PERIPLANETA AMERICANA L.*

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After stress, changes of octopamine content were observed in the antennal heart and the retrocerebral complex of cockroaches. Only individually handled animals exposed to short and extreme stress showed an alteration in the haemolymph octopamine levels. The removal of the retrocerebral complex resulted in an elevation of the octopamine content in the haemolymph. We suppose that the corpora cardiaca are not the only source for the octopamine released into haemolymph in stress situations.

Keywords: Octopamine - Periplaneta americana - stress - haemolymph - antennal heart - corpora cardiaca - corpora allata

Octopamine is suggested to function as a neuromodulator, neurotransmitter and neurohormone in insects /1, 7, 8, 12/. Especially high concentractions were found in several neurohaemal tissues /5, 6/. Octopamine seems to play a regulating role in stress situations in insects /9, 12, 13/. Octopamine is present in the haemolymph of cockroaches, where it acts as a neurohormone, controlling for instance the release of trehalose and lipids from the fat body /12/. A one- or two-minute period of excitation by handling causes an elevation of haemolymph octopamine level /3/. The cellular origin of octopamine in the haemolymph remains unclear. Therefore, experiments with retrocerebral complex extirpated animals were performed.

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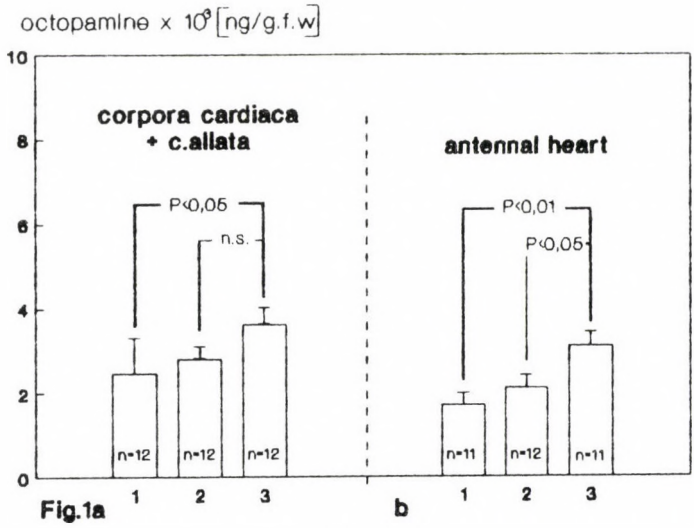

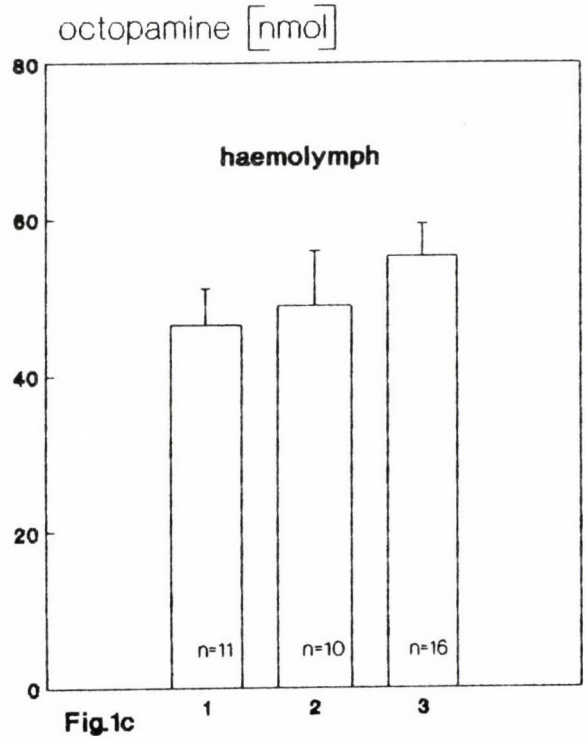

Fig. 1**a,b,c.** Influence of physical stress on the concentration of octopamine in 1. "immobilized animals", 2. "normal animals" and 3. "stressed animals". Each column represents n individual measurements (consisting of double estimations from six different experiments ± S.E.M)

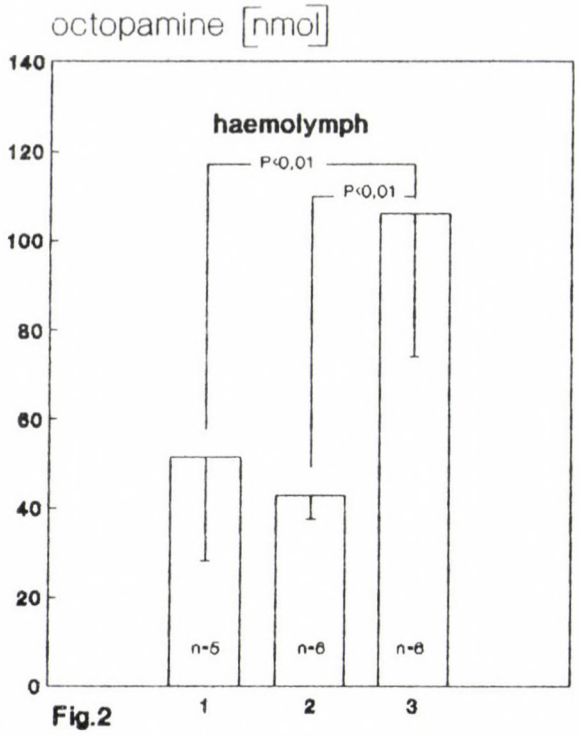

Fig. 2. Influence of physical stress of haemolymph octopamine concentration in: 1. "immobilized animals" (5 °C, 15 min), 2. "normal animals" (20 °C, 1 h), 3. "stressed animals" (increased temperature up to 40 °C, 3 min), (±S.E.M.)

We measured also the octopamine-content of different organs (antennal heart, retrocerebral complex, haemolymph) in response to different kinds of stress of longer or shorter durations.

Rearing: Cockroaches (Periplaneta americana) were raised at 28 °C and 12 h of reversed light/dark regime. Pellet food and water were provided ad libitum. Experiments were performed with 2-4-week-old males. Cockroaches were removed from the stock colony of our institute 3 h prior to experimentation and held in individual Petri dishes until the beginning of experimental treatment.

Isolation of octopamine: After stress treatment retrocerebral complex, antennal heart and heamolymph were dissected from the animal within a few seconds and immediately frozen. Organs were homogenized in a glasshomogenizer containing a defined volume of 0.04 mmol/l TRIS-HCl buffer (pH 8.6) with 1 mmol iproniazid. Samples were three times homogenized, frozen and centrifuged. Haemolymph samples were not pooled but determined separately.

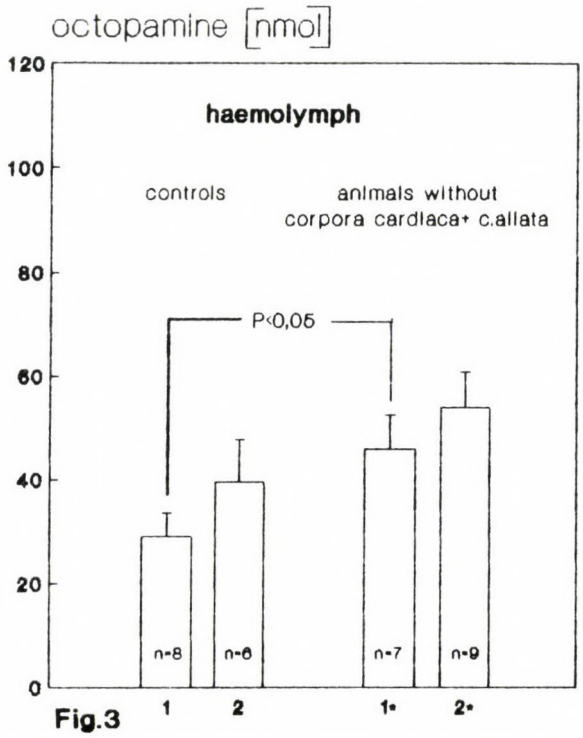

Fig. 3. Influence of corpora cardiaca + corpora allata removal on the octopamine concentration in haemolymph. 1. control animals, 2. stressed animals (2.5 min shuttle), (±S.E.M.)

Octopamine assay: Octopamine was estimated by the partly modified method of Molinoff et al., 1969 /11/. This method is based on the methylation of octopamine to H-synephrine by phenyl-ethanolamine-N-methyl-transferase (PNMT) in the presence of H-methyl-S-adenosyl-L-methionine. The enzyme PNMT was prepared from bovine adrenal glands according to Axelrod, 1962 /2/. The results were statistically evaluated with Student's t-test.

I. Stress experiments with intact animals: Six groups of five animals were immobilized by low temperature (5 ^{0}C) for 15 min ("immobilized animals"). Six other groups of five animals were prepared without any treatment ("normal animals"). Finally, six groups of five animals were mobilized by high temperature (50 ^{0}C) in a large heating box for 15 min ("stressed animals").

In the antennal heart of "stressed animals" an increase of the concentration of octopamine was registrated (Fig. 1b). In the retrocerebral complex and in the antennal heart a significant difference of octopamine-content was found between animals, stressed by cold and those mobilized by

higher temperature (Fig. 1a, b). In the haemolymph no differences between the groups were found(Fig. 1c).

II. Stress experiments with animals individually handled in small boxes: Five animals were immobilized by 5 ^{o}C for 15 min. Six animals (controls) were prepared after holding at 20 ^{o}C for 1 h. Six animals were stressed by a temperature increasing from 20 ^{o}C up to 40 ^{o}C. (These experiments were started together with Dr. P. Kestler in Osnabrück.) In the haemolymph of these animals clear differences of octopamine content between the groups were found: between animals stressed by cold and those stressed by high temperature as well as between controls and animals stressed by high temperature (Fig. 2).

III. Stress experiments with animals deprived of their retrocerebral complexes two days before: Animals were separated two hours before the experiment started. Eight unoperated animals without any treatment, six unoperated animals stressed by shaking (2.5 min), seven animals without retrocerebral complex and without any treatment, and nine animals without retrocerebral complex and stressed by shaking (2.5 min) were investigated. A higher octopamine level in the haemolymph appeared in the animals without retrocerebral complex in comparison to the controls (Fig. 3).

IV. Isolated antennal hearts were stimulated via nervus cardioantennalis by means of suction electrodes (10 V, 20 Hz) for 15 min. Controls were placed in saline without electrical stimulation. We did not succeed in finding any significant difference between the octopamine-content of antennal hearts of electrically stimulated and untreated animals.

Octopamine is thought to be responsible for many of the aspects of the classical fight or flight arousal reaction /14/. It controls the supply of energy and affects fat body glycogenolysis in cockroaches and stimulates the release of lipid from locust fat body. It functions as both a neurohormone either circulatory or locally released, and as a neuro-modulator /6/. There are a lot of observations about the increase of octopamine level in the haemolymph after several forms of stress /4/. In the present study we also found an increased octopamine level after thermal stress: 106 nM against 43 nM octopamine. Davenport et al. /4/ observed an increase of octopamine from 31 nM to 92 nM after thermal stress.

The source of the octopamine circulating in the insect haemolymph is not clear. A contribution to the increased octopamine levels may have come from a source in the head, such as the corpora cardiaca /4/. It has been suggested that octopamine is partly synthetized in the supraoesophageal

ganglion and distributed to releasing organs by axonal transport /10/. After 24 h stress (immobilization) Koszanek et al. /10/ found an increased level of octopamine in the supraoesophageal ganglion. It is known that the DUM neurons of the suboesophageal ganglion project via nervi corporis cardiaci III to the corpora cardiaca in Periplaneta americana /1/. The transversal muscle of the antennal heart is a target of two of these DUM neurons in Periplaneta americana. After stress, we observed in our experiments an increased level of octopamine in the corpora cardiaca and also in the antennal heart. It may be that the axons of DUM cells end in neurosecretory terminals within the organs investigated. However, octopamine is discussed to be a transmitter between axons of nervi corporis cardiaci II and the glandular cells of corpora cardiaca /13/. Experiments with animals without retrocerebral complex show an increased octopamine level in the heamolymph independent of stress. So corpora cardiaca cannot be the only source for octopamine in the haemolymph. Another potent source could be the neurohaemal organs of the nervous system of cockroaches and blind endings of axons of DUM neruons. This claim requires further experimental support.

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GALANIN IMMUNOREACTIVITY AND ^{125}I-GALANIN BINDING SITES IN THE BLOWFLY BRAIN*

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We have used immunocytochemical, immunochemical (RIA) and chromatogrpahic methods (HPLC, gel filtration) to provide evidence for presence of GAL-like peptide(s) in the blowfly Phormia terraenovae. HPLC indicates the presence of several forms of GAL-like peptides. Immunocytochemistry showed that there are about 160 GAL-IR neurons in the brain and subesophageal ganglia supplying the central body, superior protocerebrum, the optic lobe and tritocerebral neuropil. Autoradiography of binding with ^{125}I-labelled porcine GAL on brain sections revealed GAL binding sites in the central body complex and deutocerebrum. The presence of galanin-like peptide(s) and putative receptor sites in the fly brain suggest a role in neuromodulation in specific circuits.

Keywords: Neuropeptide - galanin - insect nervous system - receptor autoradiography - immunochemistry

Galanin (GAL) is a 29 amino acid bioactive peptide, first isolated from pig intestine /8/, later shown to be present also in the central and peripheral nervous system of mammals /5/. Preprogalanin, the GAL precursor, is a 123 amino acid protein containing a signal peptide, a single copy of GAL and a 59 amino acid peptide termed galanin message associated peptide (GMAP) /6/. Galanin has a number of biological actions in the mammalian nervous system /5/ and GAL receptors, recognizing GAL and its N-terminus fragment GAL1-16, have been characterized /1/. Galanin-like immunoreactive (GAL-IR) neurons have been demonstrated in mammals and some submammalian

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species, but to our knowledge the only report available on presence of GAL-like peptide(s) in invertebrates is our study of the blowfly nervous system /2, 3/. Here we review the available data on the distribution and partial characterization of GAL immunoreactivity and localization of ^{125}I-GAL-binding sites in the blowfly brain.

We have used immunocytochemical, immunochemical (RIA) and chromatographic methods to provide evidence for presence of GAL-like peptide(s) in the blowfly Phormia terraenovae. Combined water and acetic acid extracts of fly heads were used for radioimmunoassay, gel filtration using Sephadex G25 and G50 columns and reversed phase HPLC /2, 3/. These experiments indicated that the GAL-IR represent basic peptide(s) of about the same molecular weight as porcine galanin. Several immunoreactive components from the fly extract can be seen in the chromatograms indicating the presence of several forms of GAL-like peptides; possibly some of these are extended forms.

Immunocytochemistry (using five different GAL antisera) indicated that there are about 160 GAL-IR neurons in the brain and subesophageal ganglia (Fig. 1). In the brain GAL-IR fibers supply specific subdivisions of the central body, superior protocerebrum, medulla of the optic lobe, tritocerebral neuropil and the subesophageal ganglion. Neurosecretory cells of the median neurosecretory group (MNC) in pars intercerebralis also display

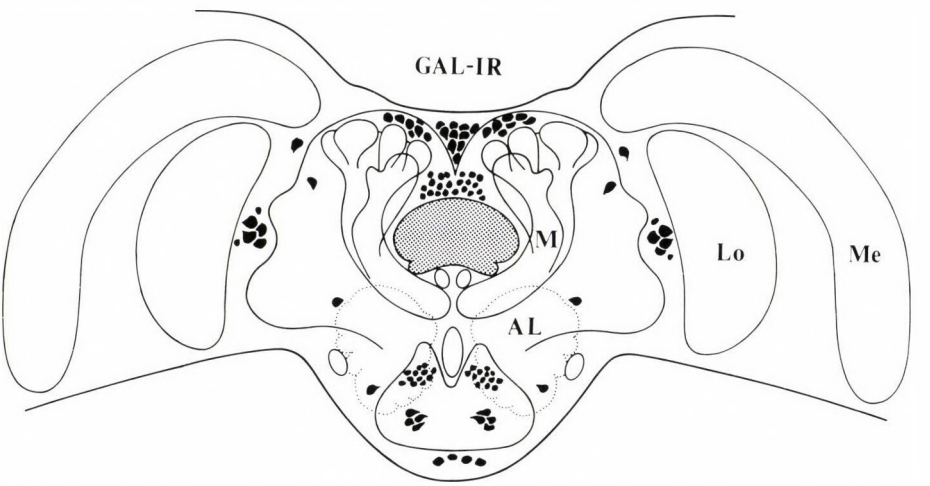

Fig. 1. Semischematic tracing of GAL-IR neurons in the brain of the blowfly Phormia terraenovae (frontal view). Both anterior and posterior cell bodies are shown in this compressed diagram. The fan-shaped body (stippled) of the central body complex is richly innervated by GAL-IR processes (from cluster of small cell bodies indicated just above the neuropil). Some other neuropils are indicated: the mushroom body (M), the antennal lobe (AL) and lobula (Lo) and medulla (Me) of the optic lobe

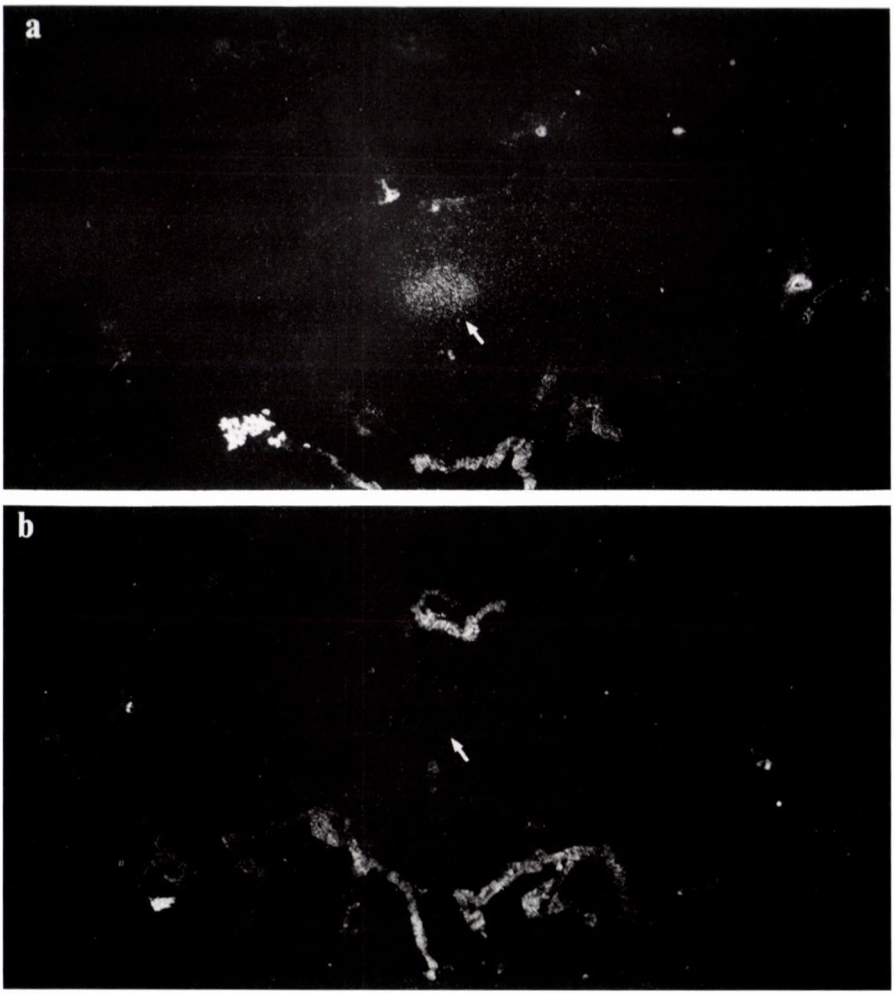

Fig. 2. Autoradiograms from frontal sections of blowfly brains. **a.** Prominent ^{125}I-galanin binding to the fan-shaped body neuropil (arrow). In no other region distinct labelling can be seen in this section. The ligand was applied in a concentration of about 0.2-2 nM (about 500 cpm/µl). **b.** Control section where ^{125}I-galanin has been displaced by $3 \cdot 10^{-7}$ M porcine galanin (GAL1-29). This micrograph shows unspecific binding. No labelling is seen in the fan-shaped body (located at arrow)

GAL immunoreactivity. Application of C-terminus specific antisera did not yield any immunolabelling in the fly brain, which is not surprising since the N-terminus is the best preserved portion of the GAL molecule in mammals /5/. Many of the GAL-IR neurons also react with an antiserum against a fragment of porcine GMAP (GMAP 19-41amide) as seen in immunocytochemical double labelling /3/.

The GAL immunolabelling seen in the central body complex is the most prominent (Fig. 1). A set of small cell bodies in the posterior protocerebrum invade the fan-shaped body where dense varicose arborizations can be seen. A set of axons leave the left and right side of the fan-shaped body and project laterally to the ventral body neuropils in each hemisphere.

We performed autoradiography of binding using HPLC purified chloramine-T-^{125}INa-labelled porcine GAL to fresh-frozen blowfly brain sections using a dip emulsion technique /2/. The experimental protocol in principle followed that of Melander et al. /4/ and Skofitsch et al. /7/. ^{125}I-GAL binding sites were found in the fan-shaped body (Fig. 2a) and in the ventral body neuropils of the central body complex. This binding was shown to be gradually displaced by increasing concentrations of porcine galanin (tested in a range of 10^{-9} - 10^{-6} M). Total displacement was obtained with $3 \cdot 10^{-7}$ M galanin (Fig. 2b).

Our findings indicate the presence of galanin-like peptide(s) distributed in a relatively small number of neurons some of which could be shown to supply processes to defined neuropil regions. Putative galanin binding sites in the central body complex indicate that a galanin-like peptide indeed may have a function as a neurotransmitter or neuromodulator in the fly brain. Possibly a neurosecretory role (release from corpus cardiacum) of the peptide(s) can be suggested since neurons of the MNC express strong GAL-IR.

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PIGMENT-DISPERSING HORMONE IMMUNOREACTIVE NEURONS IN THE BLOWFLY NERVOUS SYSTEM*

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We could demonstrate pigment-dispersing hormone immunoreactive (PDHIR) neurons in the brain and ventral ganglia of the blowfly Phormia terraenovae. PDHIR neurons were found in the optic lobe. Their processes supply the lamina, medulla and lobula complex bilaterally. Large PDHIR cell bodies in the protocerebrum have processes in the proto- and tritocerebrum and axons to the aorta wall and foregut. Eight pairs of PDHIR neurons are found dorsally and three pairs ventrally in the fused abdominal neuromeres; one pair is located ventrally in each of the thoracic neuromeres. The ventral abdominal PDHIR neurons are efferents that innervate the hindgut. PDHIR neurons may play different functional roles as neurohormones or neuromodulators in different parts of the nervous system and its peripheral targets.

Keywords: Neuropeptide - neurohormone - insect nervous system - visual system - pigment-dispersing hormone

Pigment-dispersing hormone (PDH) is a member of a family of related octadecapeptides isolated from different species of crustaceans and insects /3, 10-13/. PDH was originally isolated as a factor influencing screening pigment migration in shrimp compound eyes, but is also known to activate pigment dispersion in crab chromatophores /4, 12/. Recent immunocytochemical investigations of the central nervous system of some crustaceans and orthopteran insects indicate the presence of PDH-like peptide in interneurons, especially in the visual system /5, 6, 8, 9, 14/. These

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findings indicate that PDH in addition to being a neurohormone also may have a function as a neurotransmitter or neuromodulator in the central nervous system. The present investigation describes the distribution of PDH-like immunoreactivity (PDHLI) in the nervous system and its peripheral targets in the blowfly Phormia terraenovae.

Extracts from one hundred dissected brains of blowflies were used in a bioassay for melanophore pigment dispersion in destalked (eyestalkless) fiddler crabs, Uca pugilator. Utilizing serially diluted extracts, each dosage (in brain equivalents) was tested on five crabs. The observed pigment-dispersing responses were quantified in terms of Standard Integrated Response (SIR) values /4/ and compared with the known potency of synthetic β-PDH /12/. The blowfly extracts were shown to contain material with PDH activity at an estimated amount of about 30 fmol per brain /9/.

For immunocytochemistry tissue was fixed for four hours in 4% paraformaldehyde in 0.1 M phosphate buffer. Two rabbit antisera gainst PDH were tried: one against α-PDH /1/ and another against β-PDH /2/, both at a dilution of 1:1000 in the initial experiments. Only the β-PDH antiserum gave consistent immunolabelling. This antiserum gave strong immunostaining even at a dilution of 1:20 000 on blowfly tissue. The immunocytochemistry was performed with the peroxidase anti-peroxidase (PAP) method on cryostat sections as well as on whole tissues (pre-embedding technique) as described by Nässel and O'Shea /7/. The PDH antiserum was tested for its specificity by incubation with synthetic Uca β-PDH(10 nmol/ml diluted antiserum at 1:1000).

We could demonstrate PDHLI neurons throughout the neuromeres of the central nervous system of P. terraenovae. In the cephalic ganglion (brain, optic lobes and subesophageal ganglion) 34 PDHLI cell bodies can be seen. Of these, each optic lobe contains eight PDHLI cell bodies located at the anterior base of the medulla. These neurons supply processes to the lamina, medulla and lobula complex /8, 9/. Bundles of PDHLI axons also connect the left and right optic lobes via a posterior commissure. From the commissural PDHLI fibers collaterals innervate other regions of the midbrain such as the lateral horn adjacent to the mushroom body calyx.

In the midbrain there are eight pairs of large PDHLI cell bodies dorsally in the protocerebrum with processes in the superior protocerebrum, posterior deutocerebrum, tritocerebrum and the subesophageal ganglion (Fig. 1A). From six of these cells axons exit through the corpora cardiaca nerve (NCC 1) and through the corpora cardiaca, without forming terminals.

the corpora cardiaca, without forming terminals. These axons terminate in a region of the aorta wall and the muscles of the gastric caecum (at anterior midgut) and in the crop duct. Two additional cell bodies are found medially in the posterior protocerebrum.

Apart from the optic lobes, no glomerular neuropil region is supplied by PDHLI processes; all processes are found in non-glomerular neuropil of protocerebrum, posterior deutocerebrum and the subesophageal ganglion (Fig. 1A). In fact, glomerular neuropils such as central body and mushroom body calyces are closely surrounded by PDHLI fibers. Four brain commissures contain PDHLI fibers indicating substantial bilateral connections. Ascending PDHLI processes derived from neurons originating in the thoracic neuromeres can be seen in the subesophageal ganglion.

In the blowfly the thoracic and abdominal ganglia are fused. One pair of PDHLI neurons is located ventrally in each of the three thoracic neuromeres (Fig. 1B). In the eight abdominal neuromeres eight pairs of PDHLI neurons are found dorsally and three pairs ventrally (Fig. 1B). Immunoreactive processes are found in the neuropil along the midline in all thoracico-abdominal neuromeres. From the ventral abdominal PDHLI neuron fibers enter the median abdominal nerve from which they supply the caudal portion of the intestine: the posterior portion of the midgut and the hindgut.

So far α-PDH is found only in species of Pandalus (shrimp), whereas β-PDH and related peptides are more widely distributed among crustaceans and insects /10, 13/. The pigment-dispersing factors (PDF) from the cricket Acheta and the grasshopper Romalea are thus more closely related to β-PDH than α-PDH /13/. The PDF in Phormia may also follow this pattern since immunoreactivity was noted with the β-PDH antiserum but not with that raised against α-PDH. Results from bioassay of blowfly brain extracts for melanophore pigment-dispersion in crabs indicate the presence of about 35-40 fmol β-PDH-like material per brain /9/.

In the crustaceans Orconectes and Carcinus PDHLI was found in the neurohemal release site in the eyestalk termed the sinus gland /2, 6/ as well as in interneurons in the optic lobes, cerebral ganglia (brain) and ventral ganglia /5/. These authors suggested a neurotransmitter or neuromodulator role for PDH in the CNS in addition to its previously known hormonal role. The number of immunoreactive cell bodies in the nervous system of the crab and crayfish species investigated is lower (in the range of 16-30) than in the blowfly (about 54 cell bodies). Furthermore in the crustaceans cell bodies were only found in the optic lobes, brain, and

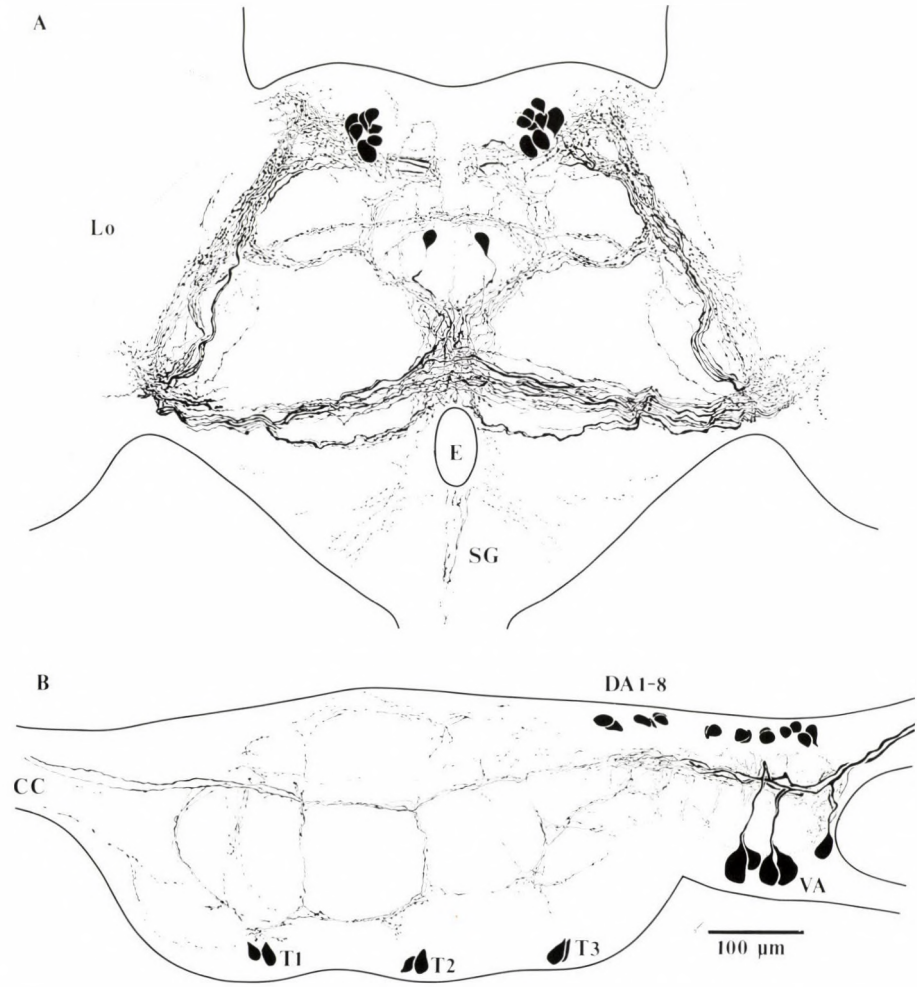

Fig. 1. Tracings of PDHLI neurons in the central nervous system of the blowfly Phormia terraenovae (from 25 µm Durcupan sections). **A.** Posterior region of the brain from frontal sections (the optic lobes are not drawn, except a small region of the lobula, Lo). All the PDHLI neurons of the midbrain are shown in this tracing. Note immunoreactive fibers in commissures and lateral bundles. Varicose terminals are found in non-glomerular neuropils only. E esophageal foramen; SG subesophageal ganglion. **B.** Sagittal view of the thoracico-abdominal ganglion with all the PDHLI neurons traced. The cervical connective, CC, is to the left. Three pairs of ventral cell bodies are seen in the thoracic neuromeres (T1-T3). Dorsal (DA1-8) and ventral (VA) abdominal cell bodies can be seen. The ventral ones form axons leaving the ganglion through the dorsal median abdominal nerve. Immunoreactive fibers are most prominent in the abdominal neuromeres. Note fibers in the cervical connective ascending towards the brain

subesophageal ganglia (in the crab neurons were also found in thoracic ganglia), but not in abdominal ganglia and no efferent PDHLI axons were detected in the nerve roots /5/ in contrast to the fly.

As in crustaceans /5/ PDH- or PDF-like peptide may act as neuromodulator, neurotransmitter and neurohormone in different neuronal systems in the blowfly. Furthermore, PDHLI neurons may play different functional roles in different parts of the blowfly nervous system. In the visual system and midbrain they may be involved in modulation of photoreceptor and interneuron properties, possibly as part of circadian regulatory circuits /8, 9/. From the aorta wall PDH-like peptide may be released as a neurohormone and at several sites the peptide may act on intestinal muscles and possibly other peripheral targets.

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STRUCTURAL PLASTICITY OF AN IMMUNOCHEMICALLY IDENTIFIED SET OF HONEYBEE OLFACTORY INTERNEURONES*

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Using a monoclonal antibody (FB 45) raised by Dr. A. Hofbauer (Würzburg) against Drosophila brain we investigated the development and plasticity of immunoreactive cells belonging to the median and lateral antennoglomerular tracts (AGTS) in the honeybee brain. In early stages of pupal development presumed AGT immunoreactivity was detected in the diffuse central neuropil of the antennal lobe as well as in the glomeruli, which differentiate at 40% pupal development. The lateral protocerebral lobe - one target area of the AGTs - is labelled throughout pupal life whereas labelling in the calyces is first restricted to the basal ring region. Although the lips of the calyces develop in middle-aged pupae, they do not show immunoreactivity until the last day of metamorphosis. Unilateral ablation performed on pupae of different stages resulted in size reduction of the antennal lobe and fusion of glomeruli. The number of labelled somata and glomeruli in the antennal lobe were reduced on the treated side. These effects were more prominent when ablation was performed in young pupae. No differences in staining intensity at the light microscopic level were found in the calyces. Therefore a pre-embedding immunohistological approach was developed to detect AGT profiles in the mushroom body at the electron microscopic level.

Keywords: Insects - brain - development - identified cells - EM-immunocytochemistry

We use a monoclonal antibody raised against Drosophila brain (FB 45) by Dr. A. Hofbauer (Würzburg) (1) to investigate developmental and injury induced plasticity in olfactory interneurones of the honeybee brain. In the bee brain various neurones are stained by FB 45 (for a detailled description, see Bicker, G., Kreissl, S., and Hofbauer, A., in prep.). Here

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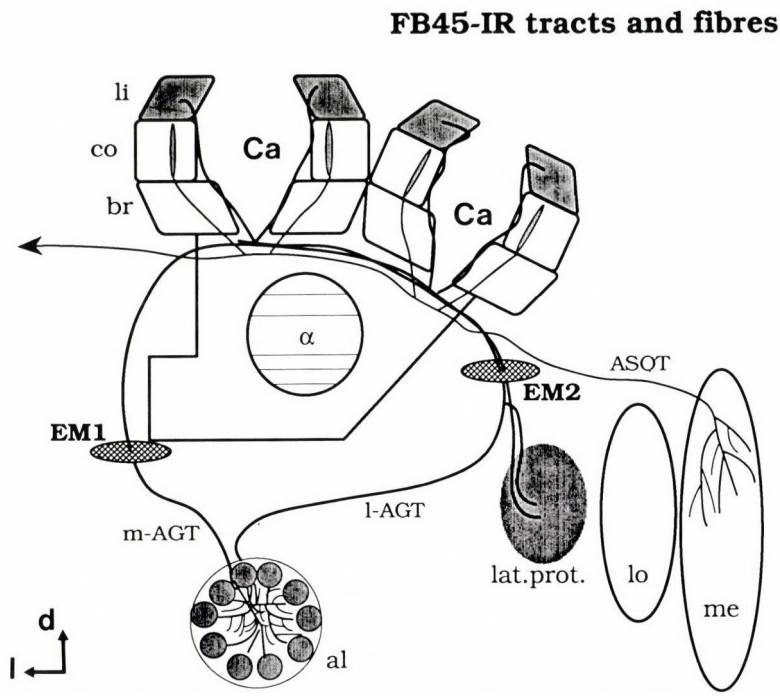

Fig. 1. Schematic of FB 45 labelled fiber tracts in the protocerebrum. Fb 45 stains two antennoglomerular tracts, m- and I-AGT, which run from the antennal lobe (al) into the protocerebrum and end in the lip neuropil (li) of the mushroom body calyces (Ca) and in the lateral protocerebrum (lat.prot). A small fiber tract originating in the medulla (me) enters the MB and terminates in the collar (co) without overlapping with the AGT endings

we focus on a subset of olfactory interneurones running in the antennoglomerular tracts (m-AGT, I-AGT). These neurones originate in the antennal lobe, project upwards to the protocerebrum and terminate in the calyces of the mushroom bodies (MB) and the lateral protocerebrum (Figs 1, 2c, f). The calyx is the main input region of the MB.It comprises three major subcompartments, lip, collar and basal ring (Fig. 1). This paper describes the pattern of FB 45 immunoreactivity during normal ontogenetic development and after deafferentation of the antennal input with the aim of examining the stereotyped plasticity in the olfactory system of the honeybee.

Honeybees and pupae of different stages were used. Operations (removing the antennal input on one side) were performed on young and old pupae.

Light microscopy: Between days 5 and 10 after emergence, dissected brains were fixed for 1 h in 4% formaldehyd in Na-phosphate buffer, 20 °C, washed in PBS (pH 7.4), dehydrated and embedded in Paraplast. Immunohisto-

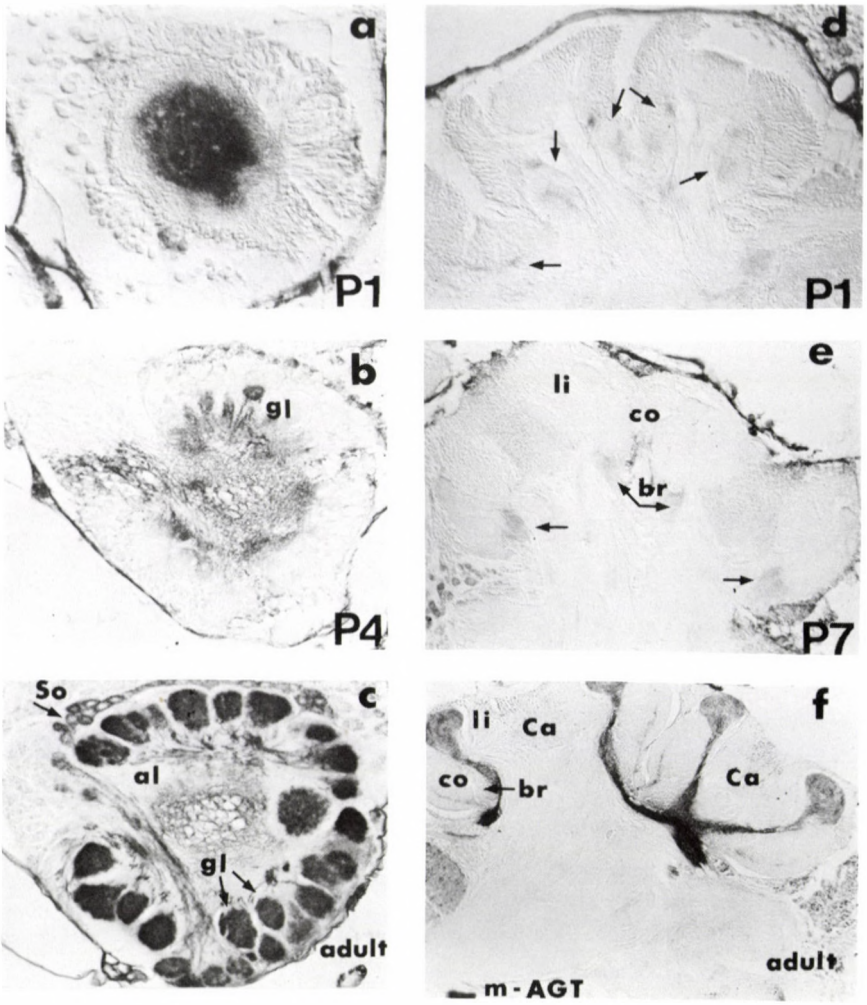

Fig. 2. Developmental changes in FB 45-IR in the antennal lobe (left) and the calyces of the mushroom body (right) for explanation, see text. P: pupa, al: antennal lobe, gl: glomeruli, So: somata, Ca: Calyx, li: lip, co: collar, br: basal ring

logical procedure were performed on 12 µm serial sections using the Avidin-Biotin-technique with diaminobenzidine (DAB) as a chromagen. The preparations were incubated overnight in primary antiserum (FB 45), 2 h in second antibody and 1 h in the ABC reagent. In all incubation steps 0.1% Triton-X-100 was added.

Electron microscopy: Immunostaining at the ultrastructural level followed the above procedure using the pre-embedding staining technique (2) on 30 µm vibratome sections but using a mixture of 0.1% glutaraldehyde

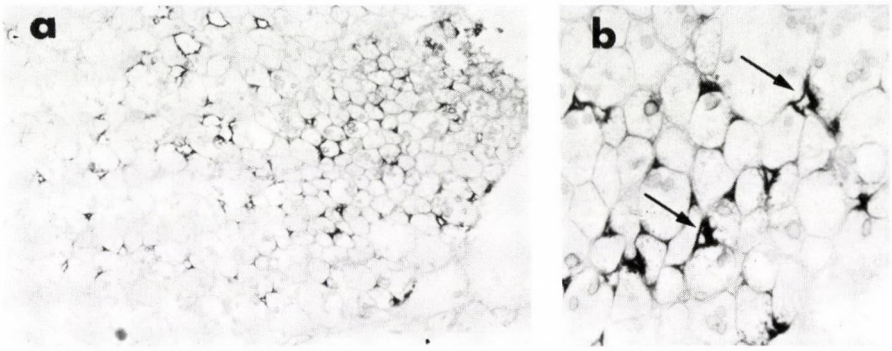

Fig. 3. Fb 45 labelling in the m-AGT at the ultrastructural level: **a**) horizontal section through m-AGT immuno-stained only part of the m-AGT fiber system arrows), **b**) higher resolution shows DAB reaction product located between the cells and on the membrane surface

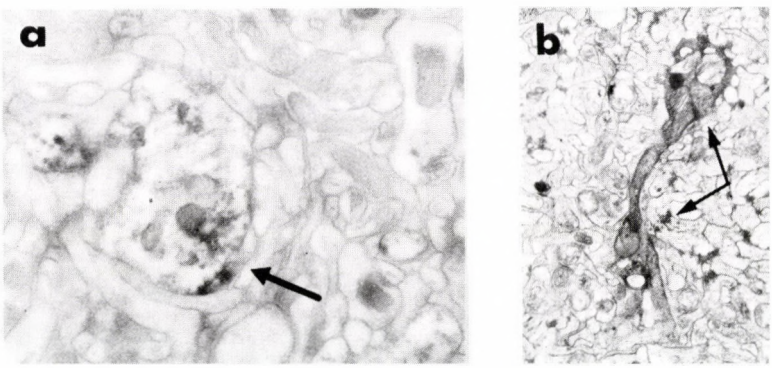

Fig. 4. **a**) in the lip of the calyces immunolabelling was predominanty found in large diameter boutons (arrow), indicating these as terminal endings of AGT fibers in the MB, **b**) a degenerated profile (arrows) of an antennal ablated animal in the same calycal region

4% formaldehyde as a fixative. After dehydration and osmication the sections were embedded in Durcupan. Ultrathin sections were contrasted with uranyl acetate/lead citrate and viewed with a Zeiss EM 10.

In one-day-old pupae (Fig. 2a), fine labelled AGT-fibres can be seen in the central neuropile of the antennal lobe (AL). AL-glomeruli appear on day 4 and already show FB 45-IR (Fig. 2b). In the adult, the central neuropile shows additional thick fibres and stronger immunoreactivity (Fig. 2c). The calyces of the mushroom bodies (MB) are not fully developed, but FB 45-IR can be seen in the presumptive basal ring (Fig. 2d). Six days later, the calyx is differentiated into basal ring, collar and lip region, but IR is still restricted to the basal ring area (Fig. 2e). Immunoreactive

fibres in the lip first appear in last day pupae. In adults the whole lip region is filled with FB 45-IR (Fig. 2f).

Unilateral antennal ablation during pupal development results in structural changes in the adult antennal lobe. The size of the antennal lobe and the number of glomeruli are reduced on the treated side, whereas the glomeruli are enlarged. On pupa days 4 to 7 old ablated animals (which were dissected between days 5 and 10 of adult stage) the central neuropile area in the AL remains as a fine and diffuse network, similar to its appearence in young pupae (see Fig. 2b). This effect is less prominent in animals operated on pupal days 8 and 9. No differences in FB-45 IR on the LM level are found in the MB calyces comparing the operated and normal sides. Therefore, ultrastructural analysis of AGT projections in the protocerebrum of normal bees were carried out in order to study the fine structure correlates of the ablation effects. Preliminary results show the following: 1. Axon countings performed in FB 45-IR areas in the protocerebrum (EM 1 and EM 2 in Fig. 1) reveal 520 profiles for the m-AGT (n=6) and 500 for the I-AGT (n=2). It is not clear yet whether all of these fibres are AGT-neurones or if the total of AGT neurones are labelled with the antibody, since only part of the fibres are labelled (Fig. 3a and b). 2. In the calyces FB-45 IR profiles are found in bouton-like structures, that are typical for extrinsic MB neurones in the lip region, indicating that these are the terminals of olfactory interneurones in the MB (Fig. 4a). 3. In a bee operated on day 4 of pupal development degenerated profiles in the lip of an adult are found (Fig. 4b). Since the structural parameter of olfactory interneurones in the protocerebrum (AGT fibres) and the MB (AGT terminals) are identified, investigations are necessary to clarify the synaptic organization and plasticity of these profiles in the mushroom bodies.

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ON THE FUNCTION OF HISTAMINE IN THE CENTRAL NERVOUS SYSTEM OF ARTHROPODS*

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Histamine is localized in the CNS of many invertebrate taxa by various methods. It is described to act as a neurotransmitter, neuromodulator, and neurohormone. Because of its distribution throughout these taxa it is tempting to forward the following hypothesis: Histamine acts as a neuroactive substance in all invertebrates. Its function as a transmitter of photoreceptors is restricted to arthropods. A special system of six large histamine-immunoreactive neurons exists exclusively in arachnids.

Keywords: Histamine - immunocytochemistry - Arachnida - neurotransmitters - visual system

Histamine is present in various invertebrates as a neurohormone, neuromodulator or neurotransmitter. In insects, histamine is located in photoreceptors and in interneurons of the visual system, the auditory system, the brain, and within the thoraco-abdominal ganglia, where histamine is considered to act as a neurohormone. There is evidence for many different functions and distributions of histamine in various taxa such as Merostomata, Insecta, Crustacea, and Mollusca; there are, however, no physiological or anatomical data on the function and localization of histamine in Arachnida.

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Animals

In all experiments, adult male and female animals of the genus Cupiennius salei Keys. (Araneae, Ctenidae) and Nephila clavipes (Araneae, Araneidae) from the laboratory stock were used. They were slightly anaesthetized with CO_2.

Vibratome sectioning

Immediate immersion of the dissected brains in freshly prepared 4% (w/v) carbodiimide (1-ethyl-3(3-dimethylpropyl)carbodiimide, Sigma) in 100 mM phosphate buffer for 16 h at 4 °C /17/ was followed by post-fixation in GPA (glutardialdehyde, picric acid, acetic acid) for 2-4 h at 4 °C. After rinsing in PBS (phosphate buffered saline) overnight at 4 °C, the brains were embedded in 5% agarose and sectioned on a vibratome to 70 µm. The sections were washed again in PBS overnight at 4 °C and then incubated in histamine antiserum (1:2000 in PBS, 10% NGS (normal goat serum, DAKO), 0.5% Triton X100) for 16 h at 4 °C. The antiserum was raised in rabbits against histamine which was conjugated to KLH (keyhole limpet hemocyanin) with carbodiimide /17/. Rising in PBS for 4 h at RT (room temperature) was followed by incubation with goat-anti-rabbit IgG (1:40 in PBS, Sigma, 3% NGS, 0.5% Triton X100) for 2 h at RT. Additional rinsing was followed by incubation with rabbit-peroxidase-antiperoxidase (PAP, DAKO, 1:100 in PBS, 3% NGS, 0.5% Triton X100) for 16 h at 4 °C. The peroxidase reaction was carried out in 0.05% DAB (diaminobenzidine, Sigma) and 0.002% H_2O_2 in 50 mM Tris buffer.The sections were dehydrated and coverslipped in Permount (Fisher Scientific).

Wholemount preparation

The wholemount staining procedure required the same fixation solutions as the agarose sections. In this procedure, postfixation with GPA was omitted because it slightly reduced the specific staining. The ganglia were rinsed in PBS for at least 16 h at 4 °C, followed by incubation with primary antiserum (1:2000 in PBS, 10% NGS, 1% TX100) for 72 h at 4°C. As a secondary antibody we used a goat-anti-rabbit FITC-conjugate (1:30, in PBS, Sigma). The brains were embedded in glycerine and photographed. In order to control specificity, the histamine antiserum was pre-adsorbed with histamine-succinylated-ovalbumin (5 µg/ml) and tested on the sections. No cross-reactivity with L-histidin-succinylated-BSA (bovine serum albumin) was found.

The coarse neuroanatomy of the CNS (fused suboesophageal ganglionic mass SOG; supraoesophageal ganglionic mass consisting of optic lobes OL and brain BR) of the wandering spider Cupiennius salei has been described earlier /2, 3/. In the present experiments we detected different classes of histamine immunoreactive (HAir) cells. Photoreceptors, visual interneurons, and a system of six large HAir cells spread over extended areas of the BR and SOG. Whenever possible, we assigned the axons of each identified cell to specific sensory- or motoric-tracts as they were described for C. salei /2/.

Photoreceptors

The photoreceptors of the eight eyes (anterior median AM, anterior lateral AL, posterior median PM, posterior lateral PL) from both species (C. salei and N. clavipes) were intensely stained. In the FITC wholemounts the immunolabelling was of equal intensity in the photoreceptors of all eight eyes, extending from the retina to the optic lamellae (Fig. 1A). Photoreceptor endings in the optic lamellae of all eyes were clearly labelled. We were unable to obtain wholemounts using the PAP-technique, as unspecific staining of the surrounding non-neuronal tissue was too intensive. The PAP-stained vibratome sections showed intensive staining in the terminals of the photoreceptor cells in the optic lamellae (Fig. 1B, C).

Visual interneurons

The labelled visual interneurons can be divided into subgroups according to shape, projection areas, neurite course, and somata position.

Monopolar cells

The so-called monopolar cells have very thin and unramified axons projecting to the optic lamellae. Their somata are located in the dorsolateral cellular cortex of the brain (Fig. 1C). They appear to function as intrinsic amacrine neurons, interconnecting other cells. The extension of the dendritic fields inside the lamellae could not be reconstructed because the intensive staining of the photoreceptor endings concealed all fine structures.

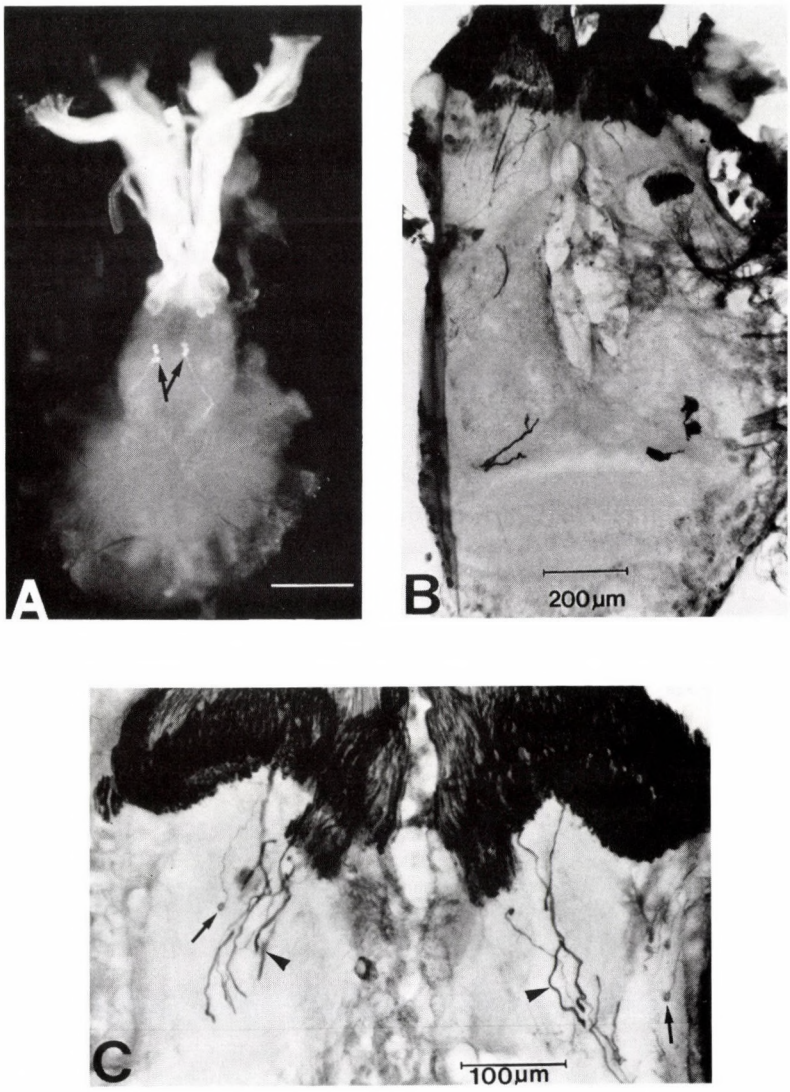
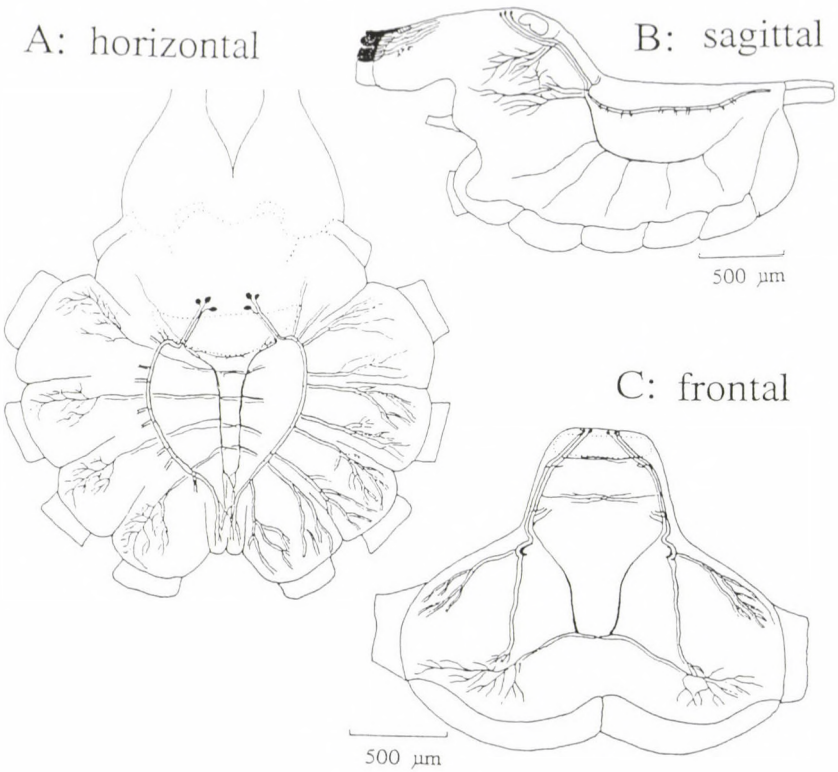


Fig. 1. **A.** Dorsal view of the CNS of a juvenile C. salei. Note intensive staining in all eight eye nerves and their respective optic lamellae. The six somata of the giant HAir-system are visible in the dorsal cellular cortex (⟶ , FITC, wholemount, scale bar 500 µm). **B.** HAir staining in the protocerebrum. The photoreceptor axons terminate in the intensively stained optic lamellae. Three somata of the right side lie directly in front of the central body. On the left side three main neurites descend in ventrolateral direction (PAP, vibratome section). **C.** HAir in the optic lamellae of the secondary eyes, with the different classes (see text) of visual interneurons (⟶, ▶, PAP, vibratome section)



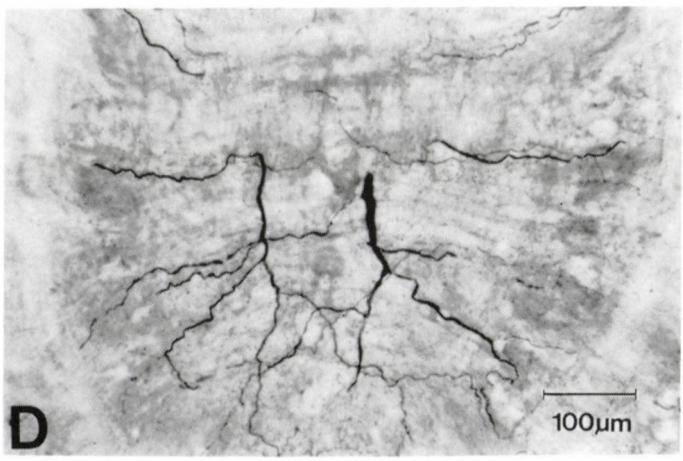


Fig. 2. Schematic views of the CNS of C. salei showing the giant HAir system in three different planes (**A, B, C**). In B only the cells of one hemisphere are shown. **D.** Dendrites of the ventral giant neurons in the sensory longitudinal tracts in the posterior part of the SOG. These plurisegmental projecting neurons have ipsi- and contralateral arborizations in the leg and opistosomal neuromeres (PAP, vibratome section)

First-order visual interneurons

This portion of the visual interneurons connect the lamellae of the secondary eyes to the glomeruli, a second-order visual neuropil. Here they have branched, small-field dendritic arborizations. Their somata are located in the cellular cortex posterior to those of the monopolar cells (Fig. 1C).

Second-order visual interneurons

The third subclass of cells comprises long-range interneurons. They connect the lamellae via the optic tract - passing the glomeruli without dendritic arborizations - with higher order visual centers.

Large HA-system

Six large HAir neurons, three per hemisphere, can be detected projecting from the brain to the SOG (Fig. 2A-C). The somata (average diameter 50 µm) of all six cells are located in the dorsal cellular cortex of the protocerebrum near the mid-sagittal plane. Their primary neurites (average diameter 10 µm, see Fig. 1B) descend ventrolaterally at the anterior side of the central body, then descend further in a dorsal tract at the posterolateral side of the tritocerebrum. All neurites have projections to the ipsilateral part of the central protocerebral area. Some of these projections have minor dendritic arborizations to the contralateral side. According to their suboesophageal arborizations, these six cells can be divided into two groups.

1. The dorsal system. Two pairs of these HAir-cells descend further ventrolaterally and join the motoric dorsolongitudinal tract to the opisthosomal neuropiles. They have ipsilateral projections at the anterior and posterior border of the pedipalpal- and leg-neuromeres to the dorsal motoric area (Fig. 2A). In addition they have a ventral projection to each neuromere which innervates the ventral sensoric area (Fig. 2C). Very tiny dendrites project to more median areas. Within the opsithosomal neuropiles they have contralateral projections.

2. The ventral system. The remaining pair of HAir neurons descends ventro-medially and joins a sensoric longitudinal tract (Fig. 2A, B). They have ipsi- and contralateral projections to the ventral sensory neuropile, where the afferent mechanosensory neurons of the legs terminate. These ar-

borizations apparently overlap on their respective sides. The contralateral projections increase in size in the posterior neuromeres of the SOG (Fig. 2D).

Histamine acts as a widespread neuroactive substance within many invertebrates. In the CNS of the sea hare and the locust (Aplysia californica, Locusta migratoria) both histamine and its receptor have been detected /10, 19, 23/. Synthesis and metabolism of histamine in the locust (Schistocerca gregaria) have also been described, as has its distribution in the locust (Locusta migratoria), the cockroach (Periplaneta americana), and the sphinx moth (Manduca sexta /8/). The autoradiographic localization of ^{3}H-histamine-binding was studied in the visual system of the locust /9/. Histamine is a neurotransmitter in the CNS and eyes of the lobster (Panulirus argus /4, 6/), and the neurotransmitter of photoreceptors in complex eyes and ocelli of Calliphora sp., Musca domestica, Locusta migratoria, and Schistocerca gregaria /11, 12, 20, 21/. Histamine has been localized immunocytochemically in the photoreceptors of complex eyes and ocelli of flies (Calliphora and Musca /14/) and the cockroach (Blaberus craniifer, /18/). Histamine is apparently the transmitter of photoreceptors in crustaceans as well (Balanus /6/). Histamine directly gates chloride channels in the lobster olfactory system (Panulirus argus, Homarus americanus /1, 5, 13, 16/. In the visual system of flies, histamine changes the chloride-permeability in lamina interneurons /12/. In crickets, histamine acts as an inhibitory transmitter in auditory interneurons /22/, and histamine-like immunoreactive neurons assume a neurohaemal function in the thoraco-abdominal ganglia of Drosophila and Calliphora /15/. In the present study histamine is localized in the CNS and the photoreceptors of two different spider species. The occurrence of histamine can therefore be divided on anatomical and physiological grounds into two components:

1. Histamine outside the visual system. In Merostomata, Arachnida, Crustacea, Insecta, and Mollusca histamine has been localized with different methods. In nearly all cases its function remains unclear. The distribution and localization often indicates a modulatory role in various sensory systems or a hormonal function as in insects.

2. Histamine in the visual system. Histamine is localized in the photoreceptors of Merostomata, Arachnida, Crustacea, and Insecta. The synthesis and localization of histamine is shown in many species; it also has light mimicking effects in insects. This indicates that histamine functions as the neurotransmitter of all photoreceptors within these taxa.

The localization and distribution of histamine as a neurotransmitter and neuromodulator in the nervous system of invertebrates suggests the following:

Histamine acts as a neurotransmitter, neuromodulator or neurohormone in all invertebrate taxa studied so far. Its function as a neurotransmitter in photoreceptors, however, seems to be restricted to arthropods. The large HAir-system we described apparently exists exclusively in arachnids and to date has no correlates in any other arthropod.

Acknowledgements

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OCCURRENCE AND DISTRIBUTION OF INSULIN RECEPTOR-LIKE IMMUNOREACTIVITY IN MOLLUSCAN BRAINS*

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Insulin receptor-like immunoreactivity (IR) was investigated in the central ganglia of different gastropod molluscs using a monoclonal antiserum raised against an epitope of the human placental insulin receptor. A well detectable and clear location of immunoreactive material was mainly found in the CNS of the freshwater snails Planorbarius corneus and Lymnaea stagnalis. This evidence correlates quite suggestively with the occurrence in these snails of a molluscan insulin peptide (MIP) previously shown. The widespread and discrete distribution of IR over a variety of physiologically distinct regions of P. corneus and L. stagnalis ganglia argues that these "receptors" may modulate multiple functions within the CNS.

Keywords: Central ganglia - insulin receptor - immunohistochemistry - gastropod molluscs

It has recently been shown that insulins and insulin-related peptides are produced in the central nervous system (CNS) of both vertebrates and invertebrates /1, 11, 12/. A family of genes that transcript for insulin-related peptides (MIPs) has been isolated and sequenced from the CNS of the snail Lymnaea stagnalis. The precursor molecules that they encode appear to be structurally similar to mammalian proinsulin, not only with respect to amino acid sequence but also as to tertiary structures /12/. Light microscopic immunocytochemical studies using antibodies raised against synthetic fragments of MIP I have shown the presence of immunore-

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activity (IR), in addition to the neuroendocrine light green cells and canopy cells of the cerebral ganglia of L. stagnalis, in homologous neurons of several other gastropod molluscs /14, 16/. The processing, storage and release of MIPs has been studied by immuno-electron microscopy in L. stagnalis /7/ and in Planorbarius corneus /14/. Whilst the role of MIPs as general growth hormones has been ascertained /5, 10/, an evidence for a specific role in the CNS is fragmentary: in P. corneus and L. stagnalis neuronal cultures, MIP(s) seem to promote neurite formation /9, 13/.

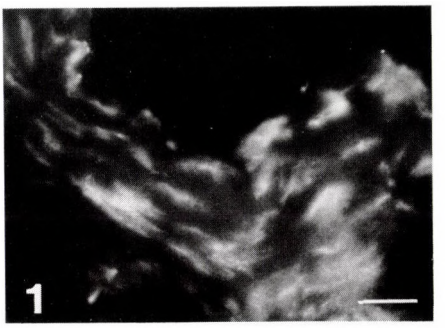
Insulin receptors in distinct regions of the mammalian brain have clearly been demonstrated in several studies employing mainly hormone binding procedures and autoradiography /3, 6, 8/. Monoclonal antibodies to insulin receptors have also been produced /4/, and recently by immunohistochemistry a distribution study of insulin receptor-like IR has been made on the rat forebrain /15/. Because no specific MIP(s)-receptors have been characterized or localized as yet in neither peripheral tissues nor the CNS, we investigated the presence and distribution of insulin-like receptors within the CNS of several gastropod species by immunohistochemistry, using a monoclonal antibody directed against an epitope located on the α-subunit of human placental insulin receptor.

We examined the CNS of gastropod species in which MIPs-IR was previously found viz. Planorbarius corneus, Lymnaea stagnalis (Basommatophoran Pulmonates), Helix pomatia, Limax maximus (Stilommatophoran Pulmonates), and some other species viz. Viviparus ater, Murex trunculus (Prosobranchs), Aplysia depilans (Opistobranchs). Cryostat sections (10 μm) of the central ganglia were fixed in chloroform:aceton (1:1) for 5 min at 4 ^{o}C. After rehydratation in PBS and blocking of non-specific binding by treating with 10% (v/v) normal goat serum diluted with PBS for 20 min at room temperature, the slides were incubated with the monoclonal antiserum (Amersham, England) diluted 1:50 with PBS containing 1% BSA overnight at 4 ^{o}C. After several rinses in PBS we processed the slides for immunofluorescence using as secondary antibody an anti-mouse IgG AMCA-conjugated (Jackson ImmunoRes., USA) or the Biotin/Avidin-peroxidase (Vector Lab., USA) method. Controls were made by omitting the primary antiserum or employing the antibody preadsorbed overnight at 4 ^{o}C with a human placental homogenate. Specificity of immunostaining was also tested on human placenta cryostat sections.

The employed antibody immunostains clearly human placental trophoblast (Fig. 1). Among the tested gastropods, a very well detectable and

reproducible pattern of distribution of insulin receptor-like immunoreactivity (IR) is found only in the CNS of P. corneus and L. stagnalis. In both of these snails the reaction product is localized in discrete clumps and bundles of fibers in the neuropils of all ganglia (Figs 2-3). Areas with an high density of insulin receptor-like IR are found in ventral sections of the cerebral ganglia near the origin of the labial nerves, but noticeable immunoreactive areas are also located in pedal and visceroparietal ganglia. In addition, IR is observed in bundles of fibers and single axons crossing the commissures and connectives and in distinct bundles of the majority of the nerves. Several neuronal somata are immunopositive in Lymnaea ganglia and more rarely in Planorbarius ganglia. Often in both the snails, immunonegative cell bodies are surrounded by immunofluorescent dots (Fig. 4). The pre-adsorption of the antibody with the placental homogenate abolishes the immunoreaction (Figs 5-6). Among the other gastropods only in L. maximus is a weak IR detectable in a bundle of fibers crossing the cerebral commissure and probably associated with a small cell cluster (Fig. 7), while in V. ater and M. trunculus signs of IR are observed in single fibers or granules without any reproducible pattern of organization. The CNS of H. pomatia and A. depilans result completely devoid of immunoreactive material (Figs 8-9).

The clear evidence of IR only in Planorbarius and Lymnaea ganglia is quite suggestive when correlated with the noticeable presence of MIP or MIP-like materials in the ganglia of these snails. The immunohistochemical data here presented are insufficient to determine if the detected IR might actually represent MIP(s) receptors. The three-dimensional model of MIP shows that a region particularly essential for the binding of insulin to its receptor differs from that of mammalian insulins, consequently it has been suggested that MIP cannot bind to the vertebrate insulin receptor. However, insulin from the gut of Lymnaea seems to bind very well to insulin receptors of rat fat cells /2/. The widespread and discrete distribution of human insulin receptor-like IR over a variety of physiologically distinct regions we found in the P. corneus and L. stagnalis ganglia argues that these "receptors" may nonetheless modulate multiple functions within the CNS.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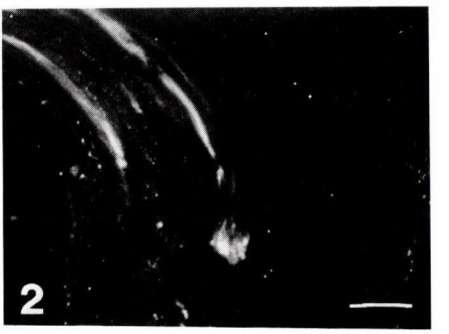
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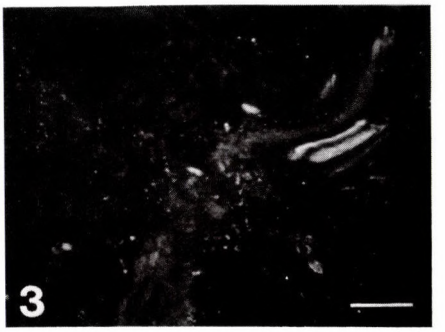
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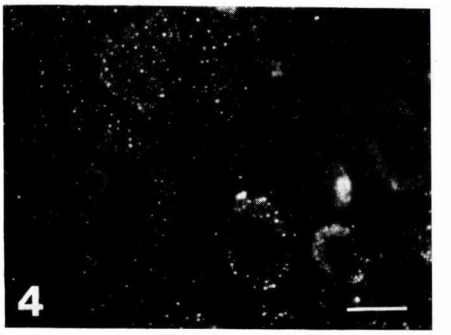
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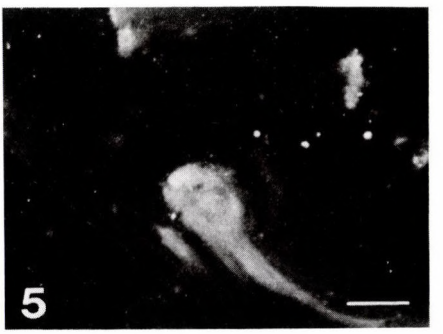
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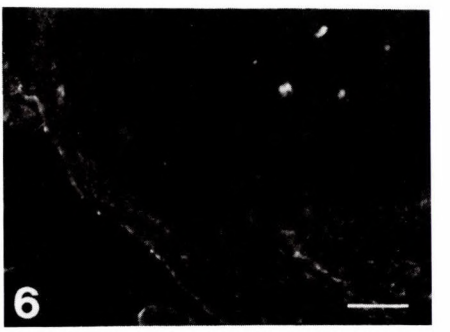
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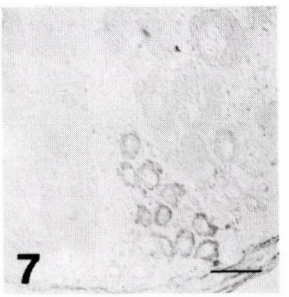
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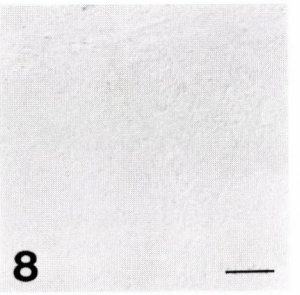
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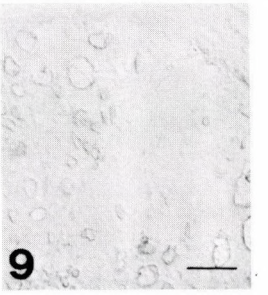
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Fig. 1. Immunofluorescent staining by the monoclonal antibody anti-human insulin receptor of a human placental trophoblast section. Secondary antibody anti-mouse IgG AMCA-conjugated. Bar: 50 µm

Fig. 2. Immunostaining as in Fig. 1 of a section through the right cerebral ganglion of L. stagnalis. Note the large bundles of immunopositive fibers crossing the commissure. Bar: 50 µm

Fig. 3. Immunostaining as in Fig. 1 of a section through the right cerebral ganglion of P. corneus. The reactivity is localized in the neuropil and in axons running to the lip nerve. Bar: 50 µm

Fig. 4. Immunostaining as in Fig. 1 of a section through the visceral ganglion of L. stagnalis. Immunonegative cell bodies are covered by immunopositive dots. Bar: 25 µm

Fig. 5. Immunostaining as in Fig. 1 of a section through the left parietal ganglion of P. corneus. Bar: 50 µm

Fig. 6. Immunostaining, after pre-adsorption of the antibody by human placental homogenate, of the section consecutive to the section of Fig. 5. The immunoreactivity is completely abolished. Bar: 50 µm

Fig. 7. Immunostaining using the biotin/avidin-peroxidase method of a section through the cerebral ganglion of L. maximus. A small cluster of weakly immunoreactive cells forms a bundle of axons crossing the commissure. Bar: 50 µm

Fig. 8. Immunostaining as in Fig. 7 of a section through the cerebral ganglion of H. pomatia. All the ganglia of this snail are completely devoid of any immunoreactivity. Bar: 50 µm

Fig. 9. Immunostaining as in Fig. 7 of a section through the abdominal ganglion of A. dépilans. No specific immunoreactivity has been detected. Bar: 50 µm

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BIOCHEMICAL CHARACTERIZATION OF A SEROTONERGIC SYSTEM IN THE NEURAL SHEATH OF HELIX GANGLIA*

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Seasonal alterations of serotonin (5HT) as well as the release of 5HT and the presence of the synthesizing enzyme of serotonin (5HTP-decarboxylase) were investigated in the neural sheath and desheathed ganglia of Helix pomatia. It has been established that i) serotonin concentration shows a seasonal variation in the sheath, ii) serotonin is not synthesized in the neural sheath, iii) K^+-dependent serotonin release occurs only in the desheathed ganglia.

Keywords: Helix pomatia - 5HT - 5HTP-decarboxylase - neural sheath - 5HT liberation

The multifunctional role (neurotransmitter, modulator and neurohormone) of serotonin (5HT), a major neurotransmitter in the snail nervous system, seems to be a general feature in invertebrates /1/. Immunocytochemistry revealed in the Helix central nervous system (CNS) that both the neuropil and the cell body layer was richly innervated by serotonergic fibers /2, 3/. In addition, networks of varicose 5HT-immunoreactive fibers was demonstrated in the neural sheath around the ganglia and peripheral nerves /3/. A role in neurohormonal regulation has been attributed to these surface elements. Earlier autoradiographic analysis /4/ showed a primary role of the neural sheath in the release and uptake of 5HT in

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gastropods. The aim of the present study was to perform a biochemical analysis of the distribution, synthesis and release of 5HT in the Helix CNS, with special attention to concentration ratios between sheath and ganglion, and to seasonal variations. All data obtained will also be compared with those on dopamine (DA), an other major monoamine neurotransmitter in the snail CNS /1/.

Snails were collected locally in Tihany and kept under moist conditions on a diet of lettuce. During the winter period, the hibernated snails were reactivated at room temperature two weeks before experiments. The circumoesophageal rings were dissected, and the central ganglia and the sheath were immediately separated without protease treatment.

The monoamine measurement was performed on individual CNSs by a Waters HPLC system using electrochemical detection. For the characterization of enzyme activity we used ganglion and sheath homogenate in 0.05M phosphate buffer pH=8.0. The concentration of 5-hydroxytryptophan (5HTP)

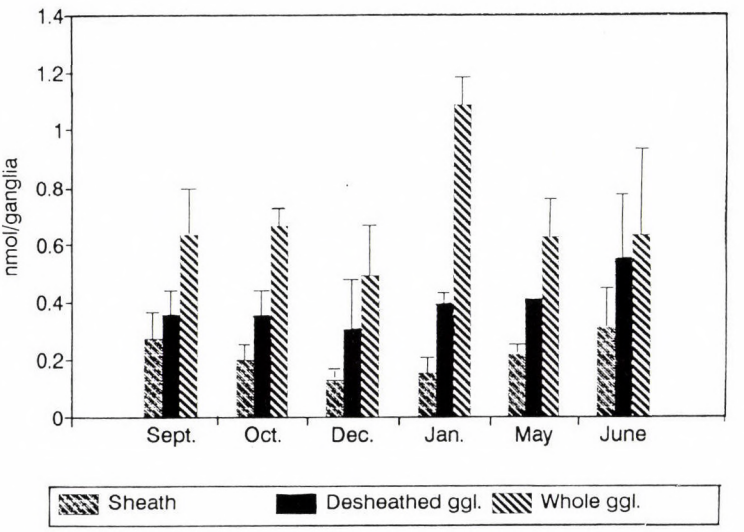

Fig. 1. Seasonal variations of the 5HT level in the snail CNS determined by HPLC-EC. Bars represent the average value of 10-12 experiments

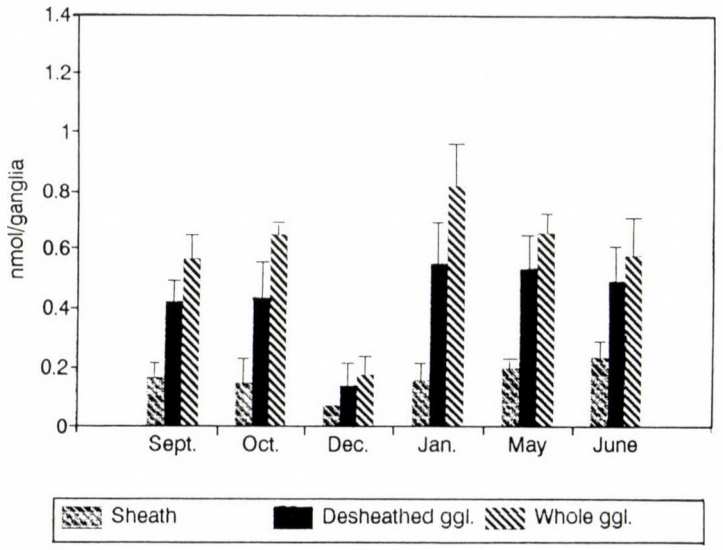

Fig. 2. Seasonal variations of the dopamine level in the snail CNS determined by HPLC-EC

was incubated with an aliquot of homogenate and 0.1 mM pyridoxal-5-phosphate cofactor at 20 ^{o}C for 20 min.

The K^{+}-evoked serotonin release was measured in Helix physiological solution, containing 0.1 M KCl and the uptake inhibitor imipramine in a concentration of 0.1 mM.

5HT is present in a relative significant concentration in the neural sheath of the Helix ganglia (Fig. 1), supporting that the 5HT-immunoreactive fiber system in the sheath /3/ represents indeed serotonin containing elements. Dopamine also occurs in the sheath (Fig. 2), but its exact cellular localization is yet to be determined.

During the active period of the annual life cycle of the animal the level of both 5HT and dopamine is higher in the central ganglia than in the sheath (Figs 1, 2). However, during winter (inactive period), not only the 5HT concentration is lower in the whole ganglion complex, but the ratio between the sheath and ganglion shows a shift toward the central ganglia.

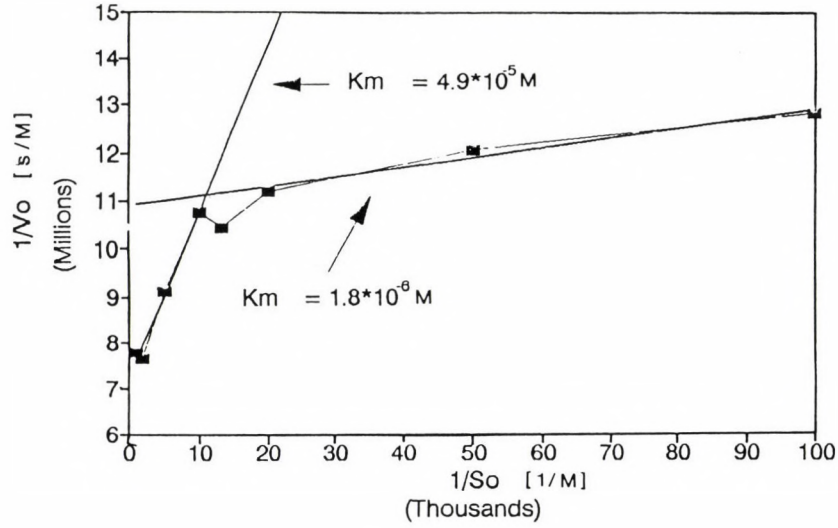

Fig. 3. The lineweaver-Bulk plot for 5HTP-decarboxylase in Helix CNS representing a low and a high affinity site

In Aplysia, it was found that /4/ K^+ evoked the release of ^{3}H-serotonin from both the ganglia and the sheath. In our experiment, however, K^+ evokes significant release of endogenous serotonin only in the desheathed ganglia, meanwhile release from the sheath does not show a K^+-dependent process (Fig. 4). These preliminary results suggest that 5HT may be released from the surface varicosities either spontaneously, or K^+ does evoke release, but the applied reuptake inhibitor fails to affect the reuptake process in the sheath.

Measurements on the synthesizing enzyme activity show that 5HTP-decarboxylase enzyme is present exclusively in the desheathed ganglia. The lack of 5HT synthesis in the sheath suggests that 5HT is synthesized by the central 5HT-ergic neurones, and then it is transported toward the sheath. Unlike the vertebrate brain /5/, the decarboxylase enzyme in the snail ganglia has a high and a low affinity component (Fig. 3). The low affinity component has a K_m value similar to that of vertebrates, however, the high affinity component has a K_m value lower by one magnitude.

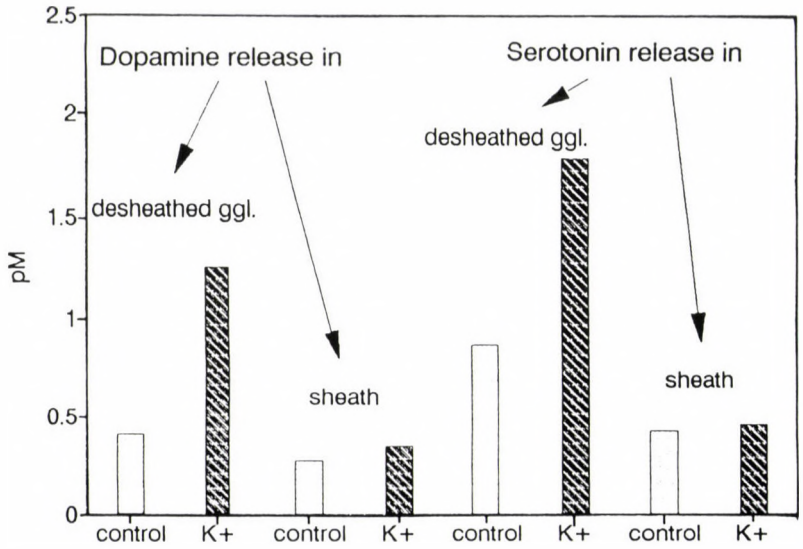

Fig. 4. K^+-evoked spontaneous release of 5HT in the sheath and in the desheathed ganglia

Acknowledgement

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PEPTIDERGIC AND AMINERGIC CENTERS IN THE HELIX CEREBRAL GANGLIA: SOMATOTOPY AND IMMUNOCYTOCHEMISTRY*

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In Helix cerebral ganglia the relationship between location of serotonin, dopamine, CARP, FMRFamide, Met-enkephalin, and substance P immunoreactive neurons and the different representation foci of the head areas was compared. The majority of immunoreactive neurons was located in representation foci, however, the extension of immunoreactive centers are larger than the representation foci. Therefore, the real extension of a representation focus with the additional non-efferent neurons might be larger than revealed by retrograde labelling.

Keywords: Amines - cerebral ganglion - immunocytochemistry - peptides - somatotopy

It has been described in the Aplysia CNS that different ganglia might be made up of a constant number of functional units consisting of a fixed but small number of large neurons and a variable number of small cells /4, 8/. It was demonstrated that a unit could be represented by a cluster of neurons where the neurons have similar physiological properties and could be immediately distinguished from each other /4, 7, 8, 12/. It was also suggested that the neurons of a cluster share the same transmitter biochemistry both in gastropods /8, 13/ and in lobster /11/. In the recent years numerous transmitter and modulator substances were demonstrated immunocytochemically in different neurons of the CNS in different gastropod

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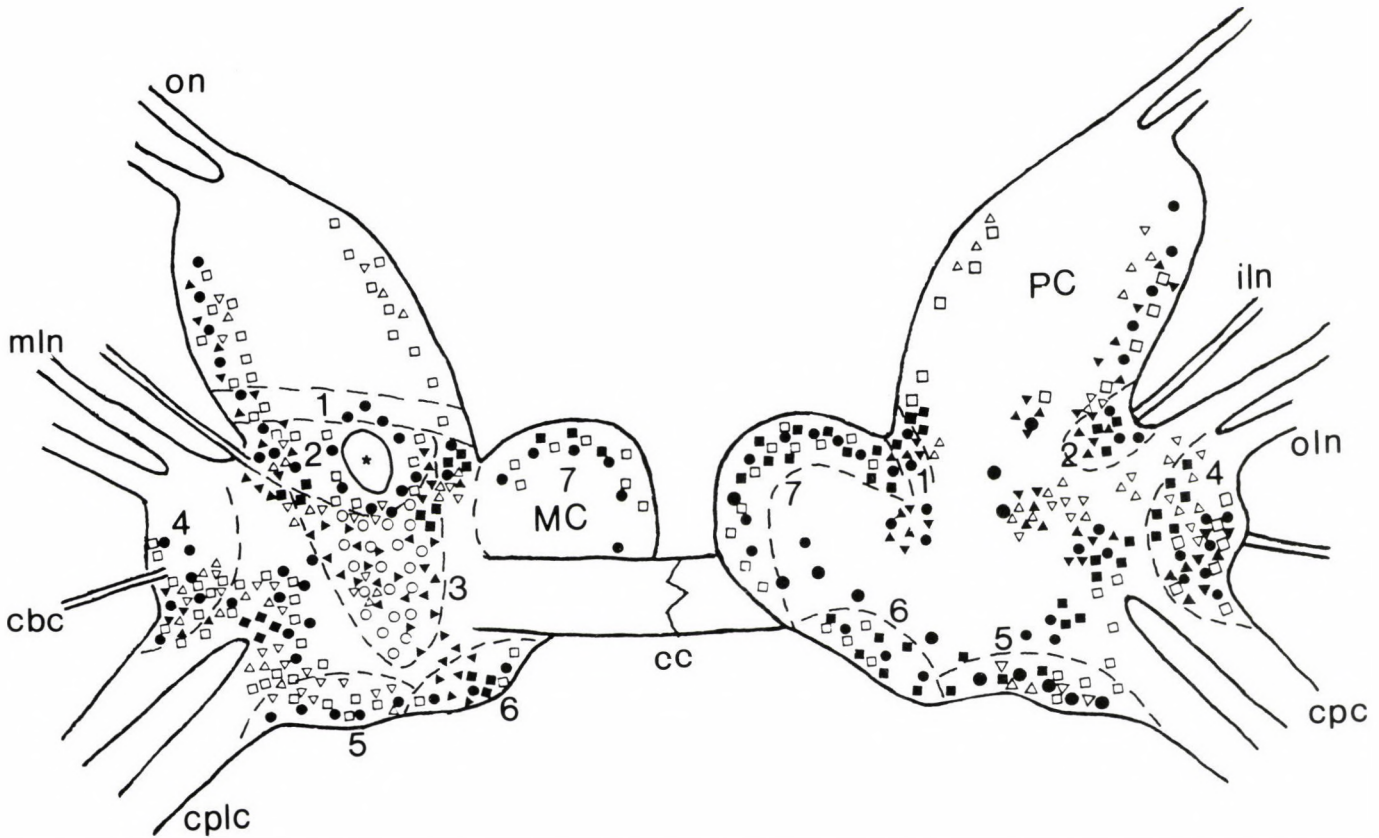

Fig. 1. The schematic drawing of the cerebral ganglion demonstrates the representation foci numbered from 1-7. The different symbols represent the distribution of neurons showing serotonin (o) - dopamine, (•) - CARP, (▲) - FMRFamide (■) - mEnk, (Δ) and SP (□) - immunoreactivity. PC: procerebrum, MC: mezocerebrum, V: ventral, D: dorsal, cc: cerebral commissure, cbc: cerebro-buccal connective, cpc: cerebropleural connective, iln: inner lip nerve, mln: medial lip nerve, oln: outer lip nerve, on: olfactory nerve, asteric: metacerebral giant cell

species such as serotonin /8, 9, 10/, dopamine /3/, GABA /1/, FMRFamide /2/. The distribution of neurons containing these substances show the characteristic feature, that they are located in distinct groups in the different ganglia of the CNS. It has been demonstrated that the different head regions of Helix are represented in the cerebral ganglion in a multifocal pattern on groups of neurons that send axonal processes to different head regions /6/. The representation of the different head regions in numerous foci shows a somatotopic order, and it was established that a representation focus is larger in extent than a cluster of neurons described in Aplysia CNS /4, 8/. This study compares the distribution of different amines-containing (serotonin, dopamine) and peptides-containing neurons (FMRFamide, catch relaxing peptide (CARP), Met-enkephalin (mENK), substance P (SP) with the location of the different representation foci described /6/ to investigate if any relationship can be established between them. The representation foci were determined by parallel cobalt-lysine and nickel-lysine backfilling of the different cerebral nerves /6/, while the distribution of the different amine and peptide containing neurons was visualized immunocytochemically. The distribution of the different amine-containing and peptide-containing neurons in the cerebral ganglia and their relationship to the different representation foci is summarized schematically in Fig. 1.

The distribution of different immunoreactive neurons shows that the majority of them are located in or close to the different representation foci. Analyzing the distribution of the different amine containing neurons it seems that the serotonin-containing and dopamine-containing neurons are separated from each other. The serotonin containing neurons are located exclusively in the representation focus 3, while dopamine-containing neurons can be found in all representation foci, except the focus 3. The different peptide containing neurons show that they cannot be clearly separated within a given representation focus.Coexistency of different substances could rarely be observed. In the representation focus 3, where somatotopy was clearly observed /6/, the unequivocal tendency of the separation of the different immunoreactive neurons could be observed. Since the immunoreactive centers are larger than the representation foci overlapped by them, the real extension of a representation focus might be larger than revealed by retrograde cobalt and nickel filling, which labels only the neurons that send axonal processes to the periphery. Therefore, it is suggested that the non-overlapping part ofan immunoreactive center re-

presents, in addition, local interneuron population of a representation focus. Furthermore, since each representation focus contains different types of immunoreactive neurons, it cannot be excluded that a focus involves different clusters of neurons that use different transmitters.

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CHARACTERIZATION OF CATCH-RELAXING PEPTIDE (CARP) IMMUNOREACTIVE NEURONS IN THE HELIX NERVOUS SYSTEM*

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Both small and large diameter of CARP immunoreactive neurons could be observed in the different ganglia of CNS of Helix but not in the foot musculature. The immunoreactivity is the strongest in the varicose segments of immunoreactive fibers. The present findings suggest a transmitter or modulatory role for CARP in both central and peripheral regulatory processes.

Keywords: Catch-relaxing peptide - CARP - CNS - foot - immunocytochemistry

The catch-relaxing peptide H-Ala-Met-Pro-Met-Leu-Arg-Leu-NH_2 (CARP) was isolated from the pedal ganglia of bivalve mollusc Mytilus /2/. The CARP proved to evoke relaxation in catch tension of Mytilus anterior byssus retractor muscle (ABRM) /4/ and proved to inhibit the contraction of different muscles in molluscs /1, 3, 4/. The presence of CARP immunoreactive neuronal elements have only been demonstrated in Rapana buccal ganglia and buccal muscles by light microscopic immunocytochemistry /1/. In this study we demonstrate the CARP immunoreactive neuronal elements in the Helix central nervous system (CNS) and in the pedal musculatur applying light microscopic immunocytochemistry in wholemount preparation of CNS as well as in paraffin sections of foot musculature according to Sternberger

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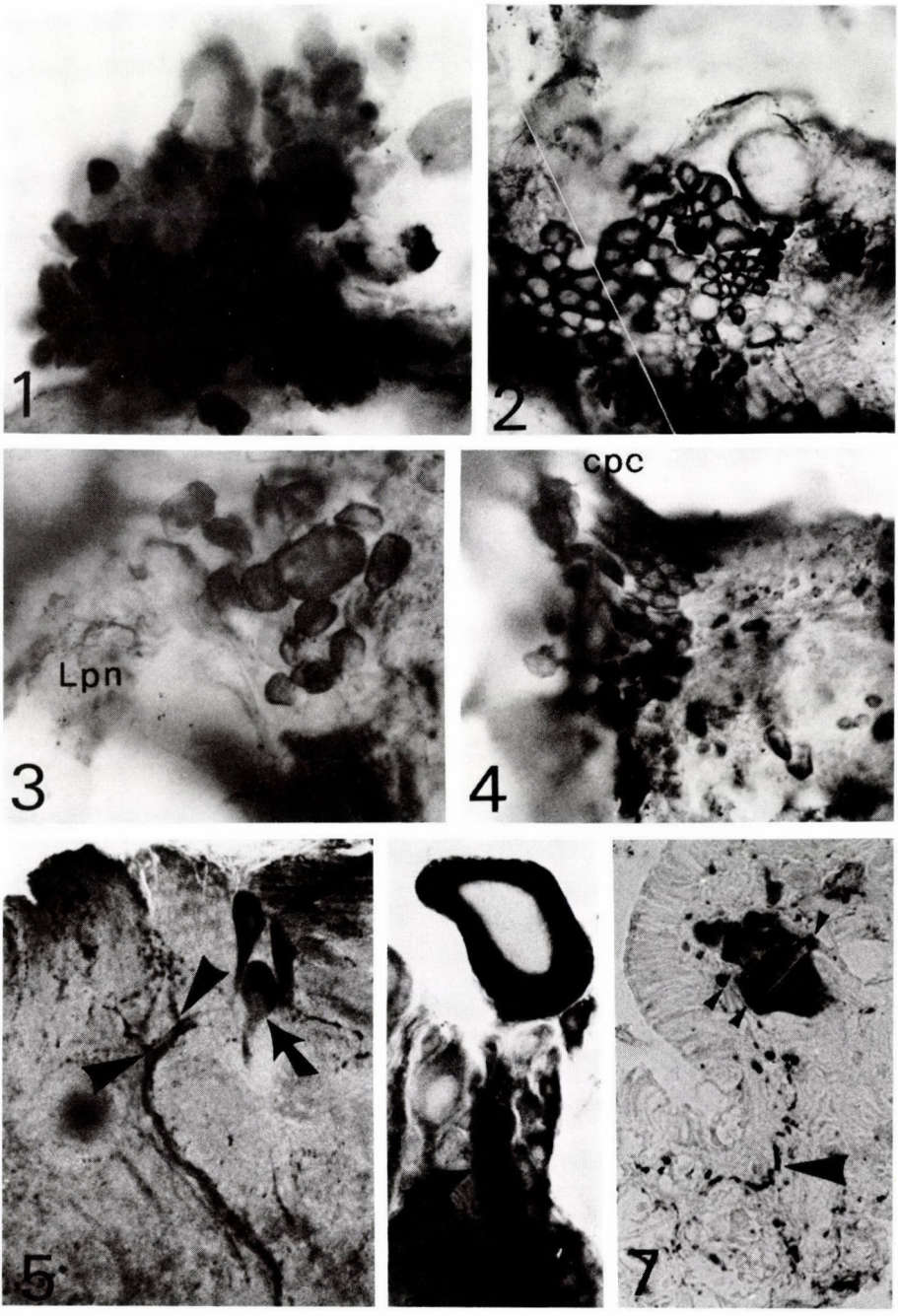
1
2
Lpn
3
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4
5
7

/5/. The immunoreactive fibers in the CNS were studied on araldite sections made from the embedded wholemount preparations. On wholemount preparations immunoreactive neurons can be observed in each ganglion of the CNS. In the buccal ganglion (Fig. 1) CARP immunoreactivity can be detected only in small-sized neurons while in other ganglia as in cerebral (Fig. 2), parietal (Fig. 3), and pedal (Fig. 4) ganglia CARP immunoreactivity can be observed in both small and large diameter neurons. In araldite sections it is demonstrated that immunoreactive fibers can be observed in the neuropil area of ganglia (Fig. 5). The fibers proximal to the cell bodies usually are thick and show week immunoreactivity (Figs 5, 6), while distally in the neuropil areas they branch and their fine branches have numerous varicosities that show intense immunoreactivity (Fig. 5). In the foot musculature numerous fine varicose fibers can be detected running over muscle cells and close to the gland cells (Fig. 7). Since CARP immunoreactive neurons can be detected throughout the CNS and immunoreactive fibers can be observed in the pedal musculature, it can be considered that the CARP immunoreactive neuronal elements play a role in the regulation and modulation of the musculature in Helix pomatia.

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Figs 1-4. CARP immunoreactive neurons in wholemount preparations from the buccal (Fig. 1), cerebral (Fig. 2), left parietal (Fig. 3) and pedal (Fig. 4) ganglia

Figs 5-6. Araldit sections demonstrate small (Fig. 5) and large diameter neurons (Fig. 6). Their proximal axon segments (arrow) have pale immunoreactivity, while their fine sidebranches have fine varicosities and show strong immunoreactivity (arrowheads)

Fig. 7. In the paraffin section of the foot demonstrate CARP immunoreactive fibers that run on muscle fibers (large arrowheads) and close to gland cells (small arrowheads)

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SEROTONERGIC INPUT ON IDENTIFIED COMMAND NEURONS IN *HELIX*[*]

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In *Helix*, serotonin evokes long-lasting alteration of activity of withdrawal triggering neurons. Cell bodies of these neurones are surrounded by a dense network of serotonin-containing fibres without any synaptic membrane specializations, which confirm the suggestion on the non-synaptic, modulatory action of serotonin on the withdrawal command elements.

Keywords: *Helix pomatia* - withdrawal reaction - serotonin - immunohistochemistry - modulatory action

In *Helix*, food conditioned aversive behavior cannot be evoked when serotonin level is decreased by toxic serotonin analogue (5,7-dihydroxytryptamine: 5,7-DHT) treatment prior to training sessions /1/. Cellular studies suggest, that aversive conditioning is dependent on the normal function of serotonergic system located presynaptically to the command neurones responsible for triggering motor elements of withdrawal behavior /2, 8/. However, it has remained to be clarified whether the serotonergic elements are connected to the command system directly or through a polysynaptic pathway. Immunohistochemical visualization of serotonin-containing elements demonstrated a rich serotonergic innervation of both cell bodies and the neuropil throughout the central nervous system (CNS) of *Helix* /3, 4/.

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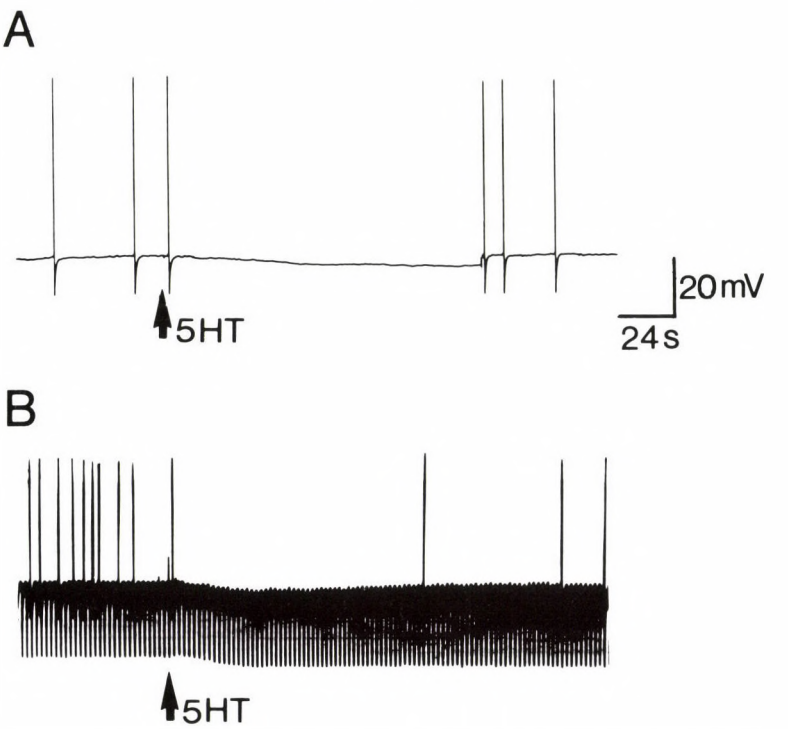
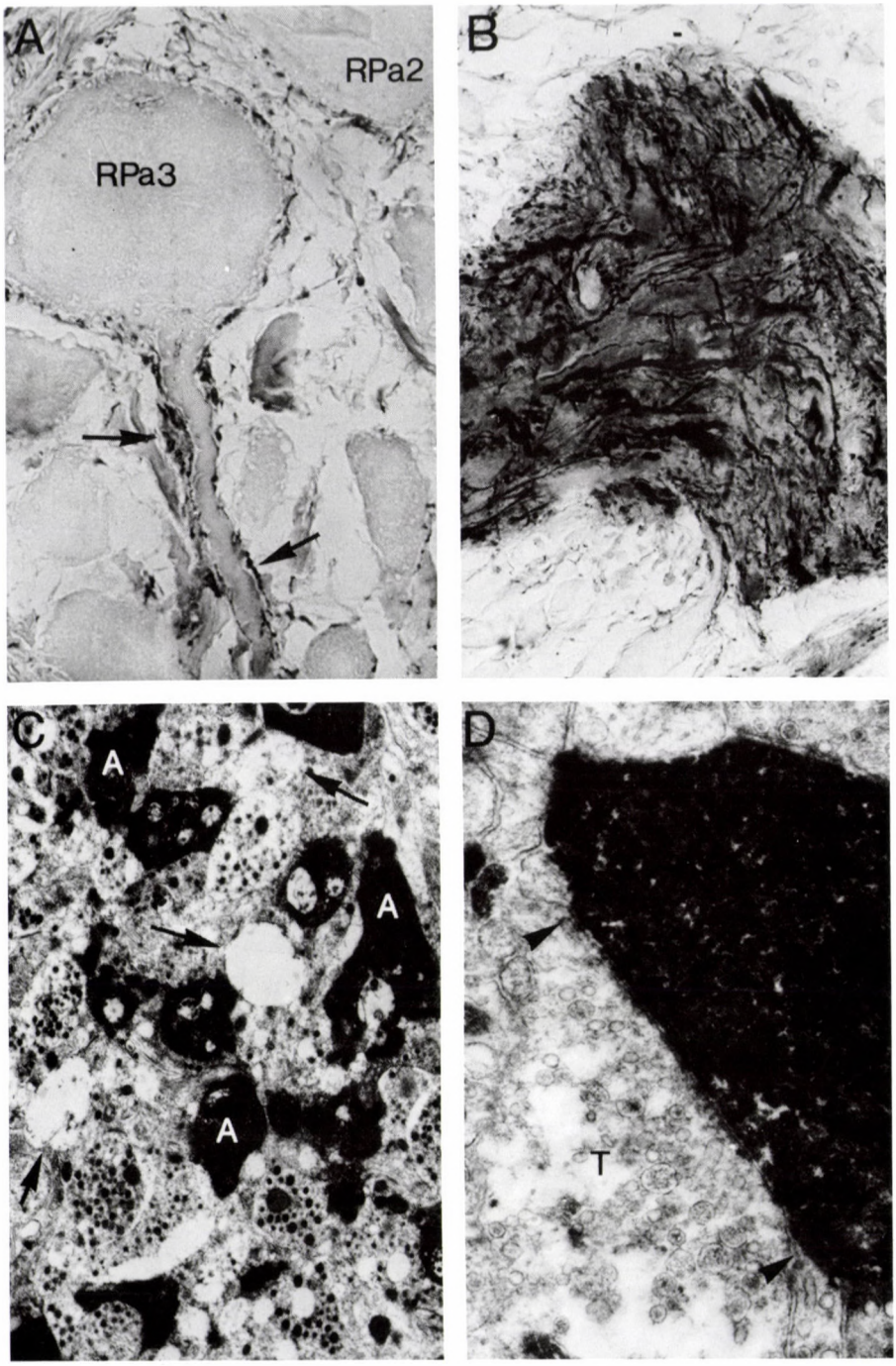


Fig. 1. **A.** Extracellularly applied serotonin inhibits the firing of the left pleural, LPl1 neurone (MP: 45 mV). **B.** Hyperpolarizing current pulses were applied through a second, independent microelectrode for detecting the changes of the membrane resistance during serotonin effect

The aim of our present study was to specify the relationship of the serotonin-containing neuronal elements to the identified neurones responsible for triggering withdrawal reactions.

Electriphysiological experiments were carried out on isolated Helix CNS preparations, using conventional microelectrophysiological method and normal Helix saline as standard /7/.

Fig. 2. **A.** In the right parietal ganglion, perikarya of RPa2 and RPa3 neurones are covered by 5-HT-IR varicosities. Immunoreactive elements form a dens network also along the primary neurite (large arrow). Paraffin section. x 500; **B.** Detail of the neuropile of the left parietal ganglion, densely supplied with 5HT-IR elements of thick axons and fine varicose fibers. Paraffin section. x 500; **C.** Low power electron microscopic detail from the neuropile of the right parietal ganglion. numerous HRP-filled RPa2 neuronal elements (A) and electron-lucent 5,6-DHT affected axon profiles occur (arrows). x 9000; **D.** 5,6-DHT affected varicosities (T) contact an HRP-filled profile of RPa2 neurone. Apart some vesicle accumulation, no typical membrane specialization can be seen on at the attachment (between arrowheads). x 20 000


A
RPa2
RPa3
B
C
A
A
A
D
T

Serotonin applied extracellularly into the bath evokes long lasting inhibition of the spontaneous activity of the identified pleural (LP11 and LP12) and parietal (LPa2, LPa3) giant neurones, accompanied with a slight, short time decrease of the membrane resistance (Fig. 1).

For visualizing serotonin-containing elements, 5HT immunohystochemistry was carried out on paraffin sections according to Sternberger /9/.

The cell bodies of all the investigated pleural (LP11 and LP12) and parietal (LPa2, LPa3, RPa2, RPa3) giant neurones were covered by serotonin-immunoreactive (5-HT-IR) elements moreover 5-HT-IR varicosities could be observed along the primary neurites of the neurons (Fig. 2A). The neuropil of the ganglia also contained 5HT-IR elements; thick axonal processes and finer varicose branches formed a dense network in the pleural and parietal ganglia (Fig. 2B).

For ultrastructural studies the double-labelling method was used /5/. Three weeks prior to the experiments serotonin neurotoxin (5,6-dihydroxy-tryptamine: 5,6-DHT; 10 mg/kg body weight) was injected into the animals, which selectively affects the serotonergic system /6/. Following this treatment intracellular HRP staining was carried out on individual, identified pleural and parietal neurones (LP11, LPa2, PLa3).

In the neuropil, many serotonin-containing (neurotoxin-affected) fibers and HRP-labelled elements of the identified giant neurones could be observed (Fig. 2C). Several axo-axonic contacts could be detected between them in high power electron micrographs. However, true synapses characterized by typical pre- and postsynaptic membrane specializations could not be observed (Fig. 2D).

Our present results proving the existence of non-synaptic serotonergic input on identified command neurons confirm the previous suggestion /10/ that the serotonergic system exerts a modulatory effect rather than a "classical" synaptic influence on the command elements, responsible for triggering the aversive reaction in Helix. This modulatory effect can be realized at both axo-somatic and axo-axonic levels.

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NEURONAL MAPPING STUDIES ON THE CENTRAL NERVOUS SYSTEM OF THE PULMONATE SNAIL HELISOMA TRIVOLVIS*

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The need for a generalized system for mapping neurons in the central ring ganglia of the freshwater pulmonate snail, Helisoma trivolvis, has recently become apparent. In the present study we have used retrograde staining of nerves to identify neurons projecting to the periphery. This map was combined with histochemical information to produce a map of neuronal clusters and landmark cells. Our mapping strategy was formulated to make direct comparison with maps already established for closely related species, i.e., Lymnaea stagnalis.

Keywords: Mollusc - CNS - map

The majority of studies carried out on the nervous system of the pond snail Helisoma trivolvis have focused on a limited number of identified neurons belonging to the paired buccal ganglia. Recently, as our interest turned toward examining behaviors mediated or modulated by neurons of other central ganglia, the need for a generalized system for mapping neurons in Helisoma became apparent. In the present study we have used retrograde filling of nerves with cobalt chloride to identify neurons which have axons projecting to the periphery. These data were then combined with histochemical information (e.g., glyoxylic acid histofluorescence of biogenic amines, serotonin and FMRFamide immunohistochemistry, etc.) to

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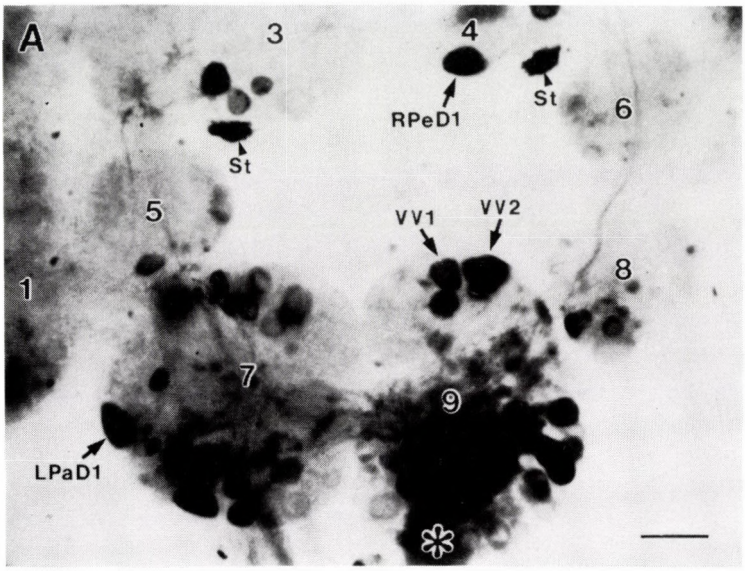

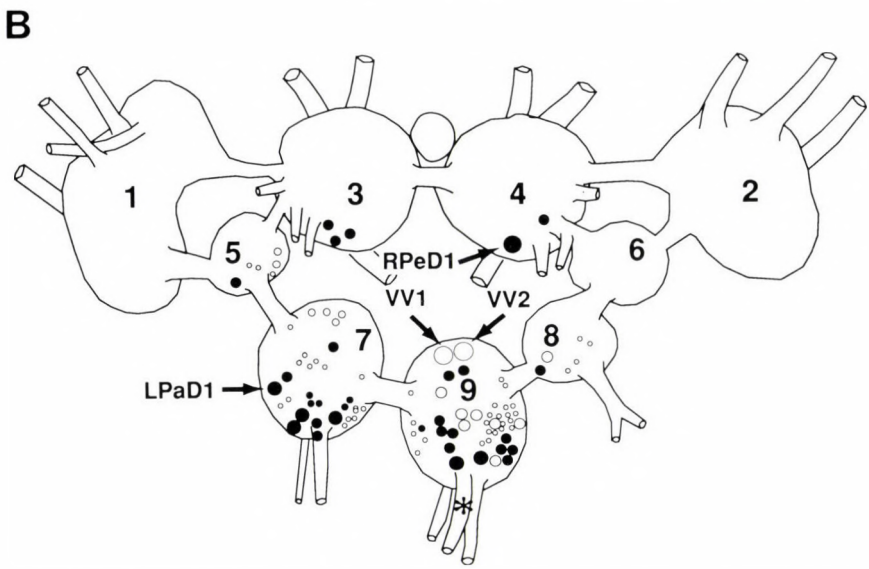

Fig. 1. (**A**) Representative cobalt chloride backfill (dorsal view) of the visceral ganglion anal nerve of Helisoma showing a large number of stained neurons, including 4 landmark cells. (**B**) Computer generated map compiled from 5 anal nerve preparations showing neurons positioned on the dorsal (solid circles) and ventral (open circles) surfaces of the central ganglia. In both (A) and (B), ganglia are numbered as follows: left and right cerebral ganglia (1, 2); left and right pedal ganglia (3, 4); left and right pleural ganglia (5, 6); left and right parietal ganglia (7, 8); visceral ganglion (9). Landmark cells: Right Pedal Dorsal 1 (RPeD1); Visceral Ventral 1 (VV1); Visceral Ventral 2 (VV2); Left Parietal Dorsal 1 (LPaD1). Other labelled structures: statocyst organs (St); anal nerve trunk (asterisk). Note: The cerebral commissure has been cut and the cerebral ganglia reflected back to expose the subesophageal ganglia. Calibration bar: 200 μm

produce a map of neuronal clusters and landmark cells. Our mapping strategy for Helisoma neurons was formulated to facilitate direct comparison with maps already established for such closely related species as Lymnaea stagnalis /1, 2, 4/.

Cobalt chloride backfilling was carried out on 23 prominent nerves emanating from the central ganglionic ring of Helisoma. For this purpose we modified the protocol of Nelson and Audesirk /3/, substituting 0.4 M $CoCl_2$ for 0.25 M cobalt hexamine chloride. After immersion of a nerve for 48-96 h in $CoCl_2$, the ganglionic rings were fixed in 10% phosphate buffered formalin (pH 7.4), developed in 7% ammonium sulphide, dehydrated in an ethanol series, cleared in dimethyl sulfoxide and mounted in methyl salicylate. Drawings of each preparation were initially made via camera lucida or from photomicrographs. The data were then compiled and maps were reconstructed using Adobe Illustrator 88 (tm) software on an Apple MacIntosh computer with the assistance of Dr. R. Hawkes (University of Calgary).

As an example of the data obtained, Fig. 1A shows a cobalt chloride backfill of the anal nerve of a Helisoma visceral ganglion (dorsal view). Information collected from 5 such preparations was compiled to create the computer generated map of neurons identified on the dorsal and ventral surfaces of the central ganglionic ring from backfills of the anal nerve, including several landmark cells (Fig. 1B). Similar maps were generated for the other 22 nerves examined (data not shown).

Maps constructed in this manner were used to identify landmark cells and to aid in the establishment of demarcation lines for neuronal clusters. The resultant generalized system of mapping Helisoma neurons is illustrated in Fig. 2. Landmark cells were chosen as those readily identifiable as individual neurons in living preparations by their position, size, and coloration within specific ganglia. Nomenclature for these cells follows that previously established for "giant cells" of Lymnaea /1, 2, 4/: 1) ganglion location, e.g., left pedal ganglion (LPe); 2) ganglionic position, e.g., dorsal (D) or ventral (V); 3) arbitrarily assigned number /1, 2, 3, etc./. Thus, landmark cell "Left Pedal Dorsal 1" is abbreviated "LPeD1"; periods between letters are optional.

Cells of similar size and coloration often lie in apparently discrete groups within the ganglia. When such information is correlated with other data (e.g., $CoCl_2$ backfilling) it is possible to partition the surfaces of the ganglia into clusters, delineated by specific landmarks such as nerve trunks, neuropil boundaries, and landmark cells. As previously done for

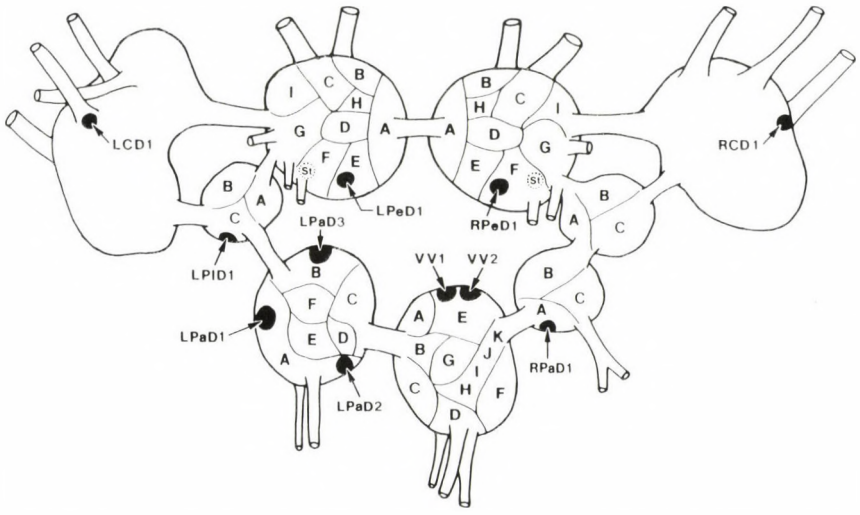

Fig. 2. Composite map showing the position of landmark cells and the demarcation of neuron clusters (A, B, C etc.) established for the dorsal surfaces of the ganglia of the subesophageal ring of Helisoma. The map is based primarily on in situ observations and $CoCl_2$ backfills of nerves. Ganglia are positioned as in Fig. 1B; ventral surfaces of the cerebral ganglia are shown (thus, cluster designations are omitted). Landmark cells include: Left and Right Cerebral Dorsal 1 (LCeD1, RCeD1); Left and Right Pedal Dorsal 1 (LPeD1, RPeD1); Left and Right Parietal Dorsal 1 (LPaD1, RPaD1); Left Parietal Dorsal 2 and 3 (LPaD2, LPaD3); Visceral Ventral 1 and 2 (VV1, VV2), Left Pleural Dorsal 1 (LPlD1). Statocyst organs (St)

Lymnaea /1, 2, 4/, we have assigned letters (A, B, C, etc.) to these clusters, preceded by the abbreviation for the ganglion in which they are located (Fig. 2). Thus, the"Right Pedal A cluster" is abbreviated "RPe-A cluster"; the dash being used to help distinguish clusters from landmark cells.

In conclusion, this study presents a method for systematical mapping of neurons within the central ganglia of Helisoma which can be used as a reference for more detailed morphological and physiological experiments. The maps can facilitate the incorporation of data obtained from many techniques (e.g., immunohistochemistry and dye iontophoresis). These maps are also useful for comparative studies, such as determining homology among neurons of different gastropod species.

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IN VITRO EVIDENCE FOR MULTIPLE NEURITOGENIC FACTORS IN THE CENTRAL NERVOUS SYSTEM OF PULMONATE MOLLUSCS*

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(Accepted: 1991-08-30)

Previous work has shown that neurons of the fresh water pond snails, Lymnaea and Helisoma, require soluble factors produced by neural tissues for neurite outgrowth to occur in vitro. In the present study, we show that mammalian nerve growth factor (NGF) stimulates neurite outgrowth of specific Lymnaea neurons. In contrast to motoneurons and interneurons, which show a robust dose response to NGF, no response was observed in neurosecretory cells. In an attempt to localize neuritogenic activity to specific ganglia or organs, we show that the dorsal bodies, endocrine structures of the cerebral ganglia, promote neurite outgrowth of specific neurons. In general, however, the spectrum of neurons that respond to dorsal body cell conditioned medium differs from that which respond to NGF. We conclude that Lymnaea neurons respond both to NGF and also to a separate factor derived from the dorsal body cells.

Keywords: Mollusc - culture - neurotrophic factor

Neurons of the freshwater pond snails Lymnaea stagnalis and Helisoma trivolvis require soluble factor(s) produced and released by neural tissues for neurite outgrowth to occur in vitro /1, 2/. To assay for the presence and specificity of neuritogenic substances in culture medium conditioned by neural tissues of molluscs we have taken advantage of the large number of neurons identified within the Lymnaea nervous system (Fig. 1). In using this assay we recently discovered that mammalian nerve growth

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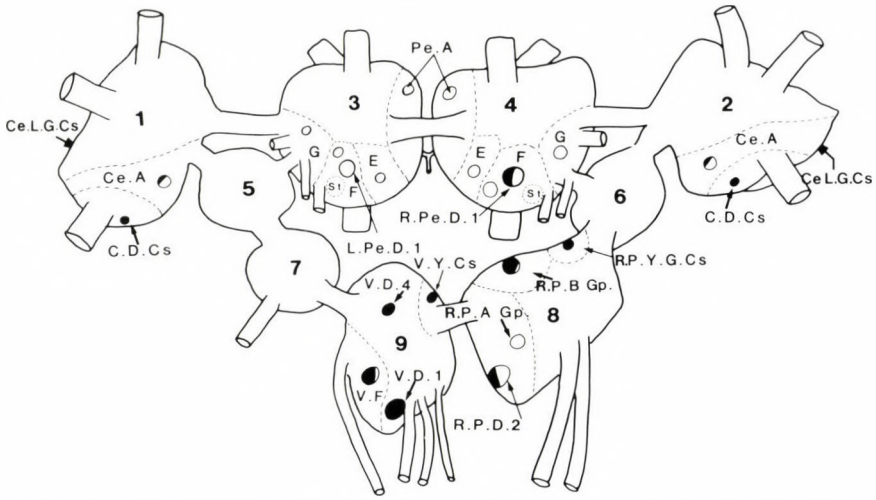

Fig. 1. Schematic diagram of the central ganglionic ring (dorsal view) of Lymnaea stagnalis showing position of identified neurons used in this study. The ganglia are numbered as follows: left and right cerebral ganglia (1, 2); left and right pedal ganglia (3, 4); left and right pleural ganglia (5, 6); left and right parietal ganglia (7, 8); visceral ganglion (9). Motor neurons: Pedal A cluster (Pe.A); Pedal E, F, and G clusters (Pe.E/F/G); Cerebral A cluster (Ce.A); Right Parietal A group (R.P.A Gp). Interneurons: Left and Right Pedal Dorsal 1 (L.Pe.D.1, R.Pe.D.1); Visceral Dorsal 4 (V.D.4). Neurosecretory cells: Cerebral Light Green Cells (Ce.L.G.Cs); Cerebral Caudo-Dorsal Cells (Ce.C.D.Cs); Right Parietal B group (R.P.B Gp); Right Parietal Yellow-Green Cells (P.Y.G.Cs); Right Parietal Dorsal 2 (R.P.D.2); Visceral Dorsal 1 (V.D.1); Visceral Yellow Cells (V.Y.Cs); Visceral F cells (V.F). Statocyst organs: (St). See Reference /3/ for further information

factor (NGF) stimulates neurite outgrowth in vitro of certain Lymnaea neurons /3/. Specifically, the neurons found responsive to mouse submandibular gland 2.5 S NGF belong to two broad categories: motor neurons and interneurons (Fig. 2). A dose-dependent increase of sprouting was observed in these cells over the range of 50-400 ng/mL, with a half-maximal response at about 150 ng/mL /3/. There was no significant response to NGF observed in identified neurons belonging to a third category: neurosecretory cells. Preabsorption of NGF-supplemented defined medium with anti-NGF serum blocked the response of Pedal A cluster motorneurons, whereas preabsorption with non-immune serum did not block the response of these cells to NGF.

In contrast to their differential responsiveness to 2.5 S NGF, isolated neurons of all three categories exhibit neurite outgrowth when cultured in medium previously conditioned for 72 h with Lymnaea central ganglionic rings (2/mL) (see Figs 2 and 3). To test for the presence of

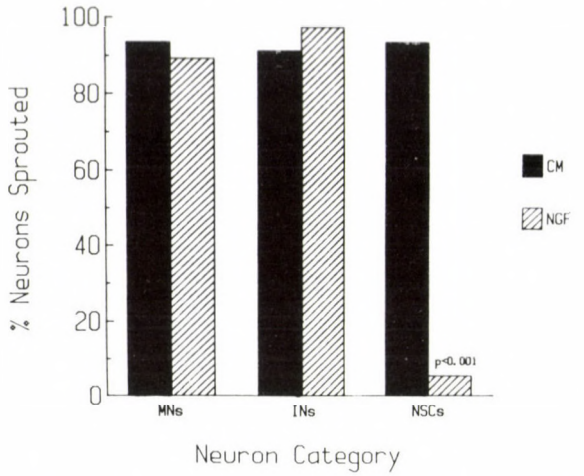

Fig. 2. Histogram comparing neurite outgrowth of Lymnaea neurons cultured in vitro either in central ganglionic ring conditioned medium (CM) or in defined medium supplemented with 400 ng/mL murine 2.5 S NGF. The neurons tested (see Fig. 1) have been pooled into 3 categories: motor neurons (MNs), interneurons (INs), and neurosecretory cells (NSCs). Neurons of all categories exhibit neurite outgrowth in CM, but only motor neurons and interneurons do so in response to NGF (n = 108, 295, 57, 39, 121, 76; from left to right, respectively)

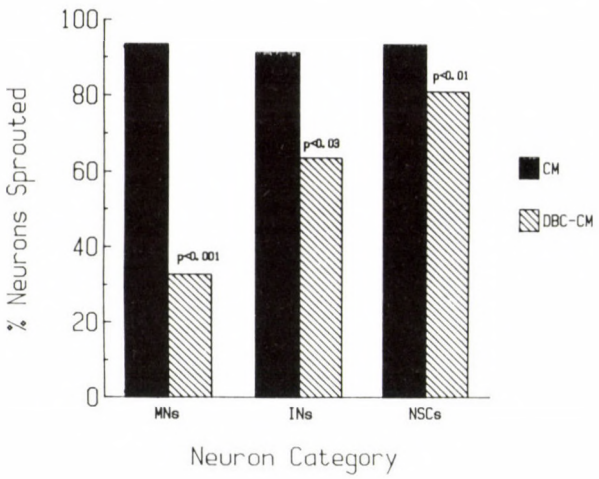

Fig. 3. Histogram comparing neurite outgrowth of Lymnaea neurons in vitro either in central ganglionic ring conditioned medium (CM) or in dorsal body cell conditioned medium (DBC-CM). As in Fig. 2, the neurons tested have been pooled into 3 categories: motorneurons (MNs), interneurons (INs), and neurosecretory cells (NSCs). Neurons of all categories exhibit neurite outgrowth in CM, but primarily neurosecretory cells and interneurons do so when cultured in DBC-CM. The difference in neurite outgrowth percentage between neurosecretory cells and motor neurons cultured in DBC-CM is significant ($p < 0.001$) by Fisher's 2x2 Exact test. (n = 108, 107, 57, 11, 121, 63; from left to right, respectively)

NGF-like molecules we preabsorbed conditioned medium (CM) with affinity purified anti-NGF serum. This preabsorption showed a dose-dependent ability to block the neurite outgrowth response of Pedal A cluster motor neurons to Lymnaea central ganglionic ring CM. However, the neurite outgrowth response of neurosecretory cells (e.g., Cerebral Light Green Cells, Visceral F and Right Parietal B group neurons) was unaffected /3/. Preabsorption with non-immune serum failed to block the neuritogenic activity of CM on any of the neuronal types examined. These experiments suggest that a NGF-like immunoreactive molecule and at least one other neuritogenic factor are present in the neural tissues of Lymnaea.

In early attempts to localize neuritogenic activity to specific central ganglia of either Lymnaea or Helisoma we observed that co-culture with dissociated cells of the dorsal bodies, endocrine structures which lie in the perineurium of the cerebral ganglia /4/, promoted neurite outgrowth of some neurons of both species /5/. In examining this activity further we have made Lymnaea dorsal body conditioned medium (DBC-CM). We have found that identified neurosecretory cells in particular and, to a lesser degree, interneurons sprout when cultured in vitro in DBC-CM (Fig. 3). Only one type of motor neurons (the Pedal A cluster motor neurons) showed significant neurite outgrowth in DBC-CM, all other types examined were unresponsive. Thus, the dorsal bodies of pulmonate molluscs appear to be a source of a neurotrophic factor which, like the postulated NGF-like molecule, shows activity limited to specific neurons. The identity of the DBC-CM factor remains to be determined.

Taken together, our experiments support the hypothesis that multiple neurite outgrowth-promoting factors are produced by the nervous systems of pulmonate molluscs. Furthermore, these endogenous factors appear to display selectivity in their ability to promote sprouting of identified neurons in vitro. The potential of these factors to influence neuronal morphology and synaptic connectivity suggests that their interactions and modulation may be important determinants of nervous system plasticity in these animals.

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IN VITRO CONNECTIONS BETWEEN LYMNAEA AND HELISOMA IDENTIFIED INTERNEURONS AND THEIR FOLLOWER CELLS*

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The mechanisms that underly the specificity of synaptic connections are poorly understood. In this study we used two homologous interneurons, the giant dopamine cell (GDC), in two species of pond snails, Lymnaea and Helisoma. We examined the ability of the Lymnaea GDC to form specific synapses with known follower or non-follwer cells in vitro. Similar tests were performed for the Helisoma GDC. Both of these interneurons form appropriate connections not only with homologous follower neurons, but also with follower neurons from the alternative species. These results suggest that common mechanisms of cell recognition and synapse formation exist in the nervous systems of these two different families of molluscs.

Keywords: Mollusc - culture - synapse - specificity

Knowledge of the mechanisms that underlie the specificity of synaptic connections is fundamental to understanding development and regeneration of the nervous system. In this study we have made use of the presence of homologous interneurons, the "giant dopamine cell" (GDC), in two species of pond snails, Lymnaea stagnalis and Helisoma trivolvis. The nervous systems of these snails are very similar, being almost mirror-images of each other. We have examined the ability of the Lymnaea GDC, also known as Right Pedal Dorsal 1 (R.Pe.D.1), to form specific synapses with known

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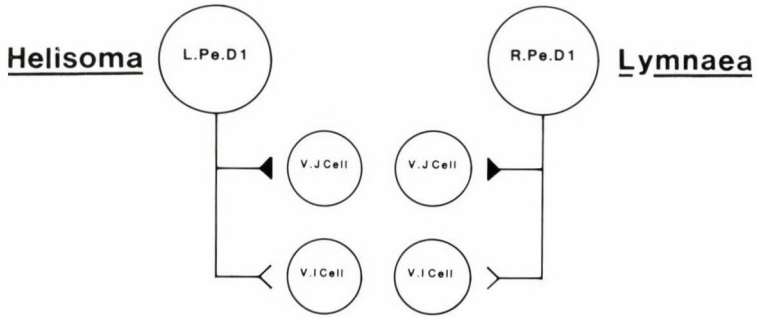

Fig. 1. Summary of the post-synaptic connections of Lymnaea R.Pe.D.1 (on right) and Helisoma L.Pe.D.1 (on left) with relevant homologous follower cells. Open and closed symbols represent excitatory and inhibitory connections, respectively

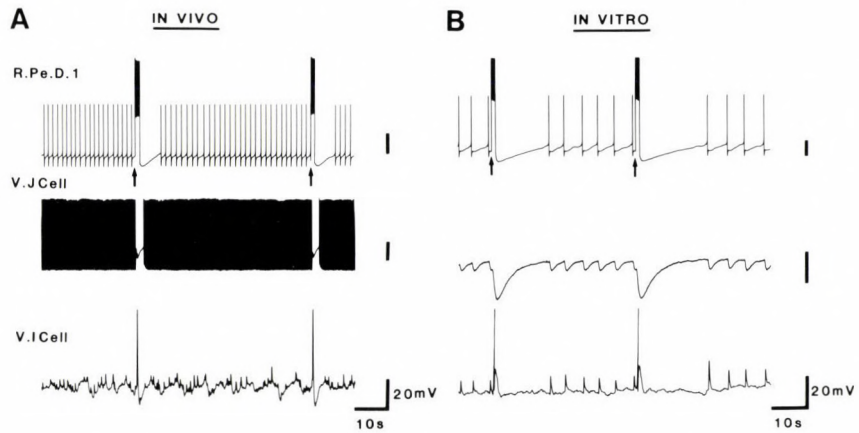

Fig. 2. In vivo and in vitro synaptic connections between Lymnaea R.Pe.D.1 and some of its follower cells. (**A**) In vivo induced electrical activity in R.Pe.D.1 (at arrows) inhibits a Visceral J (V.J) cell while exciting a Visceral I (V.I) cell. (**B**) During in vitro cell culture experiments, R.Pe.D.1 similarly formed inhibitory and excitatory connections with a V.J cell and V.I cell, respectively

follower or non-follower cells in vitro. Similar tests were performed for the Helisoma GDC, also known as Left Pedal Dorsal 1 (L.Pe.D.1), and its follower cell system. Finally, to test the ability of these homologous cells to form interspecific synaptic connections with appropriate follower cells, we substituted the Lymnaea GDC for the Helisoma GDC, and vice versa.

In acutely isolated ganglionic preparations, the GDC of Lymnaea has monosynaptic connections with follower cells of the visceral and parietal ganglia (e.g., V.J and V.I cluster cells), but does not have connections

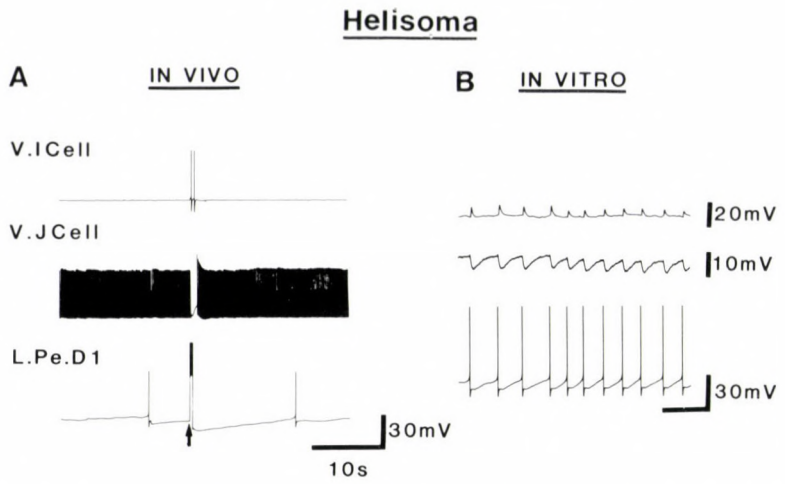

Fig. 3. In vivo and in vitro synaptic connections between Helisoma L.Pe.D.1 and identified follower V.J and V.I cells. (**A**) The induced activity of L.Pe.D.1 (at arrow) in an in vivo preparation inhibited a V.J cell while exciting a V.I cell. (**B**) In vitro, L.Pe.D.1 made an inhibitory connection with a V.J cell and an excitatory connection with a V.I cell. Spontaneous action potentials in L.Pe.D.1 produced unitary inhibitory post-synaptic potentials (IPSPs) and excitatory post-synaptic potentials (EPSPs) on follower V.J. and V.I cell, respectively

with certain other neurons (e.g., V.F cluster and R.P.B group cells) /1, 4/. We have recently identified a large number of apparently homologous follower (as well as non-follower) cells of the Helisoma GDC which are also monosynaptic in character (summarized in Fig. 1).

To test the ability of the Lymnaea GDC and Helisoma GDC to establish appropriate synaptic connections in vitro, we cultured these interneurons (together with their respective follower and non-follower cells) using previously described techniques /2, 3/. In both species, the GDC was found to make appropriate chemical (inhibitory or excitatory) synaptic connections with its follower cells (Figs 2 and 3), but did not make connections with non-follower cells (data not shown). Thus, the specificity of synapses observed in vivo is preserved when these neurons are isolated and allowed to regenerate in vitro.

When the Lymnaea GDC was cultured together with homologous follower cells of both species, it not only made appropriate connections with its own (i.e., Lymnaea) follower cells but also with the Helisoma homologues (Fig. 4A). Similarly, when the Helisoma GDC was cultured together with homologous follower cells of both species, it made appropriate connections with both its own (i.e., Helisoma) follower cells and the Lymnaea homo-

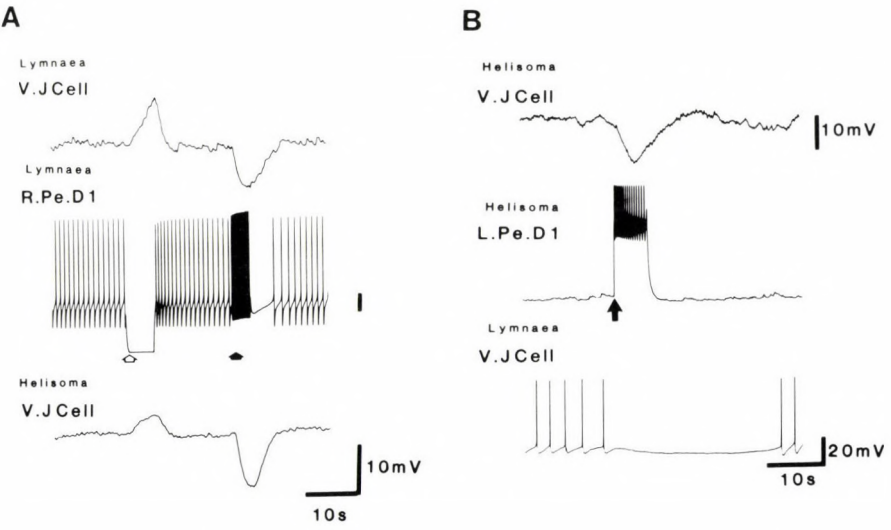

Fig. 4. The in vitro substitution of the Lymnaea GDC for that of Helisoma and vice versa. (**A**) R.Pe.D.1 from Lymnaea (middle trace) was cultured together with one of its follower cells (a V.J cell; top trace) and a homologous follower cell of the Helisoma GDC (Helisoma V.J cell; bottom trace). R.Pe.D.1 was spontaneously active, thus when it was hyperpolarized (open arrow) both the Lymnaea V.J cell and the Helisoma V.J cell were released from ongoing inhibition. When R.Pe.D.1 was further depolarized (closed arrow) both V.J cells were further inhibited. (**B**) The GDC of Helisoma (L.Pe.D.1; middle trace) was co-cultured with one of its follower cells (a V.J cell; top trace) and a follower cell of the Lymnaea GDC (a Lymnaea V.J cell; bottom trace). Depolarization of L.Pe.D.1 (at arrow) resulted in inhibition of both V.J cells

logues (Fig. 4B). Thus, the GDC of one species can substitute for the GDC of the other species.

These experiments underscore the value of using in vitro cultures to examine the synaptic specificities of identified neurons within behaviourally relevant circuits, for example those comprising the respiratory central pattern generator of Lymnaea /5/. The ability to integrate foreign, albeit homologous, cells into neuronal networks expands our conception of nervous system plasticity for it strongly suggests that common mechanisms of cell recognition and synapse formation exist in the nervous systems of different species.

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SEROTONIN IN HELMINTHS: CONTENT, SYNTHESIS, METABOLISM*

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Serotonin (5-hydroxytriptamine, 5-HT) has been identified in tissues of parasitic worms of various taxonomic, ecological and age groups (17). As compared to other helminths, representatives of the class Cestoda are investigated extensively in terms of identification and quantitative evaluation of their 5-HT content. The results of investigation of 23 representatives of cestodes belonging to four orders (Pseudophyllidea, Cyclophyllidea, Tetraphyllidea, Cariophyllaeidea) and including larval and adult forms, parasites of different hosts (fish, birds, mammals) and different localizations (body cavity, intestine) have shown that all cestodes examined contain 5-HT and concentration of 5-HT varies from 0.05 to 3.2 μg/g tissue.

Among representatives of the class Trematoda 5-HT concentration being significant (up to 6.0 μg/g tissue) in Schistosoma. 5-HT level in the other helminth classes (Nematoda, Monogenea, Amphylinida, Acanthocephala) is usually essentially lower than that in cestodes or some trematodes.

The detection of 5-HT in helminths combined with other observation such as the data about its pharmacological effect on the motor activity of parasites or the existence of the enzymic system responsible for 5-HT synthesis and inactivation allows us to view it as most probable neuro-

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transmitter /1, 10, 14, 20/. The hypothesis about the neurotransmitter function of 5-HT in parasitic worms finds support in the histochemical and immunocytochemical data confirming its localization in structures of the nervous system.

Analysis of the functional role of 5-HT in helminths shows that it may affect carbohydrate metabolism in some cestodes and trematodes /8, 9/. It has been demonstrated that 5-HT enhances adenylate cyclase and protein kinase activity in helminths, increases cAMP, phosphofructokinase and phosphorylase activity /7, 12/. It has also been shown that 5-HT may influence egg production in cestodes and nematodes /2, 3/. There is evidence indicating the 5-HT effect on the Cestoda migration in the host intestine /13/.

There are data in the literature indicating that the level of 5-HT in helminths depends on its concentration in the environment. It follows from the published data that larval and adult forms of cestodes and trematodes have the ability of actively utilizing 5-HT from their environment with the aid of a highly specialized tansport system /8, 11/. It has also been noted that there is a positive correlation between the 5-HT concentration in the host (rat) intestine and Hymenolepis diminuta tissues /4, 18/.

It has been postulated that at least a major portion of 5-HT found in Cestoda tissues is derived from the host body. If cestodes are capable to absorb 5-HT from the environment, then its concentration in tissues of helminths is obviously dependent on that in the area of their habitation. In fact, measurement of 5-HT concentration in larval and mature stages of the cestodes Triaenophorus nodulosus, Ligula intestinalis, Schistocephalus pungitii shows that 5-HT concentration in adults, occurring in the intestine is higher than in larvae occurring in the body cavity or liver of the host /17/. Comparative study of the 5-HT concentration in larvae and adults of cestodes Diphyllobothrium vogeli and in larvae of Piromicocephalus focarum also points to the fact that helminths occurring in an environment with a high level of 5-HT contain it in greater quantities.

Trematodes and nematodes also show differences in the 5-HT level in the forms residing in an environment with a different 5-HT content, e.g. Schistosoma larvae and adults, Litomosoides carinii mature stages and microfilarias /17/. The data on the presence of 5-HT in parasitic worms and their ability to actively absorb this substance from the host organism are of certain interest since they concern the possibility of using com-

pounds of exogenous origin by animals as their own physiologically active substances (neurotransmitters, neurohormones).

We investigated the ability of the parasitic flatworms Hymenolepis diminuta and Mesocestoides corti to synthetize 5-HT by means of incubation the worms in metabolic precursor of 5-HT, L-tryptophan and monoaminooxidase inhibitor, indopan, followed by spectrofluorimetric determination of the tissue level of 5-HT. To avoid possible inhibition of 5-HT synthesis by exogenous 5-HT of the host, experiments were carried out on worms in which the level of 5-HT was previously decreased by reserpine. The increase in the level of 5-HT in tissue after incubation of the worms in L-tryptophan and indopan was observed, indicating their ability to synthetize 5-HT from tryptophan. The results obtained are consistent with the data obtained by using of radioisotopes /15, 16/. Moreover, the occurrence of tryptophan hydroxylase, a key enzyme of 5-HT biosynthesis which catalyzes the conversion of tryptophan into 5-hydroxytryptophan, has been demonstrated in some helminths /5, 19/.

The investigation of 5-HT inactivation in parasites showed the presence of 5-hydroxyindolylacetic acid (5-HIAA) in tissue homogenates of cestodes (Hymenolepis diminuta, Mesocestoides corti, Triaenophorus nodulosus, Schistocephalus pungitii, Eubothrium crassum), trematodes (Haplometra cylindracea, Codonocephalus urnigerus), nematode (Rhabdias bufonis), Polystoma integerrimum (Monogenea), Amphylina foliacea (Amphylinida). This suggests that one of the mechanisms of 5-HT catabolism in helminths is its inactivation under the action of monoamineoxidase (MAO). It is known that the level of 5-HIAA is correlated with that of 5-HT metabolism. The low content of 5-HIAA as compared to the 5-HT concentration in helminth tissues is indicative of the low rate of 5-HT catabolism. It is known that the rate of 5-HT metabolism in 5-HT neurons is high and the index of its utilization (the ratio of the 5-HIAA/5-HT concentration) is greater or equal to unity. Our results give evidence that 5-HT metabolism in parasitic worms is similar to that in enterochromaffin cells of mammals, which are characterized, as follows from our own and reported data /21/, by the low index of 5-HT utilization when compared to that in the nervous tissue.

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SENSORY RECEPTORS IN THE HEAD OF STENOSTOMUM LEUCOPS. II. LOCALIZATION OF CATECHOLAMINERGIC HISTOFLUORESCENCE-ULTRASTRUCTURE OF SURFACE RECEPTORS*

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(Accepted: 1991-08-30)

The ultrastructure of three types of sense receptors in the anterior end of Stenostomum leucops is described. Type I is characterized by abundant branching microvilli and ciliary rootlets but a lack of cilia. Type II possesses a short cilium with aberrant axonema. Type III has a cilium protruding from an invagination, a long rootlet and a collar of microvilli. The glyoxylic-acid-induced fluorescence method reveals catecholaminergic (CA) fluorescence in surface cells of the anterior end. The relationship between the CA fluorescence and the localization of the sense receptors is discussed.

Keywords: Sense receptors - catecholamines - histofluorescence - ultrastructure - Turbellaria

Variations of a type of uniciliated receptor with a long rootlet and a collar of numerous microvilli have been described in sporadic reports on the ultrastructure of epidermal sense receptors in catenulids /1, 2, 3/. A similar type of sense receptor has also been observed in the pharyngeal epithelium of Stenostomum leucops /4/. Several types of surface sense receptors were observed in the "head" of S. leucops. Their ultrastructure and distribution are described in this preliminary report.

Recent studies using the glyoxylic-acid-induced fluorescence (GAIF) method have revealed catecholaminergic fluorescence in the nervous system of S. leucops /5, 6/. Additional GAIF fluorescence is observed in nerve fibres extending inwards from the surface.

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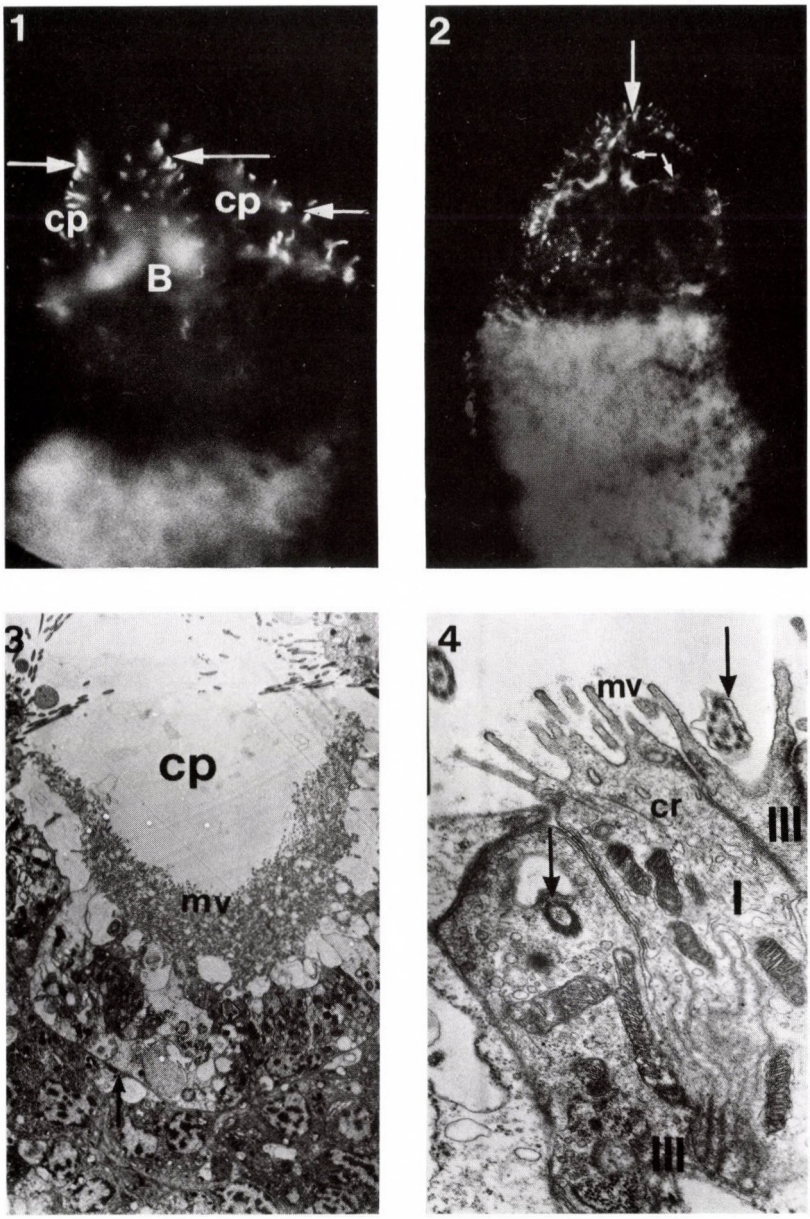
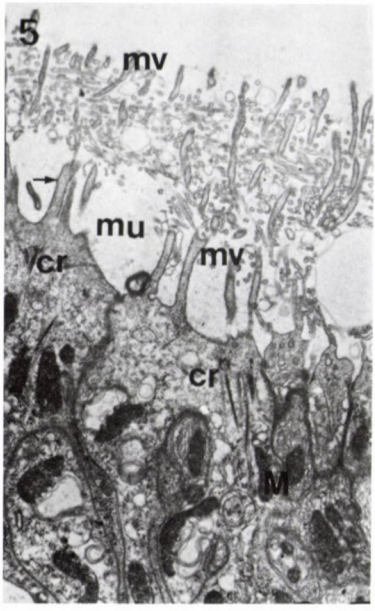
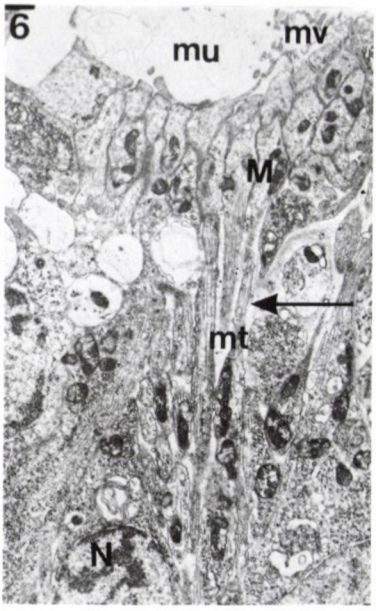


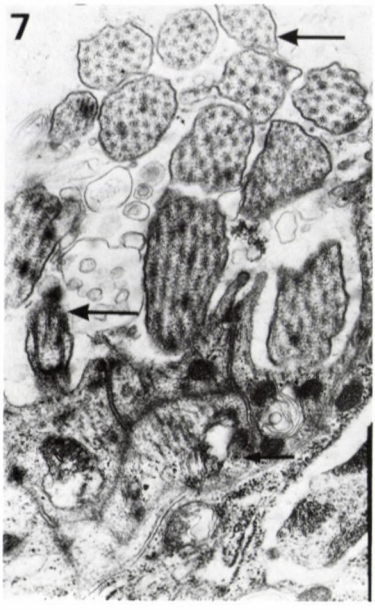
Figs 1-2. Catecholaminergic fluorescence in the head of Stenostomum leucops cells (arrows), ciliary pits (cp), brain (B), nerve fibres (small arrows). x150, x300

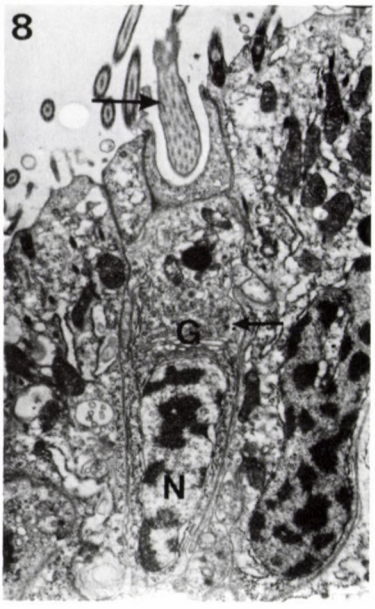
Fig. 3. Overview of ciliary pit (cp); note microvilli (mv) covering bottom of the pit, sheath of extracellular matrix (arrow). x2500

Fig. 4. Microvilli bush consisting of sense receptor types I and III (I, III), microvilli (mv), ciliary rootlet (cr), cilia (arrows). x20 000



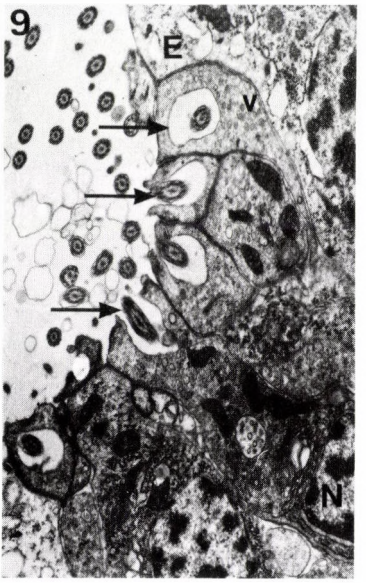


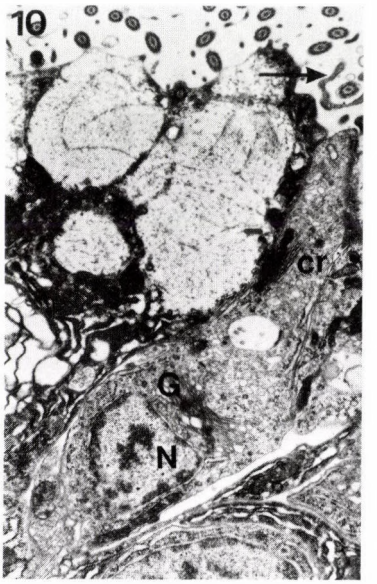




Figs 5-6. Sense receptor type I; microvilli (mv), fine filaments (small arrow), ciliary rootlet (cr), mucus (mu), microtubuli (mt), inward running cell necks (large arrow), nucleus (N), mitochondria (M). x10 000, x8000

Figs 7-8. Sense receptor type II; cilia with aberrant axonema (arrows), dense-core vesicles (small arrows), Golgi complex (G), nucleus (N) in cell soma on epidermal level. x20 000, x10 000





Figs 9-10. Sense receptor type III. Cilia protruding from invaginations (arrows), ciliary rootlet (cr), cell soma and nucleus (N) on epidermal level, epidermis (E), amorphous or dense-cored vesicles (v), Golgi complex (G). x10 000, x20 000

Specimens of Stenostomum leucops (Turbellaria, Catenulida) were collected from a small pond in the park of the Biological Institute of the Leningrad University, from Lake Littois in SW Finland and from a stock culture maintained in containers with tap water at Åbo Akademi University.

GAIF: The protocols for glyoxylic-acid induced-fluorescence described by Joffe /5/ and Reuter and Eriksson /6/ were followed. Control specimens were treated as above except that glyoxylic acid was ommitted.

Electron microscopy. Fixation for electron microscopy and further steps in the EM processing followed the method described by Reuter and Palmberg /4/.

GAIF. Spindle-shaped CA-positive perikarya are present in the epithelium of the head. They are densely distributed at the anterior pole of the body and at the borders of the ciliary pits (Fig. 1), but they are lacking in the pits themselves. Their distribution ismore sparse behind the brain. Axonal processes reach the brain separately or gather into nerves. Two pairs of nerves are present, one extending in antero-medial direction, the other in antero-lateral direction (Fig. 2). More laterally, thinner nerve fibres connect surface cells with the brain. A delicate network of

fine processes connects the positive cells near the epidermal surface. No CA-positive cells are observed in the mouth or pharynx or behind them.

Ultrastructure. The distribution of three different types of surface sense receptors shows a distinct pattern: All three types of sensory receptor cells are revealed in the ciliary pits (Fig. 3). Type I, the microvillous type, dominates, covering the bottom of the pit. Type II occurs in a very restricted area, next to the border of the pit. Type III occurs in large groups bordering the pits. A sheath of extracellular matrix penetrated by nerve processes separates the pits from the lateral lobes of the brain. Transversally and tangentially cut nerve fibres are usually observed close to this sheath. Frontally of the pit, bush-like structures consisting of sense receptors of types I and III (Fig. 4) are observed. In addition, type III is observed as single receptors scattered at the surface of the head, at the mouth opening and associated to the pharyngeal epithelium.

Type I (Figs 5-6). The microvillous type is characterized by long striated ciliary rootlets directed inward. It lacks cilia, but the apical cytoplasm is modified to numerous microvilli. Abundant mitochondria with transverse cristae occur in the apical cytoplasm. The microvilli, containing thin filaments, form a network of finer branches outside a mucus-filled area. A circumferential zone of dense material and a more distal septate desmosome are observed at the apical end of the sense receptors. Long necks supplied with microtubuli connect the apical part of the sense receptors with the deeper-lying cell bodies.

Type II (Figs 7-8). This sensilla type bears aberrant cilia, where the 9+2 pattern is disrupted and the doublets occur as random singlets in the outer segment of the cilium. A zonular density followed by a septate desmosome connects the receptor and the adjacent epidermis close to the surface. Dense-cored vesicles occur in the cell body that lies on the same level as the epidermal cells.

Type III (Figs 9-10). The uniciliated type bears a cilium with normal 9+2 axonemal pattern and a long striated rootlet. The cilium extends from an invagination forming a cylinder with the protruding cilium in the centre. The cylinder is bordered by a collar of numerous (about 10) microvilli at the apical end. Zonular dense material forms a ring next to the surface and is followed by a more distal septate desmosome where the process is attached to surrounding cells. The cell somata lie at the epidermal level. They are characterized by large Golgi complexes and numerous

vesicles containing amorphous material and a point-like core. Vesicles are observed also in the lumen of the cylinder.

Neuroactive substances. The GAIF fluorescence indicates catecholaminergic (CA) transmitters in the sensory receptors. No nerve cells other than sensory ones have been detected in the epithelial layer by electron microscopy. The distribution of the CA fluorescing cells and fibres reflects a pattern that primarly corresponds to the sense receptors bordering the ciliary pits, i.e. type III. We thus attribute CA transmitter substances to these uniciliated cells. Cells of the same ultrastructure, but without CA fluorescence, are, however, detected at the mouth opening and in the beginning of the pharynx, but not in the postoral region. A strong FMRF-amide immunoreactivity revealed in cells of the pharyngeal nerve ring /6/ points in the direction of peptidergic neuroactive substances. Colocalization of peptides and amines as well as functionally alternating states are general phenomena in the nervous systems of higher animals. Hypothetically the sense receptors in the frontal end of S. leucops could use mainly CAtransmitters while the receptors of pharynx mainly use the peptide. Diffuse FMRF-amide IR obtained in the head argues for colocalization. It must also be stressed that a similar ultrastructure does not exclude different transmitters and different functions of the sense receptors, i.e. type III receptors in pharynx and head might not be homologous. Our data introduce an interesting problem: which is the more important for elucidating the receptor homology ultrastructure or transmitter supply?

Functional aspects. A discussion of the functional modalities of the different types of sense receptors must be entirely based on morphological data. A parallelism in function and mode of origin of the ciliated pit and olfactory organs of fishes has already been stressed by Kepner and Cash /7/. The ultrastructure of the sense receptors in the bottom of the pit supports this interpretation. Olfactory dendrites bearing cilia tapering to microvilli branches surrounded by mucus, characterize the olfactory organs of vertebrates. In addition, there are structural analogies with chemosensory receptors of invertebrates, i.e. modified cilia are noted /8/. Structural analogies are, however, also present for the sensilla of the type II. Aberrant cilia with a disrupted 9+2 axonemal pattern characterize other presumed olfactory receptors of invertebrates /9/. Two different types of chemosensory receptors are therefore suggested for the ciliary pits of S. leucops.

The functional modality of the uniciliary sense receptor, type III, is difficult to base on analogies. For sense receptors resembling this type, both mechanoreception, chemoreception and combinations of these have been suggested /10/. The wide distribution of receptor type III in the head and the assumed two types of chemoreceptors in the ciliary pits, makes a mechanoreceptive function probable for the head receptors, but the hunting behaviour of the worms points strongly in the direction of a chemosensory function of the pharyngeal receptors /4/. In general, the functions of turbellarian sense receptors await for further research.

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NEUROPEPTIDES IN SENSORY STRUCTURES OF NEMATODES*

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The peptidergic innervation of sensory structures in two species of nematodes, Ascaris suum (order Ascaridida, family Ascarididae) and Cystidicola farionis (order Spirurida, family Cystidicolidae) was studied. Immunocytochemical methods were used for localization of FMRF-amide-like neuropeptides in the nervous system. Immunoreactivity to FMRF-amide, RF-amide and SALMF-amide was detected in the central nervous system of the species studied, and also in the cephalic papillary nerves, in axons of the amphids and the deirids, and in nerves innervating caudal papillae.

Keywords: Neuropeptides - sensory structures - nematodes - Ascaris suum - Cystidicola farionis

The presence of immunoreactivity to several neuropeptide antisera has recently been demonstrated in the nervous system of nematodes /5/. The distribution of FMRF-amide like immunoreactive substances, which are widespread in the nervous system of many organisms, has been described from both parasitic and free-living nematodes /1, 4, 5/, and the amino acid sequence of FMRF-like peptides of Ascaris has been determined /2/. In the present study immunocytochemistry using antibodies to FMRF-amide and related sequences were used in order to describe the neuroanatomy of peptidergic innervation of sensory structures in the parasitic nematodes Ascaris suum (order Ascaridida, family Ascardididae) and Cystidicola farionis

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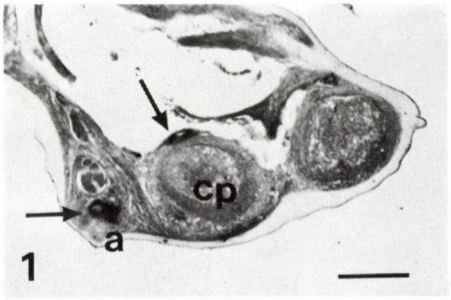
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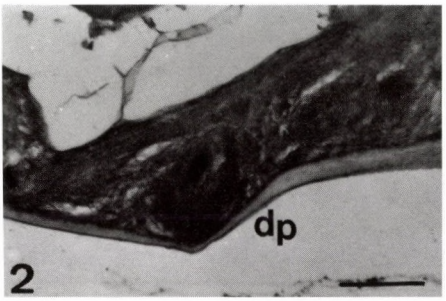
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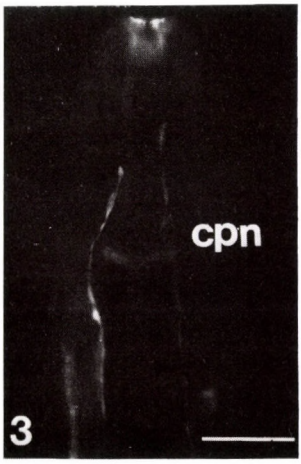
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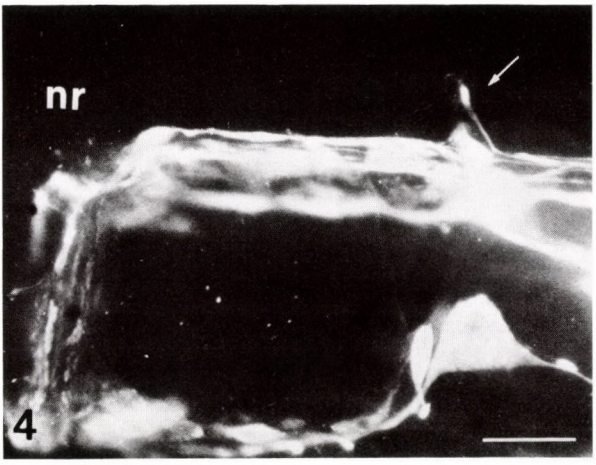
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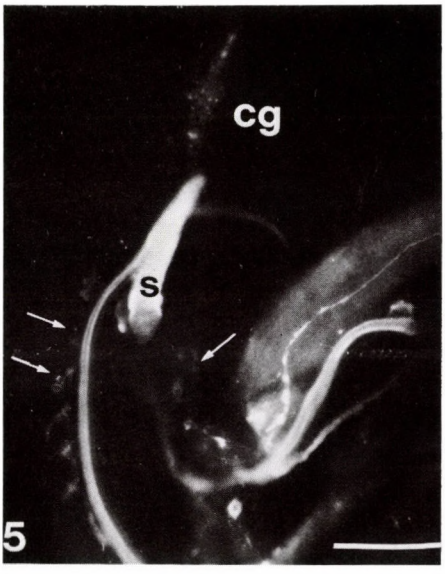
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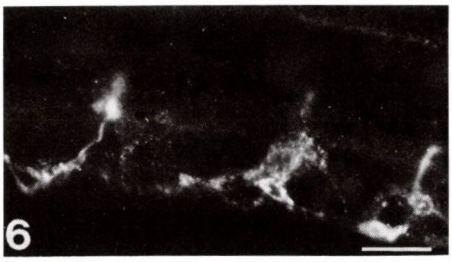
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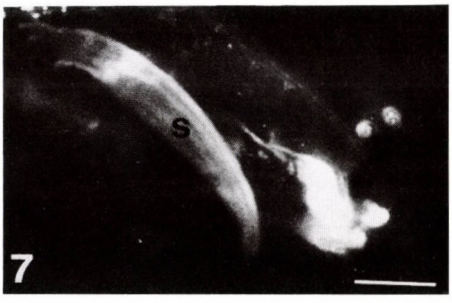
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(order Spirurida, family Cystidicolidae). This preliminary study is part of an investigation aiming at defining the morphological and functional aspects of the innervation of sensory structures in various nematode groups. The study was prompted by the systematic and taxonomic importance that has recently been attributed to sensory, especially caudal, structures in male worms of the superfamily Ascaridoidea.

Ascaris suum was obtained from the small intestine of pigs. Cystidicola farionis is a common parasite in the swim bladder of smelt, Osmerus eperlanus. Sections of Ascaris and whole mount preparations of C. farionis were stained by the peroxidase-antiperoxidase (PAP) method and the indirect immunofluorescence method, respectively. The antibodies used were raised against FMRF-amide, the sequence RF-amide, and SALMF-amide - a C-terminal analogue of a neuropeptide isolated from echinoderms /3/. The antibodies were kindly donated by Dr. C.J.P. Grimmelikhuijzen and Dr. M. Thorndyke.

In the present study, all the antibodies used gave a similar staining pattern. The distribution of immunoreactivity (IR) to SALMF-amide in flatworms has previously been shown to be similar to that of FMRF-amide /6/. FMRF-amide-like IR was abundant in the nervous system of both nematode species studied. In the central nervous system FMRF-amide-like IR was present in the nerve ring, the ganglia, and the nerve cords, as described in previous studies /1, 4, 5/.

In sections of the anteriormost end of Ascaris, positive nerve fibres were observed in the sublateral lips, innervating the sensory papillae and the amphids (Fig. 1). In the tail - the region posterior to the cloaca - of male worms of Ascaris, positive nerve fibres were observed in the distal papillae (Fig. 2).

←

Figs 1-2. FMRF-amide-like immunoreactivity (IR) in Ascaris suum. PAP staining of wax sections
Fig. 1. Positive nerve fibres (arrows) in the amphid (a) and the cephalic papillae (cp) of the sublateral lip. Bar = 10 µm
Fig. 2. Positive fibre in a distal papilla of the tail. Bar = 10 µm
Figs 3-7. FMRF-amide-like IR in Cystidicola farionis. Wholemount immunofluorescence
Fig. 3. Immunopositive cephalic papillary nerves (cpn) supplying the cephalic papillae. Bar = 50 µm
Fig. 4. Positive fibres of the nerve ring (nr). Posterior to the nerve ring a positive fibre to the deirid (arrow). Bar = 20 µm
Fig. 5. The tail region of the male worm, with autofluorescing spicules (s). Positive fibres in the proximal papillae (arrows) and the caudal ganglion (cg). Bar = 50 µm
Fig. 6. Detail of nerve fibres of the distal papillae. Bar = 10 µm
Fig. 7. Detail of the tail with the spicule and the caudal ganglion. Bar = 10 µm

In C. farionis positive IR was observed in the cephalic papillary nerves (Fig. 3). Immediately posterior to the nerve ring, a positive nerve fibre from the lateral nerve cord was observed to supply the deirid, a paired tactoreceptor (Fig. 4). In the posterior end of the male worm of C. farionis, the paired subventral proximal papillae and the postcloacal papillae (2 pairs of paracloacal and 4 pairs of distal papillae) were innervated by positive fibres (Figs 5, 6). In the tail, the cells of the caudal ganglion were strongly immunopositive (Fig. 7).

The present study shows that most of the sensory organs of the two nematode species studied are innervated by nerve fibres that are immunopositive to FMRF-amide-like and related neuropeptides. A more detailed investigation of the sensory innervation and the presence of other types of neuropeptides is in progress.

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THE DIAGNOSTIC SIGNIFICANCE OF AUTOANTIBODIES AGAINST CARTILAGE COMPONENTS OR THEIR COUNTERPARTS IN DESTRUCTIVE ARTICULAR DISEASES*

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In this work four different types of antibodies to collagens, membrane protein of chondrocytes (MP), cartilage matrix proteins (CMP) and heat shock proteins (HSP-s) were tested in order to find an available clinical laboratory method for diagnostic and monitoring purposes in patients suffering from osteoarthritis. From the point of view of diagnostic efficiency, the estimation of antibody level to MP seemed to be the best and as a supplementary method the determination of rheuma factor was recommended. The anticollagen antibody estimation is less sensitive, anti CMP antibodies are not detectable. In spite of immunological crossreaction between HSP and cartilage matrix component the antibody level against HSP has no correlation with osteoarthritis. The appearance of humoral reaction, antibodies against different cartilage specific collagens and chondrocyte membrane proteins, is an epiphenomenon, however as the supposed immune complexes, trapped in a cartilage, play an important role in the damage of cartilage which may explain the self-perpetuating and chronic nature of cartilage degradation on osteoarthritis.

Keywords: Autoantibodies — osteoarthritis

Osteoarthritis is a very common illness of the adult population. The diagnosis is based mostly on symptoms and in progressed stage on X-ray findings. The clinical laboratory methods called "cartilage markers" are the determination of detectable cartilage components in serum and urine, but

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they are less specific or difficult to carry out. Recently other approaches were introduced using immunological methods and in destructive joint disease autoimmune process was also detectable. In the present paper serum antibodies against four types of antigen were investigated: antibodies to cartilage specific collagen types II, IX, XI, to chondrocyte membrane protein (MP), to cartilage matrix protein (CMP) and to heat shock protein (HSP) 65 kD (supposed to crossreact with cartilage proteoglycans). The aim of this work was to evaluate the diagnostic efficiency of these methods.

Materials and Methods

The collagens (types II, IX and XI) were extracted from human sternocostal cartilage after mild pepsin digestion and purified by differential salt precipitation as published earlier /24/. The same method was used for detection of collagen fractions in synovial fluid /23/. The purification of antigen was proved by polyacrylamide gel electrophoresis (PAGE). The chondrocyte membrane protein (MP) extraction was carried out according to Mollenhauer and von der Mark /20/, that of cartilage matrix proteins (CMP) by the method of Paulsson et al. /30/ from fresh autopsy material of human sternocostal cartilage. The heat shock protein (HSP), recombinant 65 kD HSP of Mycobacterium bovis BCG, was kindly donated by dr. R. van der Zee (National Institute of Public Health and Environmental Hygiene, Bilthoven, the Netherlands).

For estimation of antibody level, the solid phase double sandwich ELISA technique was used according to the following protocol:

1. Coating of 1-4 μg antigen/wells, rinse	2 h at 20 °C
2. Covering the free places by goat serum, milk powder, rinse	2 h at 20 °C
3. Application of serum in 1:100 dilution, rinse	2 h at 20 °C
4. Binding of antihuman IgGAM goat serum in 1:2000-3000 dilution	2 h at 20 °C
5. Colour reaction with OPD stopped by sulphuric acid	20-30 min
Measuring by Dynatec ELISA reader MR 230 at 490 nm within 30 min.	

In the figures the OD-s of 1:100 diluted sera are given.

Standardization of the ELISA method: Each new antigen and conjugate is pretitrated. The O.D. is measured against sample blank (in a well without antigen). The O.D. values are the average of 2-3 parallels in different plates. All the series contain normal and pathological samples (pooled and stored at -30 °C).

For comparison the serum Rheuma factor (Rf) and C-reactive protein (CRP) estimation was carried out by DIALAB ELISA technique.

The osteoarthritic patients from the Orthopaedic Clinic waiting for replacement of hip or knee joints were suffering from severe osteoarthritis. The age-matched controls were hospitalized patients free from any arthritic symptoms and other younger persons of our outpatient department. The number of patients in each investigation are given in Figs 1--8.

Results and Discussion

I. Anticollagen antibodies

In the synovial fluid of intact joint (not only in the macrophages) collagen type II and alpha chain supposed to be part of collagen XI were found /24/. In osteoarthritic synovial fluid degraded collagen peptides were detectable. This degradation could be prevented by administration of Arteparon /34/ (a polysulphated proteoglycan).

With respect to the breakdown of collagen in OA the study of the serum anticollagen to types II, IX and XI /25/ was carried out. In 40% of our OA cases (n=42) an elevated anticollagen level especially of minor collagens was found and in 15% of normal cases (n=17) an elevation collagen type II/25/. The same results were published by Charriere et al. /3/. The detection of anticollagen antibody method was criticized for a number of reasons: (a) IgG has a spontaneous tendency to bind to and aggregate on collagen. (b) The source of collagen could also be the ingested collagen peptide and only the pathological cartilage. (c) Collagen sample as antigen contains the rest of pepsin, because during the preparation pepsin is used to release collagen from tissues. (d) Solid-phase immunoassays tend to detect only antibodies of high avidity.

Due to the low diagnostic effectivity of anticollagen determination and concerning the methodical problems, instead of collagen other cartilage matrix components of higher antigenicity were studied as antigens.

II. Antibodies to chondrocyte membrane protein (MP)

The serum antibody level to MP is elevated in RA and osteoarthritis /21, 28/. In destructive joint diseases high frequencies of T-cells reactive against MP were detected /2/. The antigen epitope has not been clarified so far.

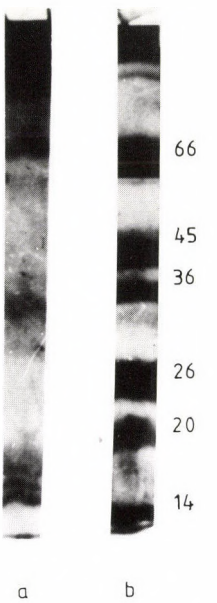

Fig. 1. SDS-10% PAGE slab gel electrophoresis of chondrocyte membrane protein. At least four bands are present. a = membrane; b = standard

The MP antigen extracted from sternocostal cartilage obduction material of newborn was less than 0.5% of the original weight. Reduced by mercaptoethanol, it has at least four peptides of 16, 21, 34 and 60 kD molecular weight (Fig. 1). In patients suffering from severe OA (n=86), before replacing the femoral head, the serum antibody level to this human MP was found to be raised significantly in comparison with control persons (n=44). The reaction could be inhibited by antigen /28/. From patient serum a concentrate of IgG fraction was obtained by affinity chromatographic method. This serum using Western immunoblotting technique reacts mainly with the subunit of 60 kD (Fig. 2).

Some characteristics of antibody determination

Changing with age: 92 persons of different ages from 2 to 90 years, clinically free from any arthritic symptoms, were investigated. The serum

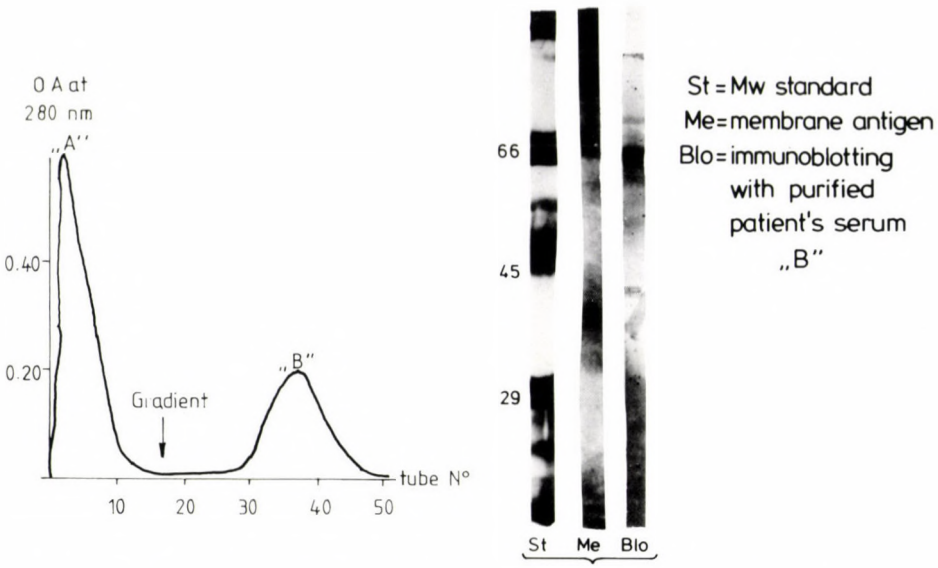

Fig. 2. Immunoblotting of chondrocyte membrane antigen (right) by purified patients's serum (left) marked with peak "B". The patient's serum showed the highest reaction to Western immunoblotting by band 60 kD. St = Mw standard; Me = membrane antigen; Blo = immunoblotting with purified patient's serum "B"

antibody level to MP, if there was any, was very low. Plotting the results to the age, a very low curve was found ($y = 0.07 x + 0.040$). With age there is a slight increase of the antibody level (Fig. 3).

Antibody level in OA patients: 39 patients with clinical diagnosis of OA and 39 age-matched controls (the female : male ratio was: 2.8:1, 3:1, respectively). The antibody level to MP of OA patients was 0.288, that of controls was 0.060. The difference was significant ($p < 0.001$) according to Student's t-test (Fig. 4).

The standard deviations in intra- and interassay are acceptable: Sera of controls: 0.060 ± 0.004. Sera of OA patients: 0.298 ± 0.269. Control pathological interassay: 0.800 ± 0.100. Patients sampling for 2.5 years: 0.250 ± 0.150.

Longitudinal study: 7 selected OA cases (suffering from monoarticular OA) were monitored after the removal of the femoral head. The antibody level decreased within months. These results point to the fact that with the removal of the major part of pathological cartilage, the tissue causing the autoimmune reaction was transitorily eliminated (Fig. 5).

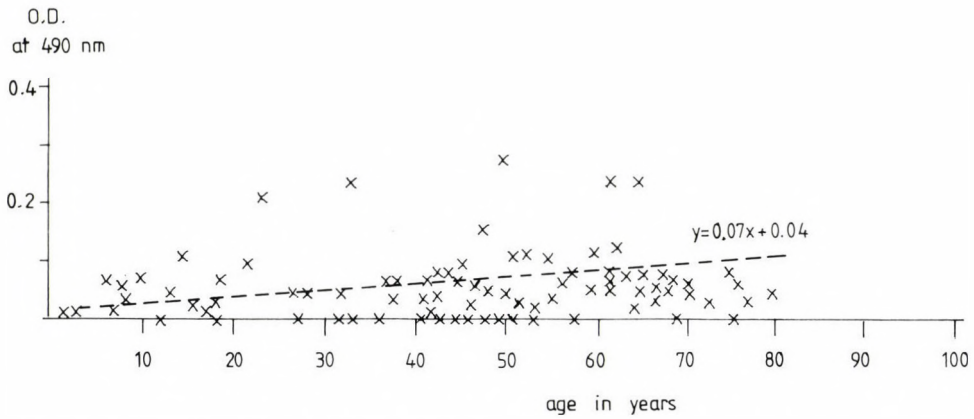

Fig. 3. Serum antibody level to chondrocyte membrane protein. Alteration with age in controls (n=92). A slight increase of antibody level was found with aging

Antibody levels to MP and to HSP to Rheumatoid factor (Rf) and to C-reactive protein (CRP) were correlated. Listing out the patients (n=24) with the highest antibody to MP level: most of the illnesses were osteoarthritides, several had necrosis of femoral head, and only 4 with other diagnoses. There was a very low correlation to CRP, Rf level and no correlation was found with anti HSP level.

Alsalameh et al. /2/ showed that the T-cell reaction of peripheral T-cells and synovial mononuclear cells to MP was significantly increased in OA-des patients by measuring the ^{3}H-thymidine uptake of cells in the presence and absence of MP. So it is the evidence of cellular immunological reactions of MP effect in RA and OA as well.

The antigen used has not been characterized exactly: it consists of at least four peptides. By immunoblotting the patients' sera did not react with the purified 34 kD peptide (named Anchorin CII by the authors /20/), but they did with the 60 kD fraction of antigen. The closer characterization of epitope and arthritogen character of antigen remained to be elucidated.

III. Antibodies to cartilage matrix protein (CMP)

The antigen of 148 kD in reduced form of 94, 60, 34 kD peptides occurs in tracheal and in nasal, sternal, auricular and apiphyseal cartilage /29/. The serum level of CMP increased in RA and polychondritis /35/. CMP was recommended as a marker for involvement of cartilage in disease. Its epitope has not been clarified.

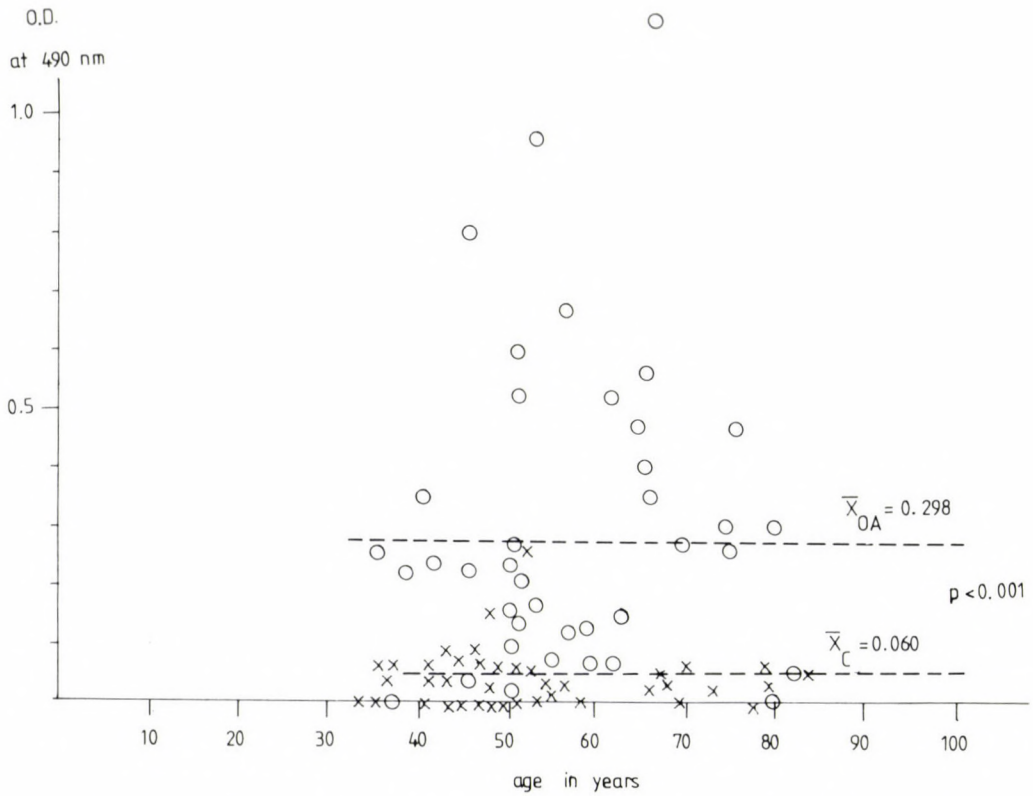

Fig. 4. Serum antibody level to chondrocyte membrane protein in osteoarthritic and control patients. A significant difference was found between the two groups. o = osteoarthritic (n=39); x = controls (n=39)

Table 1

The diagnostic efficiency of anti-MP and anti-collagen methods in OA patients and control persons

	Anti-MP (n=70)	Anti-collagens (n=59)
Sensitivity	0.64 (64% —)	0.52 (52% of patients)
Specificity	0.87 (87% —)	0.76 (76% of controls)
Efficiency	1.51	1.28

The routine methods used in clinical pathology showed higher diagnostic efficiencies (from 1.71 to 1.9).

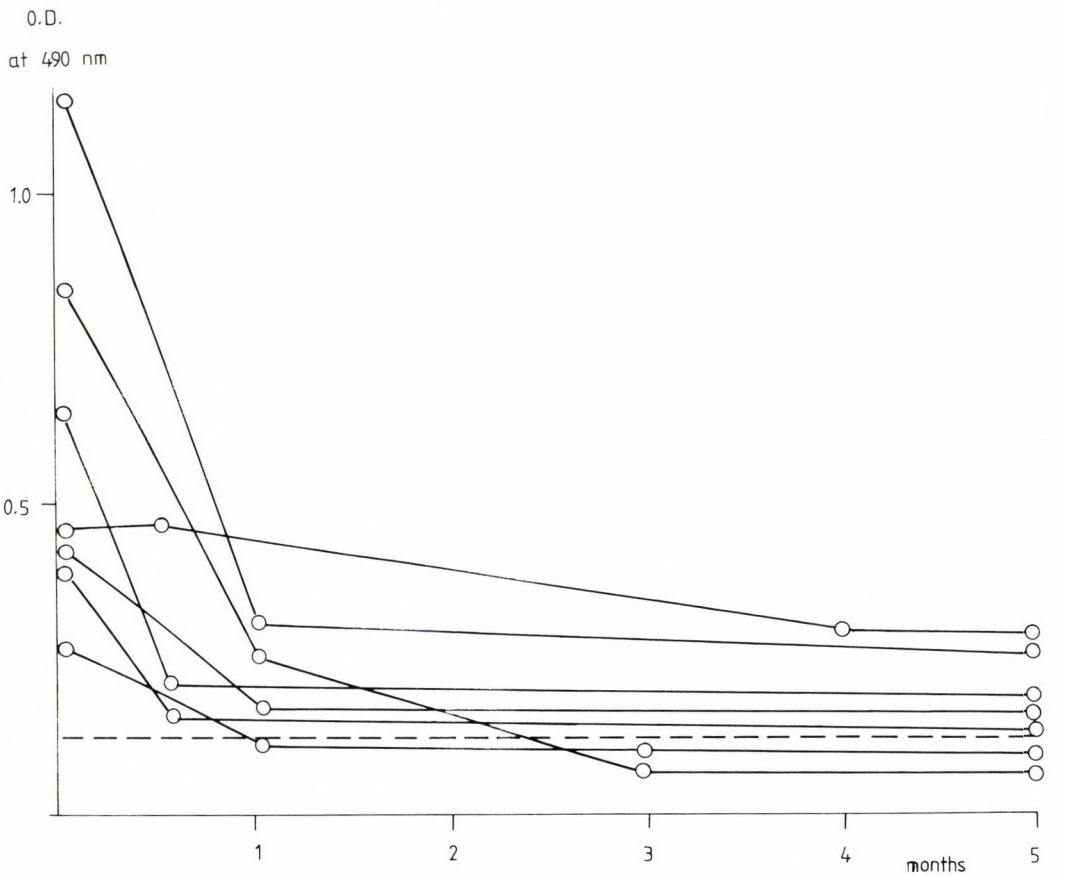

Fig. 5. Changes of serum antibody level to chondrocyte membrane protein in patients of dominant monoarticular manifestation after the removal of the arthritic femoral head. Within 1-2 months a transient drop of antibody level was detectable. o——o monoarthritic patients (n=7); ----- healthy subjects (n=8)

The cartilage matrix protein (CMP) was extracted from the same source as the MP, from obduction material of newborn sternocostal cartilage. The 6 M guanidium chloride extract was fractionated by cesium Cl gradient ultracentrifugation, the floating fraction, free of proteoglycans was used as antigen (Fig. 6). The antibody level against this CMP was studied in sera of 71 controls and 39 OA patients.

Antibody was very rarely found, in less than 10% of the investigated persons. There was no correlation with age, and there was no difference between control (n=71) and OA (n=39) patients (Fig. 7).

The release of CMP with age in serum and in polychondrotic patients was determined by RIA technique /30, 35/. There were no data on antibody

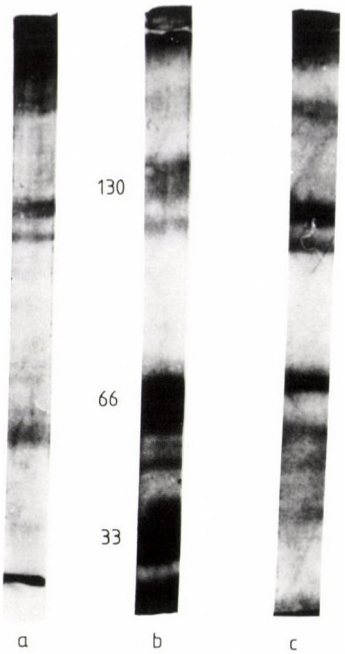

Fig. 6. SDS-6% PAGE slab gel electrophoretic pattern of cartilage matrix in reduced and non-reduced form. With reduction the peptide bands of low molecular weight are dominant. a = nonreduced; b = standard; c = reduced

level. The lack of detectable antibody level to CMP related to age or to osteoarthritis points indirectly to the specific antigen character of MP extracted from the same source.

IV. Antibodies to heat shock protein 65 kD

HSP related antigens are expressed by arthritic synovial membrane, in subcutaneous nodules /13, 17/ of RA patients, by osteoarthritic chondrocytes /18/. The antibody level against HSP antigen is raised in RA patients, especially in the early stage. The amino acid sequence 180-188, of the main epitope is a nonapeptide /7, 37, 38/: Thr-Phe-Gly-Leu-Gln-Leu-Gln-Leu-Thr /35/ is recognized by T-cell and induced resistance against experimental adjuvant arthritis (AA).

It is notable that the experimental adjuvant arthritis could be produced also by the epitope of Mycobacterium bovis, and the main amino acid sequence is a nonapeptide. The HSP 65 analogue could be detected in RAA

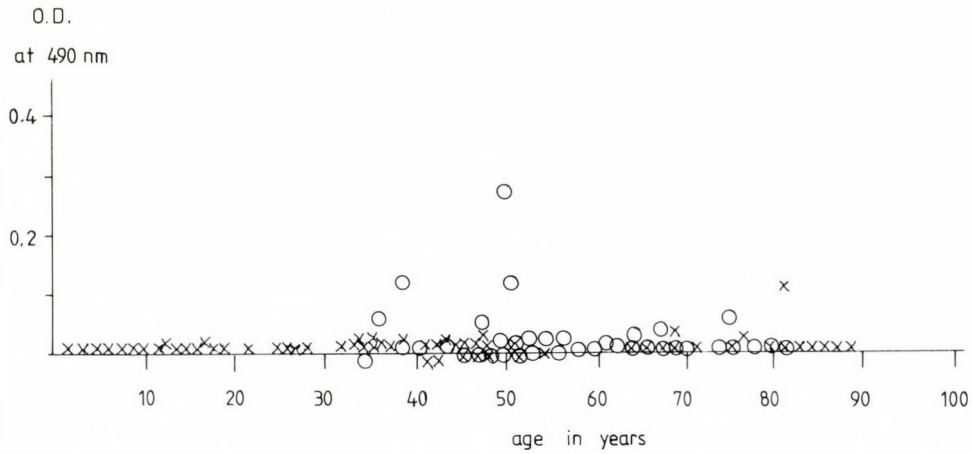

Fig. 7. Serum antibody level to cartilage matrix protein in osteoarthritic and control patients. Only in 4 four patients was antibody level measurable. o = osteoarthritic (n=39); x = controls (n=71)

subcutaneous nodules, and in serum antibodies to HSP. The HSP expression was revealed by Kubo et al. /18/ in tissue culture of chondrocytes from OA cartilage.

The putative role of HSP or its counterparts in the pathogenesis of RA and other kinds of arthritis was based on the following observations: 1. T-cells reactive with HSP can induce AA. 2. T-cells reactive with HSP have been detected in the synovial fluid of patients /33/. Antibodies reactive with HSP recombinant 65 kD were found in patients' sera /13/.

It was considered that HSP would crossreact with cartilage proteoglycan with its potential counterpart expressed during the disease /6/.

Mollenhauer reported that in AA induced by HSP there was a significant elevation of antibody level to MP which showed a good correlation with the manifestation of the disease in rats (Personal communication).

Knowing all these results, it seemed to be interesting for us to investigate all patients, altogether 100 persons (controls and OA patients), concerning their antibody level to HSP as well.

Antibody level was measurable only in 20% of the cases. There was no correlation with age or illness between controls and OA group (see Fig. 8).

The clinical diagnosis of the 22 patients with elevated anti-HSP level in order of the decreasing values are myocardial infarction, alcoholism, RA, hepatitis alcoholica, sinusitis maxillaris, pulmonary carcinoma, pneumonia,

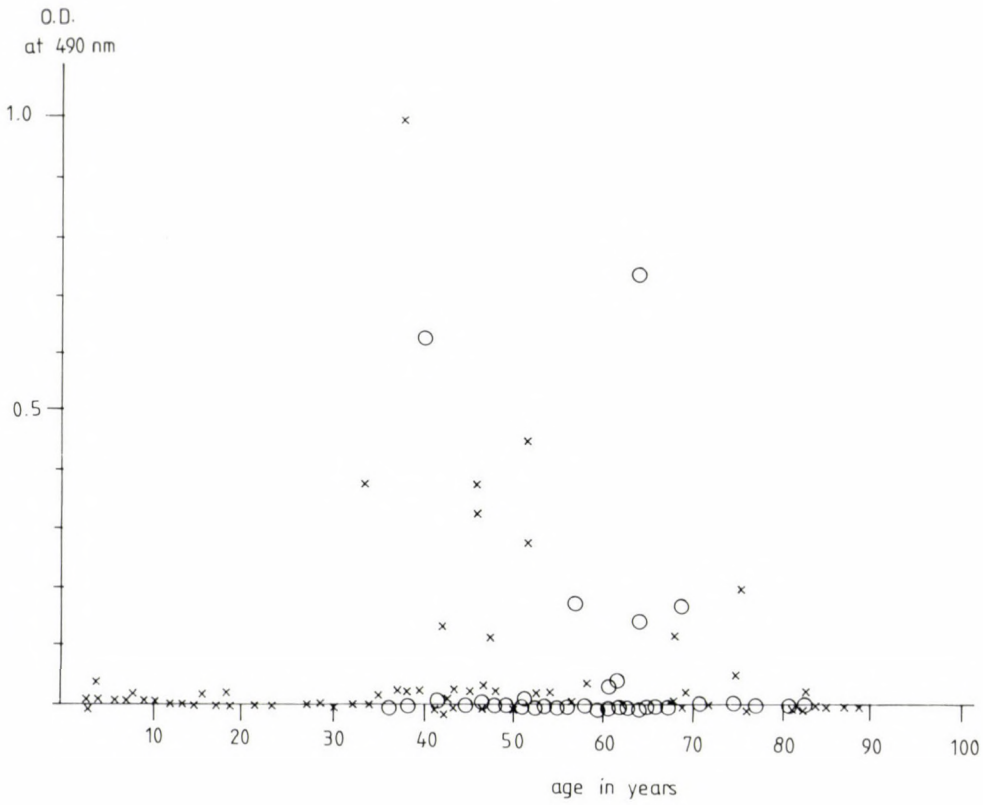

Fig. 8. Serum antibody level to heat shock protein 65 kD of Mycobacterium bovis BCG in osteoarthritic and control patients. No correlation was found between antibody level and osteoarthritis. o = osteoarthritic (n=39); x = controls (n=71)

hyperlipidaemia, pyelonephritis, etc. There was no dominant illness in the group of patients.

General Discussion

Osteoarthritis is generally accepted as a degenerative disease in which there is a secondary alteration of the cartilage tissue. Recently some new data have emerged that point to an autoimmune character of the illness as well: (i) there are some forms of OA, and some cases where there are direct evidences to an autoimmune process; (ii) there are clear-cut genetic molecular disorders which facilitate an autoimmune process and mean predis-

position to the development of familial OA; (iii) molecular mimicries were detected between arthritogen antigen of adjuvant arthritis (the epitope is called HSP 65 kD) and cartilage matrix proteins /8/.

ad (i) Autoimmune reaction against cell surface proteins in human degenerative tissue disorders, like myasthenia gravis, diabetes, chronic myopathy, have been described. In OA, especially in the later stage, there is direct evidence that activated enzymes /19/ destroy the matrix and the exposed collagen and proteoglycan epitopes induce antibody production and this leads to a vicious circle /5, 15, 32/. So the humoral immunological changes could be the indicators of the ongoing of the autoimmune process, its self-perpetuating and chronic nature /16/.

ad (ii) There are ever more data on the molecular mutant of alfa-1 chain of collagen type II /27/, or the mutation of codon arginine for cystein which mean predisposition to OA /11/. It was available with genetic fusing technique of the MHC class I. B27 with the β_2-microglobulin determinant to produce experimental spondylarthritis /14/. It is also known that inborn errors facilitate the autoimmune process and form a predisposition to arthro-ophthalmopathia, etc.

ad (iii) The collagen induced experimental arthritis (CIA) model has produced much new evidence on the causal role of collagen, more exactly on its epitope of CB 11 peptide in mice /36/ and CB 10 in rats /10/ consisting of 21-26 amino acids /22/. CIA can be prevented with the epitopes administered before the immunization. It is also known that the adjuvant arthritis (AA) can be produced and prevented by main epitope of heat shock protein, or with its reactive recombinant peptides HSP 65 kD it cross-reacts with cartilage proteoglycans. The proteoglycan core protein and link protein have also been described as causal agents in arthritis and spondylarthritis /6, 12, 26/.

There are many evidences that autoimmune process occurs also in some cases of osteoarthritis. The most sensitive method would be the detection of antigen sensitive T-lymphocytes or the detection of autoantibody—IgGAM-complex in situ immunohistofluorescent methods. From technical point of view the humoral immunological reactions are easier to carry out and the automatization of these methods is already solved. The method of choice is the antibody determination against membrane protein. It has the highest diagnostic efficiency. The determination of anticollagen level is less sensitive using native collagen types II, IX and XI as antigens. In experimental collage-induced arthritis it is a sensitive method. But in human diseases it

Table 2

The correlation of different parameters ("r", the coefficient of correlations is given)

	MP	HSP	Rf	CRP
MP	—	0.105	0.250	0.156
HSP	0.232	—	0.370	0.024

was found less sensitive by Charriere et al. /3/ as well. There are some technical methodological problems mentioned above, but the propeptide of these cartilage specific collagens might offer more sensitive antigens in analogy to N-procollagen (III). Their Gly-Leu-Gln amino acid sequence occurs in the nonapeptide epitope of HSP 65 kD. (Personal communication of K. van der Mark.) Against cartilage matrix protein no autoantibody building was found. It should be stressed that the membrane extract from the same source showed high reaction.

The correlation of different methods (expressed as correlation coefficient of the results) was very low (see Table 2).

The lack of correlation between antibody to HSA and OA seems to be in contradiction with the results of Kubo et al. /18/. They found positive correlation between the expression of HSP of cultivated chondrocytes from osteoarthritic cartilage and the severity of the illness. The reason might be the fact that the protective /31/ role of HSP (against stress, superoxide radical) "Chaperone" activity described by van Eden /9/ is predominant only at cellular level /4, 37/. The monoclonal antibody to HSP 65 kD did not show any affinity to the peptides of MP by immunoblotting technique as reported by Mollenhauer et al. /21/. Different source of monoclonal antibodies to HSP 65 kD did not react with ELISA technique with human MP antigen used by us (details of these results are not given here).

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EVALUATION OF EXERCISE INTENSITY INDICATED BY SERUM LACTATE DEHYDROGENASE ACTIVITY IN HEALTHY ADULTS

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Noting relationship between exercise and serum LDH and α-HBD activities, we prescribed 3 exercise regimens of various intensities for 22 healthy adult subjects over predetermined periods. Variations in the serum enzyme activities were determined in relation to these exercise programs. We studied whether these enzyme levels may serve as new indices to evaluate the amount of exercise. The test subjects were divided into 3 groups to perform 3 types of exercise (30%, 50%, 70% of VO_{2max}) for 6 weeks. The serum enzyme activities at the start and completion of the experiment were determined and compared. The results obtained were as follows:

A rise in the serum LDH activity was noted in association with exercise. The mean increases for these groups were 10.2%, 7.5% and 23.5%. A statistically significant decrease in the serum α-HBD activity was noted in association with exercise.

It has been suggested that by observing variations in these enzyme levels in normal individuals for an extended period, change in the intensity of physical exertion within the range of daily activities can be estimated.

Keywords: Serum LDH activity — serum α-HBD activity — exercise intensity — VO_{2max}

Introduction

In our days it is extremely important to evaluate the appropriate amount of daily physical activity (including work) to promote health and prevent hypertension, diabetes and other diseases of adulthood /1, 4, 9/. Unfortunately, research in this field has been insufficient and no suitable

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methods to determine the appropriate amount of physical activities have been found. We attempt in this work to show that serum lactate dehydrogenase (LDH) activity may serve to evaluate physical activities.

In clinical medicine, serum LDH activity is used effectively for the diagnosis of heart diseases such as myocardial infarction and hepatobiliary diseases such as liver cirrhosis and hepatitis. It is believed that exercise is one of the factors responsible for physiological changes in normal individuals and that serum LDH activity is closely correlated with physical activities /6, 11/. In the present study, healthy adult test subjects were engaged in exercise programs of 3 different intensities for a predetermined duration, and change in LDH and related α-hydroxybutyrate dehydrogenase (α-HBD) in their serum were observed to evaluate the suitability of using serum LDH activities as a new index for physical activity.

Methods and Subjects

The test subjects were divided into 3 groups to perform 3 types of exercise for 6 weeks. The serum enzyme activities at the start and completion of the experiment were determined and compared.

1. Subjects

The test subjects, 22 dietitian college students, were divided into group A (8 students), group B (8 students) and group C (8 students originally but 2 dropped out during the experiment. To select healthy individuals, the following were excluded:

1) Those who were currently under medical treatment.
2) Those who were questioned and found to have past records of cardiac, muscular or hepatobiliary disease or abnormal subjective symptoms.
3) Those who exhibited anomalies when examined by internists (in routine examinations such as blood pressure determination).
4) Those who were engaged in particularly demanding daily exercise.

Table 1 shows the ages and physical data of the test subjects.

2. Amount of exercise

The amount of exercise corresponded to 30% of the maximum oxygen uptake (VO_{2max}) for group A, 50% of the VO_{2max} for group B and 70% of the VO_{2max} for group C. At this intensity, the exercise was carried out for 30 minutes, 3 times a week for 6 weeks.

Table 1

Physical data of subjects

Item	Group A	Group B	Group C
No. of subjects	8	8	6
Sex	female	female	female
Age (year)	20.3 ± 0.5	20.6 ± 0.7	20.6 ± 0.5
Height (cm)	156.8 ± 9.2	156.7 ± 5.7	162.3 ± 6.8
Weight (kg)	53.2 ± 10.8	54.7 ± 3.2	56.8 ± 4.0
Broca index	93.6 ± 12.9	96.4 ± 11.3	91.1 ± 8.6

Broca index: [Weight/(Height-100)]x100

Table 2

Serum LDH before and after 6 weeks of training (units)

Group	Subjects	Before	After
A VO_{2max}: 30%	No. 1	391	379
	No. 2		
	No. 3	240	367
	No. 4	321	440
	No. 5	310	318
	No. 6	356	340
	No. 7	260	250
	No. 8	383	398
	Mean	323.3	356
	S.D.	58.2	61.1
B VO_{2max}: 50%	No. 9	342	376
	No. 10	323	393
	No. 11	261	306
	No. 12		
	No. 13	369	323
	No. 14	290	303
	No. 15	327	352
	No. 16	383	415
	Mean	327.8	352.5
	S.D.	42.5	43.9
C VO_{2max}: 70%	No. 17	354	432
	No. 18	317	391
	No. 19	306	367
	No. 20	312	466
	No. 21	367	360
	No. 22	331	437
	Mean	331.1	408.8
	S.D.	24.5	42.5

Table 3

Serum α-HBD activity before and after 6 weeks of training (units)

Group	Subjects	Before	After
A VO_{2max}: 30%	No. 1	322	233
	No. 2	303	229
	No. 3	282	198
	No. 4	356	257
	No. 5	325	241
	No. 6	325	236
	No. 7	282	220
	No. 8	332	257
	Mean	315.8	233.8
	S.D.	25.4	19.3
B VO_{2max}: 50%	No. 9	303	252
	No. 10	310	252
	No. 11	255	143
	No. 12	298	227
	No. 13	354	241
	No. 14	303	220
	No. 15	310	217
	No. 16	392	267
	Mean	315.6	227.3
	S.D.	40.8	38.2
C VO_{2max}: 70%	No. 17	330	191
	No. 18	342	226
	No. 19	383	274
	No. 20	335	257
	No. 21	322	255
	No. 22	332	312
	Mean	340.6	252.5
	S.D.	21.7	41.2

3. Blood samples

Blood sample collection was scheduled on the initial and last days of experiment. On these days, the subjects were instructed to fast from the preceding night. The blood was collected from the cubital vein and the serum was immediately isolated. The haematological examination was scheduled so that it could be completed on the day of specimen collection. The test items included serum LDH activity /2/ and serum α-HBD activity /12/.

Results

Table 2 shows variations in the serum LDH activity for each group. A rise in the serum enzyme activity was noted in association with exercise

Table 4

α-HBD/LDH ratio before and after 6 weeks of training

Group	Subjects	Before	After
A VO_{2max}: 30%	No. 1	0.82	0.61
	No. 2		
	No. 3	1.17	0.53
	No. 4	1.10	0.58
	No. 5	1.04	0.75
	No. 6	0.91	0.69
	No. 7	1.08	0.88
	No. 8	0.86	0.64
	Mean	1.00	0.67
	S.D.	0.13	0.11
B VO_{2max}: 50%	No. 9	0.88	0.67
	No. 10	0.95	0.64
	No. 11	0.97	0.46
	No. 12		
	No. 13	0.96	0.74
	No. 14	1.04	0.72
	No. 15	0.94	0.61
	No. 16	1.02	0.64
	Mean	0.97	0.64
	S.D.	0.05	0.09
C VO_{2max}: 70%	No. 17	0.93	0.44
	No. 18	1.07	0.57
	No. 19	1.25	0.74
	No. 20	1.07	0.55
	No. 21	0.87	0.81
	No. 22	1.00	0.71
	Mean	1.03	0.64
	S.D.	0.13	0.14

as follows: 323.0 ± 58.2 to 356.0 ± 6.1 units for group A; 327.8 ± 42.5 to 352.5 ± 43.9 units for group B; 331.1 ± 24.5 to 408.8 ± 42.5 units for group C. The mean increases for these groups were 10.2%, 7.5% and 23.5%, respectively. Only group C showed a statistically significant increase ($p < 0.05$).

Table 3 shows variations in the serum α-HBD activity for each group. A decrease in the enzyme activity was noted in association with exercise as follows: 315.8 ± 25.4 to 233.8 ± 19.3 units for group A; 315.6 ± 40.8 to 227.3 ± 38.2 units for group B; 340.6 ± 21.7 to 252.5 ± 41.2 units for group C. The mean values of decrease for these groups were 26.0%, 28.0% and 25.8, respectively. Each was a statistically significant decrease.

Table 4 shows variations in the α-HBD/LDH ratio for each group. A decrease in the ratio was noted in association with exercise as follows:

1.00 ± 0.13 to 0.67 ± 0.11 for group A; 0.97 ± 0.05 to 0.64 ± 0.09 for group B; 1.03 ± 0.13 to 0.64 ± 0.14 for group C. The mean decreases for these groups were 33.1%, 33.7% and 38.1%, respectively. Each was a statistically significant decrease.

Discussion

In recent years, the rapidly increasing use of automobiles and modernization of industry have resulted in reduced physical activity. On the other hand, caloric intake has increased with the consumption of larger quantities of animal fat. These life style changes resulted in a rapid rise in the prevalence rate of adult diseases such as hypertension, diabetes and ischaemic heart diseases. A detailed examination has revealed that appropriate exercise serves to prevent or alleviate the conditions of these diseases. Thus there is an increasing public interest on exercise to maintain and promote health. Yet research has been inadequate in finding an objective index to evaluate the amount of physical activity /8, 13, 14/.

Maximum oxygen uptake (VO_{2max}) /3/ and serum lactate level have been used to determine the intensity of exercise but they cannot be used as indices for fluctuations in physical activity over extended periods. The relationship between serum enzyme activities and exercise has been studied only with regard to a factor for deviations in clinical data in the area of clinical pathology and in relation to the effect of transient excessive training in the field of sports medicine /5, 7, 10/. There have been no studies on the applicability of serum enzyme activities as indicators of physical activity in daily life.

To establish an objective index of physical activity, serum LDH activity, which is frequently examined in blood chemical analyses in such procedures as mass examinations, was selected. The reason for this selection was that LDH derived from the skeletal system is known in the field of clinical pathology to be released into the serum as a result of physical activities, thus raising the serum content above a reference level. This phenomenon is explained by the following process: a rise in the intracellular osmotic pressure causes ATP consumption, glycolysis and other metabolic processes associated with exertion; consequently cells swell, enhancing the escape of intracellular enzymes into the serum; thus serum enzyme activity is raised.

In view of the serum enzyme dynamics described above, we investigated fluctuations of the serum LDH activities for use as an index of changes in physical exertion in normal individuals engaged in ordinary daily activities. The observation lasted for 6 weeks, during which the test subjects were instructed to engage in physical activities of light and medium intensities. Consequently it was found that only exertion of medium intensity produced a statistically significant increase in the enzyme level. Many investigators reported that the maximum safety threshold in the exercise test approximates 70% of the maximum oxygen consumption. The results of the present study suggest that a statistically significant increase in this enzyme activity (detected through continuous follow-up examinations by procedures such as mass screening) indicates that, except when a disease develops, the test subject has continued physical activities beyond the maximum safety threshold. In addition, we investigated the relationship between α-HBD, an enzyme related to LDH, and exertion. α-HBD is not a single enzyme: among LDH isoenzymes, LDH-1 and LDH-2 reduce α-ketobutyric acid and their enzyme activities are collectively called α-HBD for convenience. Conditions such as myocardial infarction are often reported to cause increases in the activities of this enzyme group. The exercise test of the present study produced statistically significant reductions at all 3 exertion intensities (a significant reduction was noted even with a light exercise intensity that corresponded to 30% of VO_{2max}). A similar tendency was also noted in the α-HBD/LDH ratio, suggesting reductions in the LDH-1 and LDH-2 fractions among LDH isoenzymes. It was suggested that changes in the intensity of physical exertion within the scope of daily activities may be determined through the combined observation of serum LDH and α-HBD activities at general physical examinations.

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L-SELECTINS REVEALED BY IMMOBILIZED ANALOGUE MOLECULES ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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The lymphocyte-endothelial interaction is initiated by selection type adhesion molecules on the surface of the lymphocytes (L-selectins) and by their carbohydrate ligands (addressins) in the glycocalyx of the endothelial cells. Under experimental conditions they can be substituted by analogue sugars, such as polyphosphonomonoester core polysaccharide and fucoidin. In this study, the expression of phosphomannosyl and fucoidin receptors is demonstrated on lymphocytes from human peripheral blood by polyacrylamide immobilized analogues. Based on adhesion and sugar inhibitory experiments, authors suggest that lineage and probably species specific differences exist in the expression and activity of the selectins responsible for binding of the two analogue molecules.

Keywords: Selectins — PPME — fucoidin — carbohydrate immobilization — lymphocyte-endothelial interaction

Introduction

Binding of lymphocytes to endothelial cells requires a sequence of adhesive interactions between different adhesion molecules. The adhesion molecules of the lymphocytes (L-selectins) are homologous to C-type (CA^{2+}-dependent) animal lectins, and they play a pivotal role in lymphocyte recirculation /10, 13/. Their carbohydrate ligands (vascular addressins) are part of the endothelial glycocalyx moiety /4, 17/.

In experimental models of lymphocyte-endothelial interactions analogue molecules are used to substitute the ligands for L-selectins /1, 17/. These

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molecules are the polyphosphomonoester core polysaccharide (PPME) from the yeast Hansenula holstii and the commercially available fucoidin from Fucus vesiculosus /2, 3, 6, 11/. These carbohydrate analogues bound to solid surfaces may serve as cytochemical probes /1, 17/.

In the present study we sought an answer, whether these analogue molecules immobilized to polyacrylamide/bisacrylamide gels may serve as tools to measure the activity of L-selectins of human peripheral blood lymphocytes. We have found an avid binding of lymphocytes both to PPME and fucoidin derivatized gels. Certain mono- and polysaccharides have shown an inhibitory effect, and it has been possible to saturate the carbohydrate binding receptors of human peripheral blood lymphocytes with both PPME and fucoidin.

Materials and Methods

Healthy blood donors served as the source of human peripheral blood lymphocytes. The lymphocytes were separated from whole blood by standard Ficoll-gradient centrifugation. The standard cell suspension medium was Dulbecco's minimum essential medium (H-DMEM).

The majority of chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Crude phosphomannan was kindly provided by Dr. M. Slodki (Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill.).

The preparation of fucoidin, PPME and carbohydrate-derivatized polyacrylamide gels was based on the methods of Brandley et al. /1/.

Briefly, fucoidin was treated by a protease, then dialyzed against running and distilled water. The dialysate was applied to Sepharose 4B gel filtration, and a high molecular weight carbohydrate peak was collected.

The PPME preparation started by a mild acid hydrolysis of H. holstii phosphomannan, then saturated by barium hydroxide, and the barium salt of high molecular weight PPME was precipitated by ethanol. After the removal of barium by a cation exchange resin, a sodium salt was obtained. The acid hydrolysis resulted in a pentasaccharide phosphomonoester (Pent), which was also isolated from the supernatant of barium precipitation /8/.

Prior to reduction with sodium borohydride and mild periodate oxidation, PPME and fucoidin were derivatized with hexanedianine.

The resulting amino sugars were coupled with a spacer molecule N-succinimidyl ester of 6-acrylamidohexanoic acid, and the product was copolymerized with acrylamide/bisacrylamide as described by Pless et al. and Guarnaccia and Schnaar /5, 7, 9/.

The cell adhesion was performed in conventional ELISA plates with 96 wells. 150 µl of 3.3×10^6 cells/ml in H-DMEM were centrifuged to the gel surfaces (50 g, 5 min, at 4 °C). After 1 hour of incubation at 4 °C, the wells were immersed in HEPES buffered saline at 0 °C, covered with a plastic tape, then centrifuged at an inverted position for 10 minutes (50 g for PPME, and 150 g for fucoidin activated gels). Cell adhesion was determined by the lactate dehydrogenase activity of the adherent cells /1/.

Results

Lymphocytes isolated from the peripheral blood of healthy human donors adhered specifically to both PPME and fucoidin derivatized acrylamide gels. The background adhesion was determined by inhibition experiments, i.e. lymphocyte suspensions were preincubated with PPME and fucoidin solutions of 250 μg/ml concentration.

Specific adhesion, after subtracting the background adhesion, was 14 and 11% of cells added to the wells for PPME and fucoidin derivatized gels,

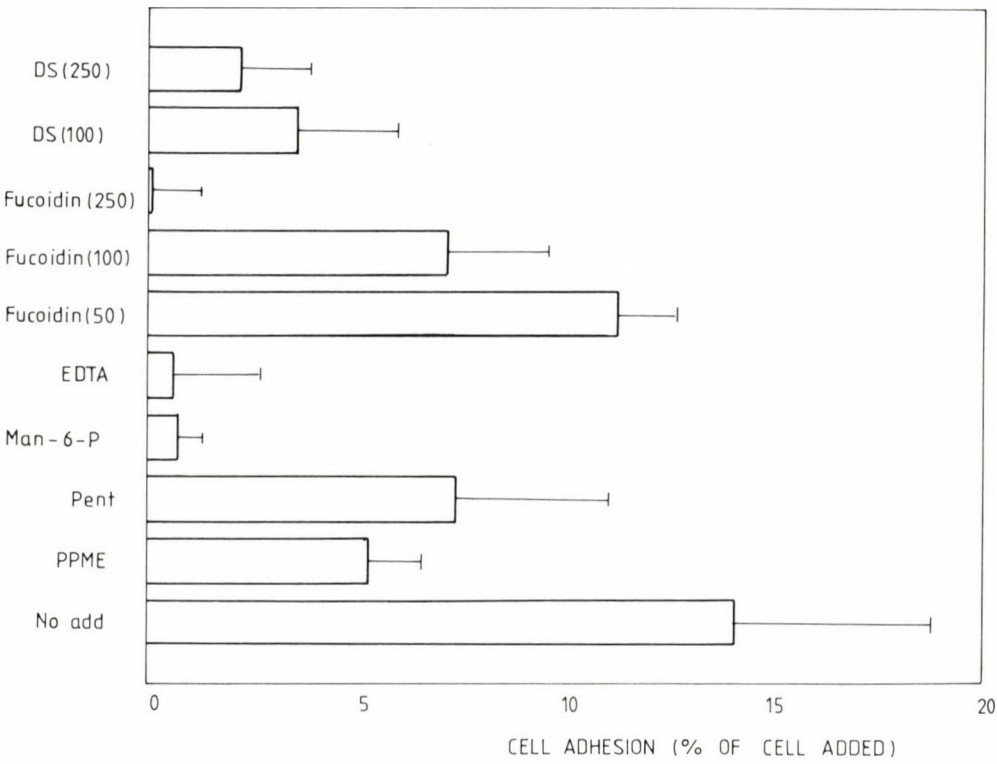

Fig. 1. Adhesion of human peripheral blood lymphocytes to PPME derivatized gels. Inhibition of cell adhesion by soluble carbohydrates and by removal of divalent cations. The background adhesion (10-18%) determined by saturation of the receptors (250 μg/ml PPME) was subtracted. Note that 250 μg/ml fucoidin also decreased the adhesion to the background level. Mannose-6-phosphate at a 50 mM concentration, as well as depletion of divalent cations (4 mM EDTA) resulted in a strong inhibition. Concentration of sugars necessary for 50% of adhesion were as follows, PPME: 50 μg/ml, Pent: 200 μg/ml, fucoidin: 100 μg/ml. Abbreviations: no addition (no add), dextran sulfate (DS). Figures in parentheses represent concentrations in μg/ml. Data are presented as the mean ± SEM (from minimum three experiments in duplicates)

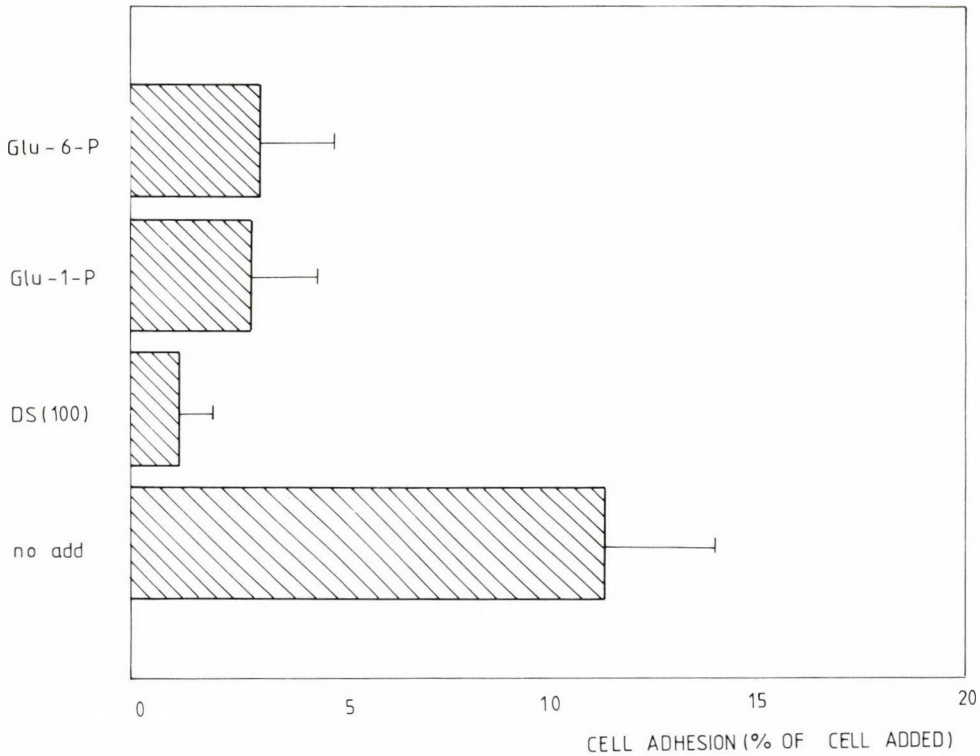

Fig. 2. Adhesion of human peripheral blood lymphocytes to fucoidin derivatized gels. The background adhesion (12%, 250 μg/ml fucoidin) was subtracted. Note that glucose-1 and glucose-6-phosphate (50 mM) as well as dextran sulfate are potent inhibitors of fucoidin receptor mediated adhesion

respectively. Sugars having an inhibitory effect on lymphocyte adhesion to PPME derivatized gels are as follows: PPME, Pent, fucoidin, dextran sulfate and mannose-6-phosphate. Binding to fucoidin derivatized gels was inhibited by fucoidin, dextran sulfate, glucose-1-phosphate and glucose-6-phosphate. In experiments where lymphocytes were suspended in EDTA supplemented with HEPES-buffered Dulbecco's minimum essential medium (H-DMEM), the binding to PPME derivatized gels was reduced to background level. PPME and EDTA supplementation did not result in an inhibitory effect on binding of cells to fucoidin derivatized gels. Our experiments are summarized in Figs 1 and 2.

Discussion

Carbohydrates serve as ligand molecules in many cell--cell and cell--matrix interactions, since their innate variability provides the kind of flexibility needed to control these interactions /14/. Their actions can be studied in some experimental settings, where purified carbohydrates and their analogues interact with viable cells /1, 17/.

Carbohydrate copolymerization in acrylamide/bisacrylamide gels seems to be an excellent tool to study the molecular mechanism of lymphocyte-endothelial interaction. We found an equally stable adhesion of human peripheral blood lymphocytes to polyacrylamide/bisacrylamide gels derivatized with analogue sugars PPME and fucoidin. Moreover, we revealed many similarities in the inhibitory experiments as well. L-selectins are known to interact with both PPME and fucoidin, however, there are some additional receptors on lymphocytes for fucoidin /16/. Our experiments show that cell lineage and probably species differences exist in the phosphomannosyl and fucoidin receptor expressions, since in their study with peripheral lymph node suspension of rats Brandley et al. /1/ demonstrated that only one-third of cells competent to bind fucoidin were also competent to bind PPME derivatized gels.

The study of adhesion-analogues and their inhibitors is a promising field of research, since the inhibitors could be applied as anti-inflammatory and anti-thrombogenic drugs. Tumours express carbohydrate ligands for different selectins, and such drugs might also be anti-metastatic /14/. Moreover, some lymphoma/leukaemia cell lineages express L-selectins at a high level, and mitogens are able to stimulate the expression of these molecules /15/. Investigations of human lymphoma/leukaemia cells with immobilized analogue carbohydrates may reveal many interesting aspects of lymphoma/leukaemia dissemination.

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ION UPTAKE AND GROWTH OF WHEAT SEEDLINGS TREATED WITH NITRITE AT DIFFERENT pH VALUES

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Increasing concentrations of $NaNO_2$ caused decreases in the uptake of K^+, Na^+ and SO_4^{2-} by wheat roots at pH 4. At pH 6 or 7, the detrimental effect was much less. The translocation of nutrients to the shoots was also impaired. The root growth was strongly inhibited by increasing $NaNO_2$ concentrations, but the shoot development was little affected. At low pH, however, the inhibitory effect was much higher.

Keywords: Wheat seedlings — K^+ uptake — potassium — sodium and sulfur contents — NO_2^- — pH effect

Introduction

Plants usually take up nitrogen in the form of NH_4^+ or NO_3^-. These forms may undergo several transformations. In aerated soils, NH_4^+ is oxidized by bacteria to NO_3^- in two steps:

a) $NH_4^+ + 3/2\ O_2 \xrightarrow{6\ e^-} NO_2^- + 2\ H^+ + H_2O$

b) $NO_2^- + 1/2\ O_2 \xrightarrow{2\ e^-} NO_3^-$

The oxidation of NH_4^+ to NO_3^- generates 2 H^+ and may cause acidification of the soil. There are five genera of Nitrobacteriaceae able to oxidize NH_4^+ to NO_2^-: Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus and Nitrosovibrio. Only one genus, Nitrobacter, is able to oxidize NO_2^- to NO_3^- /6/.

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In waterlogged soils and soils lacking O_2, NO_3^- is used by certain bacteria as an oxygen source and denitrified:

c) $NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2$

The complete reduction of 2 NO_3^- to N_2 generates 2 OH^-, which may lead to an increase in pH. Many bacteria are able to reduce NO_3^- or NO_2^-. Examples of such denitrifying bacteria are to be found in the genera Pseudomonas, Micrococcus and Thiobacillus.

While many studies have been made of NH_4^+ and NO_3^- uptake by plant roots /5, 7, 10/, relatively little is to be found in the literature on NO_2^- uptake. Opinions differ as to whether or not NO_2^- and NO_3^- are transported by the same systems in algae and higher plants. Cordoba et al. /4/ exclude a common carrier. Ullrich /11/ found that NO_3^- and NO_2^- compete for the same site. The classic literature on this subject, and also the molecular approaches, have recently been reviewed by Clarkson and Lüttge /3/.

The process of nitrification or denitrification may be interrupted, resulting in the production of NO_2^- /6/. Thus, plant roots may be confronted with varying concentrations of NO_2^- in the soil solution.

In a preliminary study with rice seedlings, we demonstrated that the H^+ ion concentration of the external medium influenced the toxic effect of NO_2^- extremely /14/. The results obtained suggest that the lower the pH in the root environment, the higher the uptake of NO_2^- by the roots, resulting in an inhibition of ion uptake and growth even at low concentrations of NO_2^- which are otherwise non-toxic to the roots of seedlings /13/.

The aim of the present paper is to report results on the processes of ion uptake regulation, growth and dry matter yield of NO_2^--treated wheat seedlings.

Materials and Methods

Winter wheat (Triticum aestivum L. cv. GK Szeged) was used as test plant. Seeds were washed for 3 h in running tap water and then left to germinate at 25 °C on wet filter paper in Petri dishes. After 1 day, seeds were placed on stainless steel screens over glass beakers, transferred to a controlled growth room and cultivated in 0.5 mmol/l $CaSO_4$, in full nutrient or in nutrient-deficient solutions. The composition of the nutrient solution was as follows: $NaNO_2$ from 0.1 to 10.0 mmol/l, KH_2PO_4 1.0 mmol/l, Na_2HPO_4 0.5 mmol/l, $CaCl_2$ 0.5 mmol/l, $MgSO_4$ 0.5 mmol/l and micro nutrients as described earlier /12/. There were usually 20 plants on a screen. The plants were illuminated, receiving 60 W m^{-2}. When used for the experiment, the

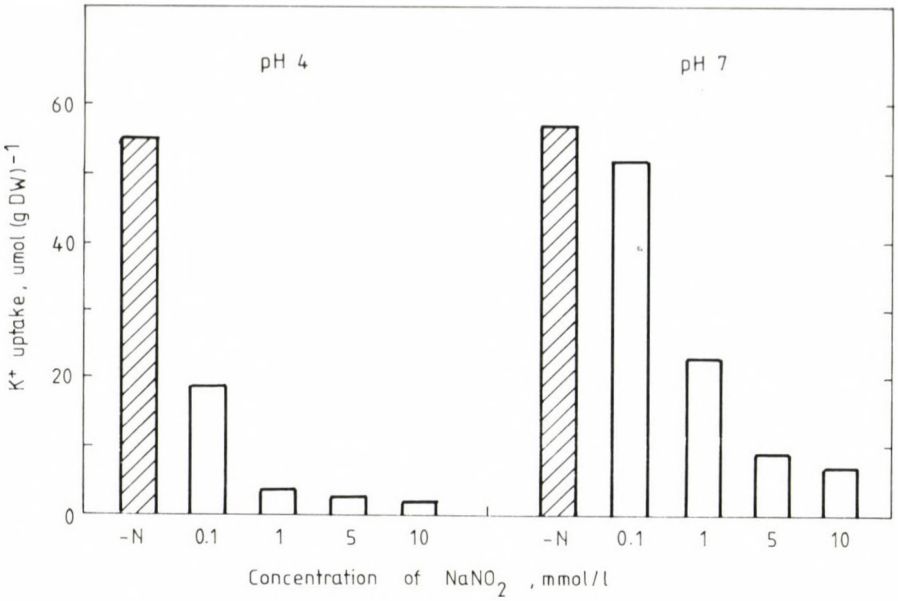

Fig. 1. Effects of pH and increasing $NaNO_2$ supply on the K^+ uptake of roots of 6-day-old wheat seedlings grown in 0.5 mmol/l $CaSO_4$ solution. Uptake solution: 1 mmol/l K(^{86}Rb)Cl + 0.5 mmol/l $CaCl_2$ + $NaNO_2$ as indicated. Uptake time: 1 h. Each value is the mean of 3 replicates. The SE did not exceed ±8%

plants were 6 days old. At the start of the experiment, the screens with plants were placed on beakers containing the nutrients under study. Each beaker contained 300 ml of growth solution.

The short-term K^+ uptake experiments were performed with intact plants in isotope-labelled absorption solutions (740 kBq l^{-1} ^{86}Rb). After a 1 h uptake time, the roots were rinsed three times for 1 min each in 400 ml distilled water, and their activities were then determined. ^{86}Rb activity was measured directly in the roots by γ-spectrometry. The pH of the growth and absorption solutions was adjusted to the appropriate values with 0.1 mol/l HCl or 0.1 mol/l NaOH, as needed.

The dry weights of the shoots and roots of all plants were determined on harvesting. The K^+, Na^+ and S contents were determined by inductive coupled plasma emission-spectroscopy (Perkin--Elmer Plasma-II), as described earlier /15/.

A typical series of results from three independent experiments is presented in Figs 1--6. All experiments were carried out with three parallel samples, and the data given below are averages. The deviations between the data from the individual determinations were within 8% of the arithmetical mean value.

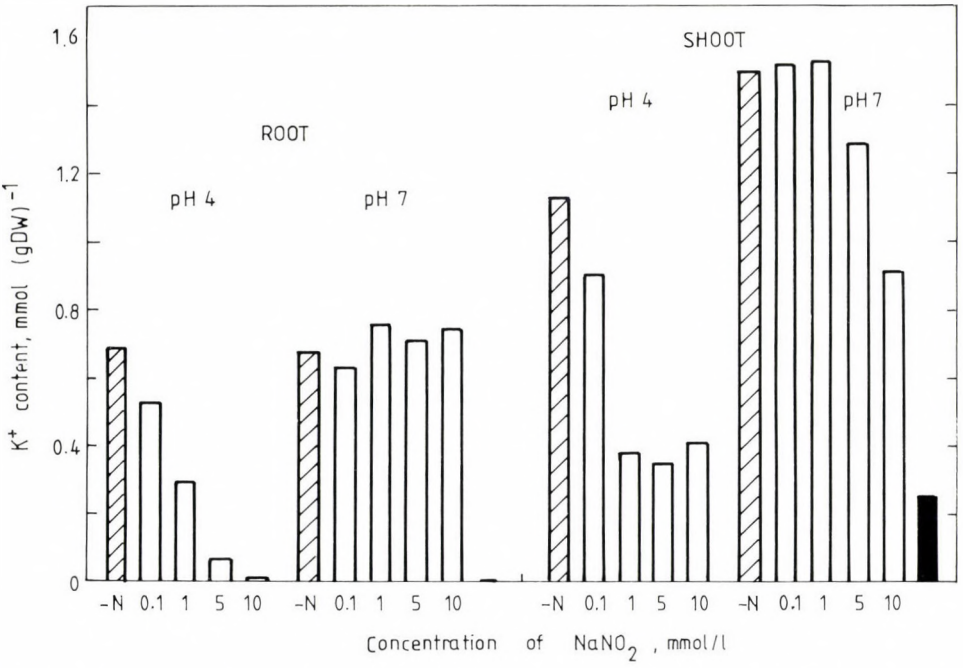

Fig. 2. Effects of pH and increasing $NaNO_2$ supply on the K^+ content of 6-day-old wheat seedlings from grown in nutrient solution. Full colums: control plants grown in 0.5 mmol/l $CaCl_2$ solution. Otherwise as in Fig. 1

Results

The uptake of K^+ during 1 h from a solution at pH 4 or pH 6 of 1 mmol/l KCl (labelled with ^{86}Rb), 0.5 mmol/l $CaSO_4$ and increasing concentrations of $NaNO_2$, decreased as the NO_2^- concentration increased (Fig. 1). The decrease was more pronounced at lower pH, as clearly seen if one compares, for example, the treatments with 0.1 or 1 mmol/l $NaNO_2$.

In a parallel experiment with inactive nutrients, the K^+ content of plants showed a similar pattern at pH 4 (Fig. 2). Increasing $NaNO_2$ concentrations caused a decrease in K^+ content. At pH 7, the content of K^+ was not affected or was only slightly influenced. The translocation of K^+ to the shoots was lower at pH 4 than at pH 7 and decreased with increasing $NaNO_2$ supply.

The Na^+ content (Fig. 3) at low pH was much lower than the K^+ content. There was little uptake at pH 4 as compared to pH 7. While the Na^+ content slightly increased with increasing $NaNO_2$ supply at pH 4, at pH 7 this effect

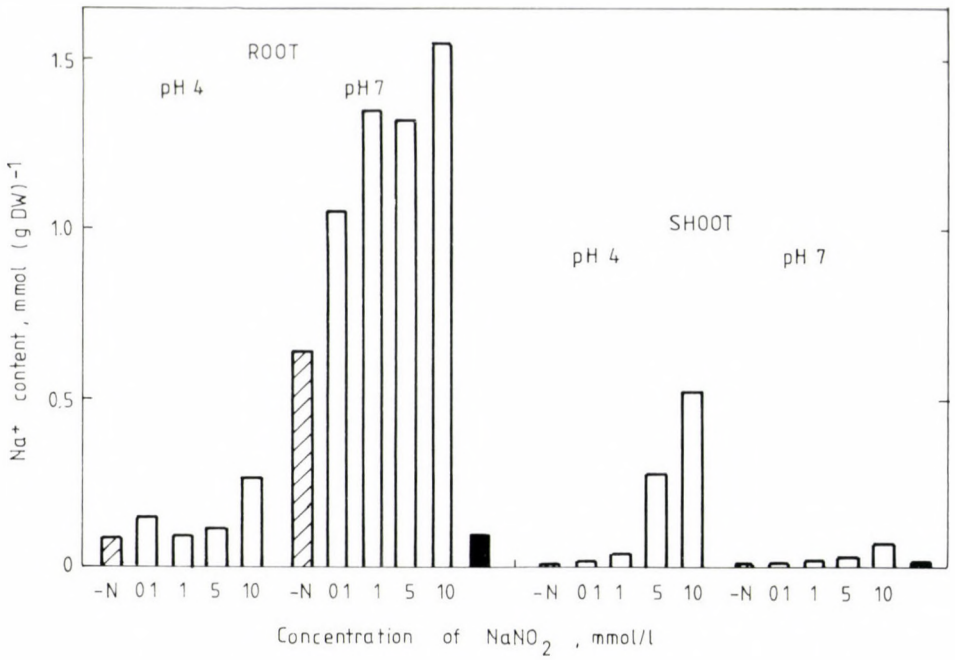

Fig. 3. Effects of pH and increasing $NaNO_2$ supply on the Na^+ content of 6-day-old wheat seedlings grown in nutrient solution. Otherwise as in Figs 1 and 2

was more pronounced. The translocation of Na^+ to the shoots was generally low, but it increased with the $NaNO_2$ concentration.

The S content (Fig. 4) of the roots at pH 4 and 7 were similar in magnitude. There was a decrease at pH 4 with increasing $NaNO_2$ supply, whereas at pH 7 the S content increased, as was found for K^+ and Na^+.

The translocation to the shoots was similar at the two pH values. Since relatively high amounts of S were found in the control plants grown in 0.5 mmol/l $CaCl_2$ solution, there was little uptake of S in the treatments.

When plants were grown for 6 days in a complete nutrient solution the root development was less with $NaNO_2$ as compared to $NaNO_3$. The root growth decreased at pH 4 with increasing $NaNO_2$ supply, but increased when $NaNO_3$ was supplied (Fig. 5). The shoot growth was inhibited at higher $NaNO_2$, but enhanced when $NaNO_3$ was supplied. At pH 6 (Fig. 6), a moderate decrease in root development was noted with increasing $NaNO_2$ supply, but the shoot growth was similar as in the case of $NaNO_3$ treatment, with the exception of 10 mmol/l $NaNO_2$ treatment.

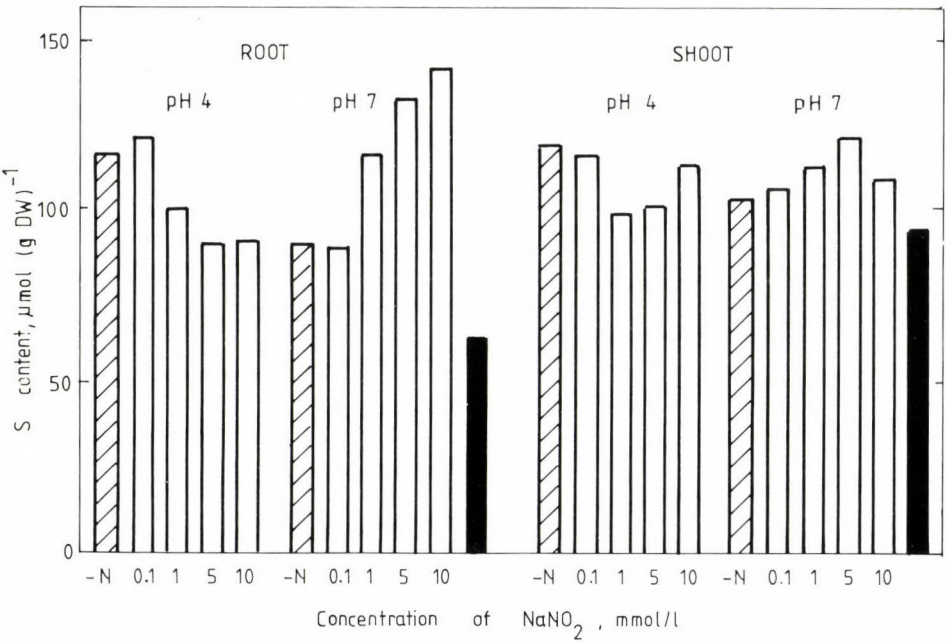

Fig. 4. Effects of pH and increasing $NaNO_2$ supply on the S content of 6-day-old wheat seedlings grown in nutrient solution. Otherwise as in Figs 1 and 2

Discussion

In all the studies conducted, a negative effect of NO_2^- on the ion uptake by wheat roots was observed; the growth of the roots was also impaired. Our results clearly show that the low K^+ concentration in the roots at higher $NaNO_2$ concentration and at low pH is due to the toxic effect of NO_2^- and not to the high Na^+ concentration of the growth solution. Such an effect, i.e. a retarded K^+ uptake into the roots at higher Na^+ concentration, cannot be excluded in the absence of NO_2^- treatment /16/.

In most soils, NO_2^- is usually present in negligible amount, but it may accumulate in the soil solution under certain environmental conditions and be toxic to plants, as found by Cawse and White /2/, Haynes /6/ and Marschner /8/. In higher plants, NO_2^- is reduced by nitrite reductase to NH_4^+. The enzyme is associated in roots with proplastids and in leaves with chloroplasts /1/. Certain herbicides, such as diuron, strongly and selectively inhibit nitrite reductase in leaves and correspondingly increase the NO_2^- concentration in the tissue /9/.

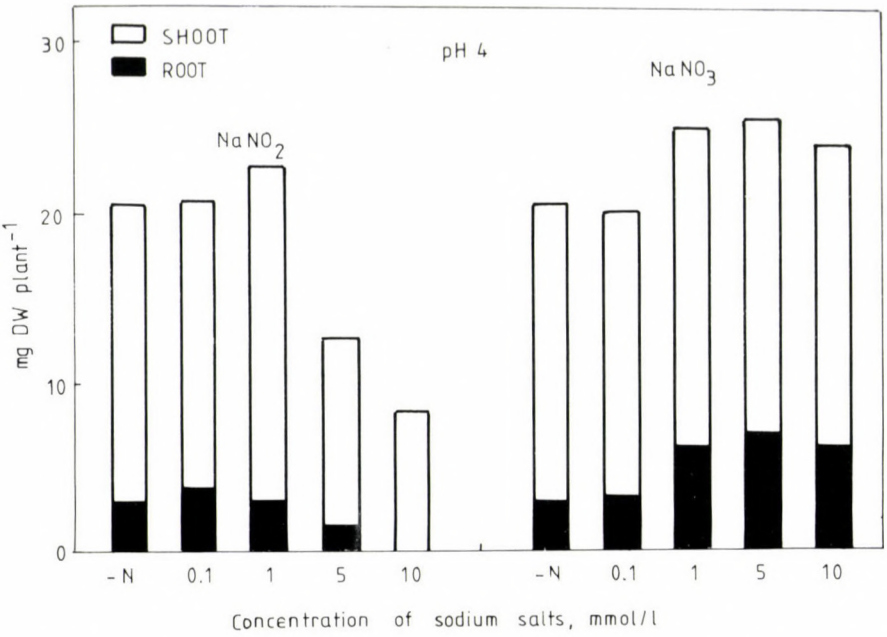

Fig. 5. Effects of pH 4 and increasing $NaNO_2$ supply on dry weight of 6-day-old wheat seedlings grown in nutrient solution. Otherwise as in Fig. 1

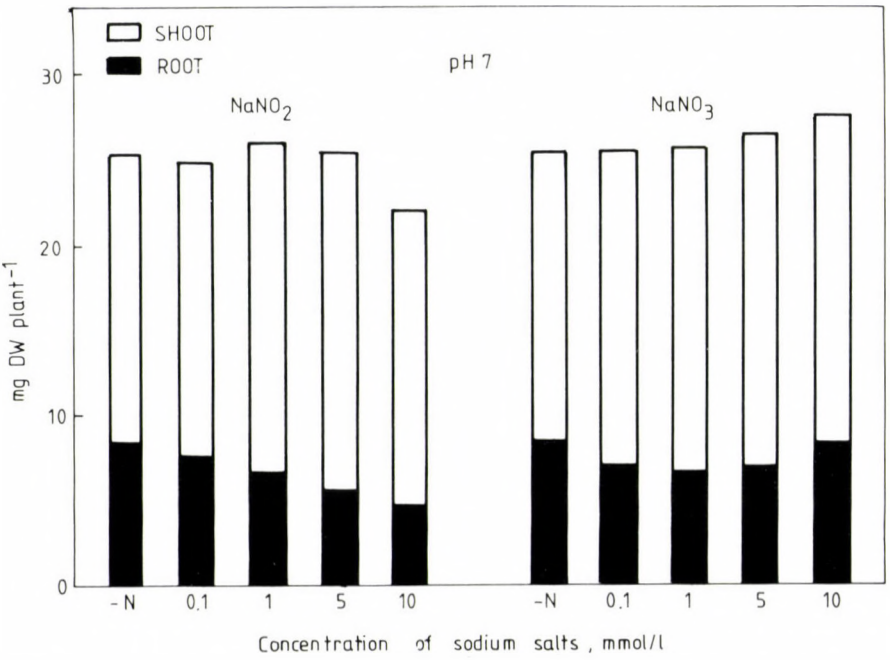

Fig. 6. Effects of pH 6 and increasing $NaNO_2$ supply on dry weight of 6-day-old wheat seedling grown in nutrient solution. Otherwise as in Fig. 1

To avoid NO_2^- accumulation in soils, which might lead to impaired plant growth, good soil management has to be practised. Acidification of soils should be avoided by liming if necessary, and aeration of the soils by ploughing and other mechanical practices should be ensured in cases of soil compression.

Acknowledgement

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EFFECTS OF NITRITE AND SULFATE ON K^+ UPTAKE AND GROWTH OF WHEAT AND CUCUMBER SEEDLINGS

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The uptake of K^+ ion was studied in the roots of wheat (Triticum aestivum L. cv. GK Szeged) and cucumber (Cucumis sativus L. cv. Budai csemege) seedlings grown in nutrient solution under nitrite and sulfate stress conditions. Seedlings pretreated with 1 or 10 mM $NaNO_2$ absorbed more $K^+(^{86}Rb)$ than those treated with 0.1 mM $NaNO_2$. However, the posteffect was influenced considerably by Na_2SO_4 treatment. The results suggest that at least a partial feed-back regulation of K^+ uptake may occur. However, due to the high Na^+ content of the roots and the possible toxicity of NO_2^-, effects of Na^+ and NO_2^- in this process cannot be excluded. The dry matter yields of the roots and shoots were strongly influenced by the NO_2^- supply of the plants. However, appreciable differences were observed between wheat and cucumber seedlings. The optimal SO_4^{2-} and NO_2^- concentrations of the growth solution for maximal growth differed considerably for the two species, and also for the roots and shoots in a given species. The root growth was strongly inhibited by increasing NO_2^- concentrations, but the shoot development was favourably affected, indicating that plants can assimilate NO_2^- taken up by the roots.

Keywords: Wheat and cucumber seedlings — $K^+(^{86}Rb)$ uptake — potassium, sulfate and sodium contents — effects of NO_2^- and SO_4^{2-} — NO_2 assimilation — shoot and root growth

Introduction

In an earlier study we demonstrated that seedlings grown under conditions of S deficiency or high (supraoptimal) levels of NO_3^- absorbed more K^+ than those pretreated with normal levels of SO_4^{2-} or NO_3^- /22/. It was also demonstrated that the growth and dry matter yields of the seedlings, espe-

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cially at higher NO_3^- concentrations, were strongly influenced by the SO_4^{2-} supply of the plants. When plants are S-deficient, the net uptake of NO_3^- and the transport of N to the shoot both decline, the protein synthesis is inhibited and non-protein nitrogen (e.g. amides, NO_3^-, etc.) accumulates /6, 12, 18, 20/. From these data it was concluded that the higher the N concentration in the root zone, the more important is the optimal S supply for the plants.

Plants obtain nitrogen required for their metabolism from the soil solution. The predominant form of N available to plants is NO_3^-, for under most soil conditions NH_4^+ is rapidly nitrified via NO_2^- to NO_3^-. The rate of conversion of NH_4^+ to NO_3^- is affected by several soil environmental conditions, including oxygen supply, population of nitrifying organisms, soil pH, temperature, soil moisture, amount of NH_4^+ in the soil, and man-made nitrification inhibitors /10, 11/.

In most soils NO_2^- is usually negligible. Nevertheless, some NO_2^- is always present and affects plant growth and metabolism to generally unknown degrees /1/. Further, endogenous NO_2^- can be generated during the NO_3^- assimilation of plants /4/. Detailed investigations of the effects of NO_2^- on plants are therefore of importance from both theoretical and practical aspects.

Recently, we found that increasing concentrations of $NaNO_2$ in the absorption solution caused decreases in the ion uptake and growth of wheat seedlings, especially at low pH /9/. This prompted us to study the effects of increasing concentrations of NO_2^- and SO_4^{2-} on the K^+ uptake regulation and growth of wheat and cucumber seedlings.

Materials and Methods

Winter wheat (Triticum aestivum L. cv. GK Szeged) and cucumber (Cucumis sativus L. cv. Budai csemege) were used throughout. Seeds were washed for 3 h in running tap water and then left to germinate at 25 °C in the dark on wet filter paper in Petri dishes. After one day, the seedlings were placed on stainless steel screens over glass beakers, transferred to a controlled growth room and cultivated in modified Hoagland solution /21/ with different NO_2^- and SO_4^{2-} concentrations for 14 days. Only NO_2 was used as N source. Each beaker contained 600 ml of growth solution and 10 seedlings. All nutrient solutions were renewed every 3 days. The seedlings were illuminated for 16 h, receiving 60 W m^{-2}.

The short-term K^+ uptake experiments were performed with intact plants in isotope-labelled absorption solutions (740 kBq l^{-1} ^{86}Rb). The pH of the absorption solutions was initially adjusted to 6.5, and was checked after

the absorption period. After a 1 h uptake time, the roots were rinsed three times for 1 min each in 400 ml distilled water, and their activities were then determined. ^{86}Rb activity was measured directly in the roots by γ-spectrometry.

The dry material (shoots and roots) of the seedlings was wet-digested in 1 ml H_2SO_4 and 2 ml H_2O_2 at 280 ^{o}C for 3 h. Samples were then made up to 10 ml with triple-distilled water. The K^+, S and Na^+ contents were determined by inductive coupled plasma emission spectroscopy (Perkin--Elmer Plasma-II), as described earlier /23/.

A typical series of results from three independent experiments is presented in Figs 1--5. All experiments were carried out with three parallel samples, and the data given below are averages. The deviations between the data from the individual determinations were within 8% of the arithmetical mean value. Curve fitting was performed graphically.

Results

Figure 1 shows the posteffects of an increasing Na_2SO_4 supply at three $NaNO_2$ levels on the short-term K^+ influx of wheat and cucumber roots. The data clearly show that the K^+ uptake by intact roots is markedly influenced by NO_2^- and SO_4^{2-} pretreatments. For cucumber, the higher the $NaNO_2$ concentration in the growth solution, the more rapid is the K^+ influx into the roots. As concerns wheat, the highest K^+ influx into the roots was experienced on 1 mM $NaNO_2$ treatment. Increase of the Na_2SO_4 supply in the growth solution resulted in an increase in the K^+ influx too, except at very high (10 mM) Na_2SO_4 concentration.

The K^+ concentration in the roots was higher in seedlings pretreated with low levels of $NaNO_2$, which indicates a direct influence on the net K^+ uptake (Figs 2 and 3). A slow growth of the shoots with little dilution of K^+, however, may also induce this. The effects of increasing $NaNO_2$ and Na_2SO_4 supply on the K^+ content of the roots are inhibitory, which strongly indicates a Na^+ inhibition of K^+ uptake. However, the toxic effect of NO_2^- in this process cannot be excluded.

As expected, the higher the S and Na^+ concentrations of the growth solution, the higher the internal S and Na^+ contents of the roots of the seedlings (Figs 2 and 3). However, very definite differences can be detected between the roots of wheat and cucumber seedlings, especially in response to extreme (10 mM) Na_2SO_4 treatment. It is noteworthy that the K^+, S and Na^+ contents of the shoots practically correspond to those in roots (data not shown).

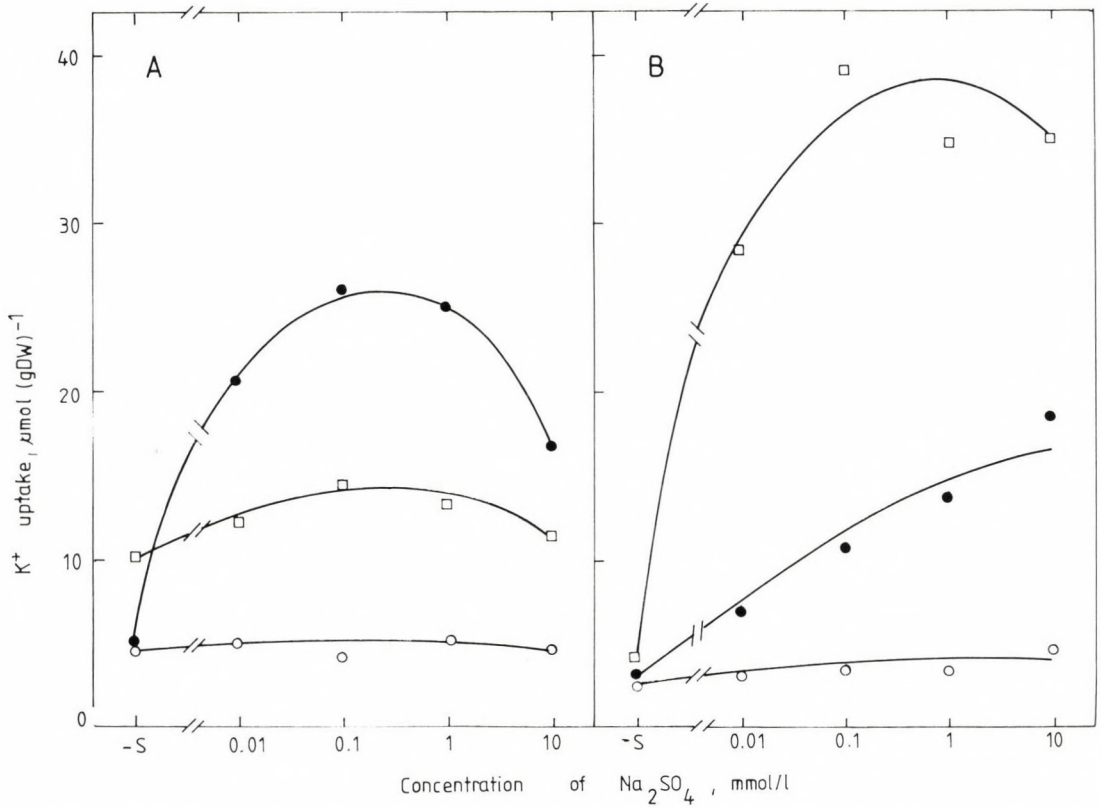

Fig. 1. Posteffects of increasing SO_4^{2-} supply at three $NaNO_3$ levels on the $K^+(^{86}Rb)$ uptake of roots of 14-day-old wheat (A) and cucumber (B) seedlings grown in nutrient solution. Symbols: 0.1 mM $NaNO_3$ (○), 1.0 mM $NaNO_3$ (●), 10 mM $NaNO_3$ (□). -S, control without SO_4^{2-}. Uptake solution: 1 mM $K(^{86}Rb)Cl$ + 0.5 mM $CaCl_2$; uptake time 1 h. Each value is the mean of 3 replicates. The SE did not exceed ±8%

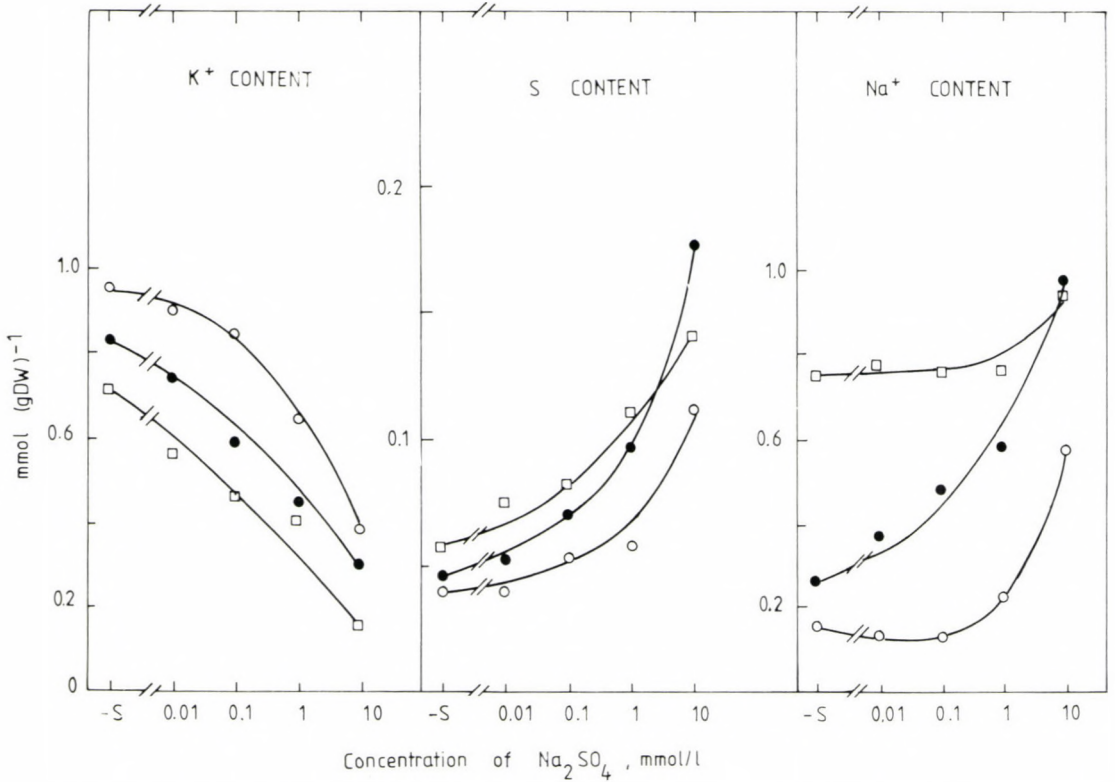

Fig. 2. Posteffects of increasing SO_4^{2-} supply at three $NaNO_2$ levels on the K^+, S and Na^+ contents of roots of 14-day-old wheat seedlings grown in nutrient solution. Otherwise as in Fig. 1

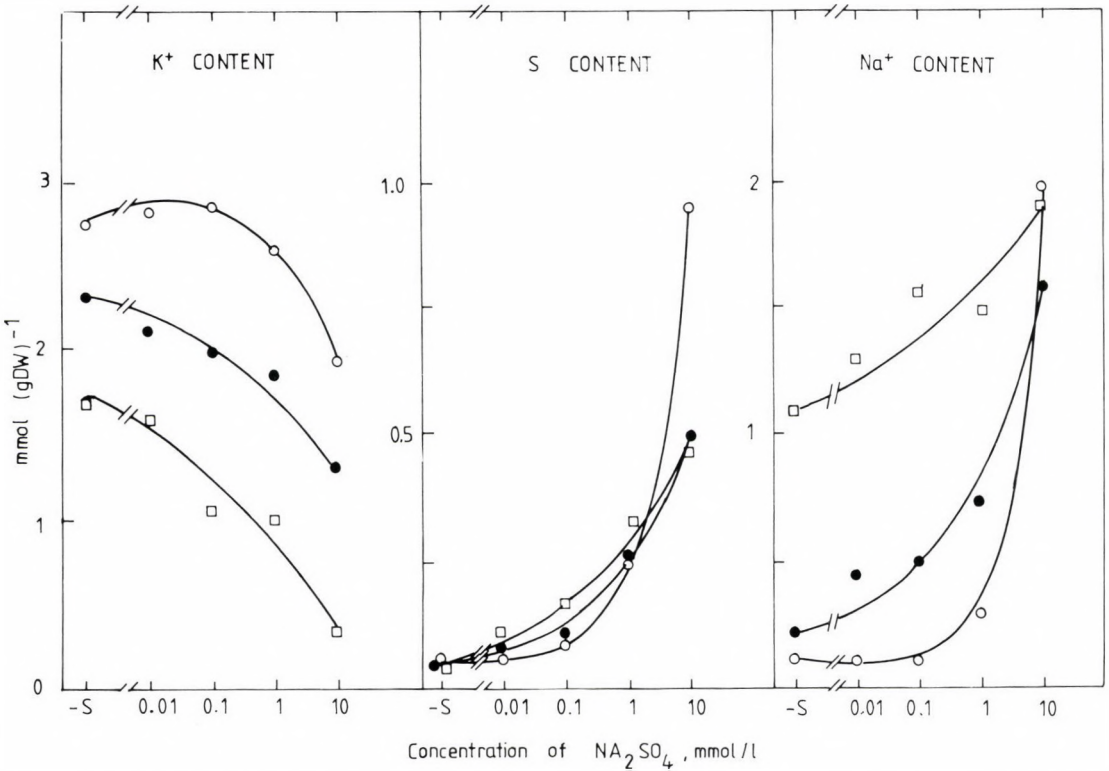

Fig. 3. Posteffects of increasing SO_4^{2-} supply at three $NaNO_2$ levels on the K^+, S and Na^+ contents of roots of 14-day-old cucumber seedlings grown in nutrient solution. Otherwise as in Fig. 1

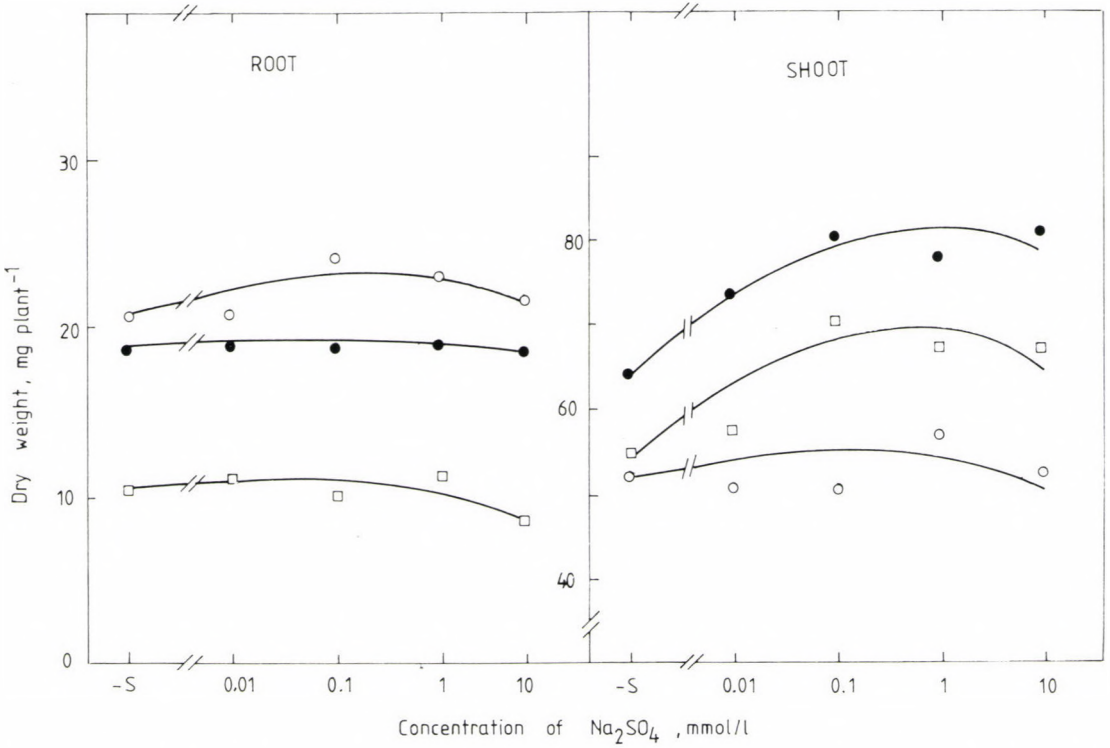

Fig. 4. Effects of increasing SO_4^{2-} supply at three $NaNO_2$ levels on dry weight of 14-day-old wheat seedlings grown in nutrient solution. Otherwise as in Fig. 1

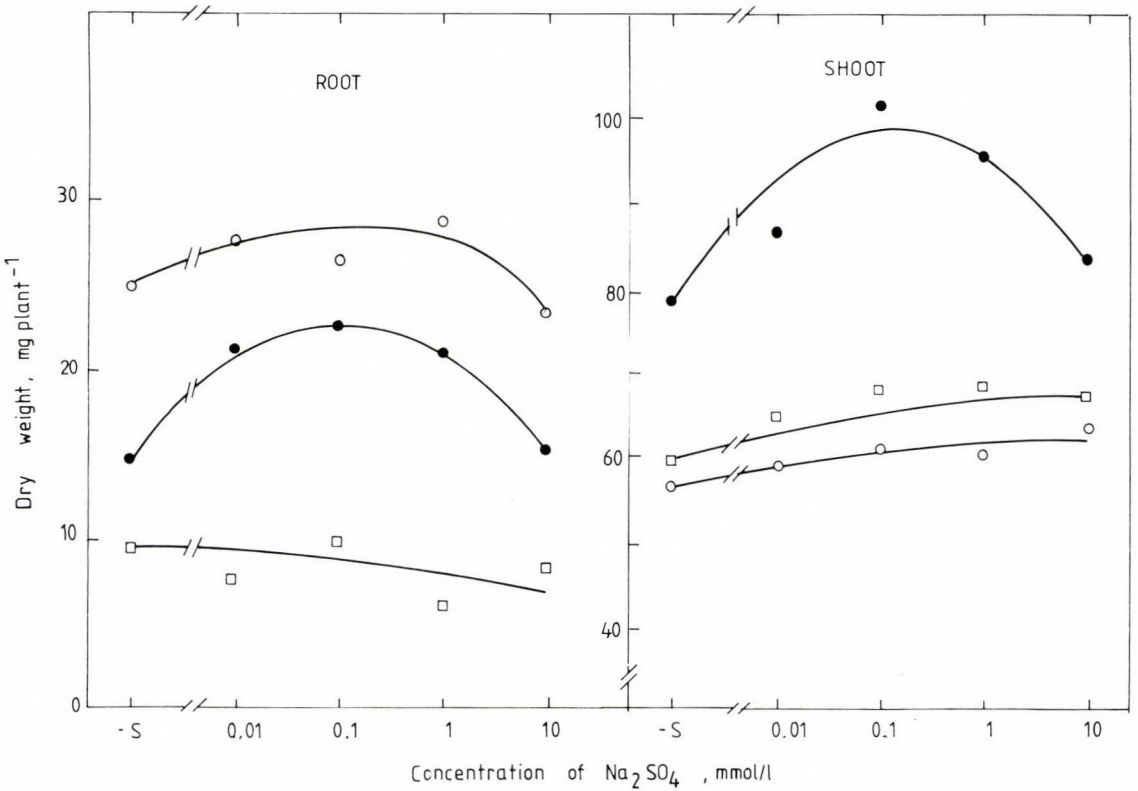

Fig. 5. Effects of increasing SO_4^{2-} supply at three $NaNO_2$ levels on dry weight of 14-day-old cucumber seedlings grown in nutrient solution. Otherwise as in Fig. 1

The dry matter yields of shoots and roots for wheat and cucumber seedlings grown in nutrient solution with different NO_2^- and SO_4^{2-} treatments are shown in Figs 4 and 5. In the presence of 0.1 mM NO_2^-, SO_4^{2-} treatment had no special effect on the dry matter production of 14-day-old seedlings. The most favourable treatment for the dry matter yield of cucumber seedlings proved to be 0.1 mM SO_4^{2-} and 1 mM NO_2^- (shoots), or 0.1 mM NO_2^- (roots). In the case of wheat seedlings, the most favourable SO_4^{2-} treatment was a concentration of 1.0 mM. Otherwise, the results for wheat seedlings correspond to those for cucumber.

Discussion

In well-aerated soils, the autotrophic oxidation of NO_2^- to NO_3^- proceeds at a faster rate than the conversion of NH_4^+ to NO_2^-. Consequently, NO_2^- is not normally present in amounts greater than 1 µg/g soil /11/, but it may accumulate in the soil solution under certain environmental conditions and be toxic to plants, as found by Cawse and White /2/, Haynes /10/ and Marschner /13/. There are reports that certain herbicides and heavy metals at higher concentrations, high soil-pH, etc. can inhibit the activity of the NO_2^- oxidizer (Nitrobacter) more than that of the NH_4^+ oxidizer (e.g. Nitrosomonas), resulting in an accumulation of NO_2^- /8, 10/.

Uptake studies with roots in different ion states have shown that the ion influx is dependent on the ionic relations in the external solution, on the tissue ion concentrations and on the requirements of the plant /3, 7, 16, 19/. However, relatively little is known about the ion uptake when plants are grown in a nutrient solution with NO_2^- as N source /9/.

The present experiments show that the K^+ uptake by wheat roots is stimulated by 1 mM $NaNO_2$ treatment in the presence of a 0.1 mM Na_2SO_4 supply. In the case of cucumber, however, such a stimulation in K^+ uptake was detected only on 10 mM $NaNO_2$ treatment (Fig. 1). A retarding posteffect was found for the K^+ uptake of roots when seedlings were grown at 0.1 mM NO_2^-.

Our results suggest that at least a partial feed-back regulation of K^+ uptake may occur and that NO_2^- pretreatment or a high Na^+ content has a key role in this process. It seems that the low K^+ uptake in plants under very low NO_2^- and SO_4^{2-} deficiency conditions could be explained by the well-known feed-back mechanism /5, 7, 17/. This assumption is supported in both cases by the pattern of change of the internal K^+ concentration of the roots

(Figs 2 and 3). Because cation uptake and anion uptake are regulated differently, direct interactions between them at low external concentrations do not necessarily occur /13/. However, at high external concentrations, SO_4^{2-} with a lower uptake rate could depress the rate of K^+ uptake. Naturally, at high Na^+ content in the seedlings, a Na^+-induced K^+ uptake inhibition may also occur. Further, on 10 mM $NaNO_2$ treatment, a possible toxic effect of NO_2^- in this process cannot be excluded /9/.

The difference in dry matter yield of 14-day-old seedlings clearly shows that the NO_2^- effect is influenced by the SO_4^{2-} supply of the plants (Fig. 4). The most favourable treatment for the dry matter yield of seedlings proved to be 0.1 mM SO_4^{2-} and 1 mM NO_2^-. This means that 10 mM NO_2^- treatment, in contrast with NO_3^- /22/, has a very definite retarding effect on the growth of the shoots.

The inhibitory effect of NO_2^- varied considerably between the species and was also different for the roots and shoots in a given species. It is noteworthy that the roots, and especially the root hairs (data not shown) are unusually sensitive to NO_2^- treatment. The growth of root hairs is more sensitive than root elongation to environmental stress factors /14/. Therefore, observation of the changes taking place in the root hairs could provide valuable general information about the physiological condition of plants.

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DEVELOPMENTAL ANALYSIS OF PRIMARY AND SECONDARY SOMATIC EMBRYOGENESIS IN SOYBEAN TISSUE CULTURE

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Somatic embryogenesis from immature soybean cotyledons was induced on Murashige & Skoog medium, supplemented with 2,4-dichlorophenoxyacetic acid. The full developmental analyses of primary and secondary somatic embryogenesis was monitored by scanning electron microscopy. The developmental stages of embryogenesis from the first unequal cell division through the three- and four-cell embryos, and multi-cell globular and heart shape embryos, to the fully developed plants were determined. When the globular stage embryos were incubated on the embryo maturation medium for 4 to 6 weeks a secondary embryogenesis was observed and scanned. Chimeric embryos with epidermis zones similar to mature leaf were characterized.

Keywords: Somatic embryogenesis — scanning electron microscopy — soybean

Introduction

The process of plant regeneration in tissue culture can follow two different paths, namely organogenesis (caulogenesis or rhizogenesis), or embryogenesis /1, 12, 25/. Of the two, somatic embryogenesis has greater importance due to its one-cell origin /10/. Successful soybean somatic embryogenesis, documented by its certain stages, has been reported by Kiss et al. /14/, Finer /6/, Barwale et al. /2/, and Christianson et al. /4/, but

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Abbreviations: BA: benzyladenine, CH: casein hydrolysate, 2,4-D: 2,4-dichlorophenoxyacetic acid, GA3: gibberellic acid, IBA: indolebutyric acid, LM: light microscopy, SEM: scanning electron microscopy

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none of these included full developmental analysis. In the present study we conducted the characterization of soybean somatic embryogenesis from the first unequal cell divisions to plant regeneration. To our knowledge, this report is the first to give a full developmental analysis of primary and secondary somatic embryogenesis in soybean tissue culture.

Materials and Methods

Tissue culture

For callus induction, cotyledons (721 cotyledons of 5 mm in size) of immature soybean (Glycine max L., Merr.) embryos were excised after surface sterilization of the pods in 0.2% $HgCl_2$. Cotyledons were placed with their adaxial surface onto callus initiating medium (M-1), containing Murashige & Skoog /19/ salts and organics, supplemented with 2,4-D (10, 20 and 40 μM), and sucrose (3%). The callus cultures were incubated at 24 ^{o}C, at a light intensity of 1000 lux, under a 16 h photoperiod for 28 days. From the 28th day, cotyledons with embryos at the globular stage were subcultured onto embryo-maturation medium (M-2) containing MS-salts and organics, IBA (0.6 μM) and ABA (1 μM). Fully developed cotyledonary embryos, two weeks after transfer, were subcultured onto plant regeneration medium (M-3) containing MS salts, charcoal (0.1%) ascorbic acid (0.1 g/l), 1.7 μM BA and 0.2 μM IBA. Regenerated clones were micropropagated on hormone-free, half-strength MS medium (M-4). For the induction of secondary embryogenesis, primary somatic embryos were kept on M-2 embryo-maturation medium for 4 to 6 weeks. The developed clusters of secondary embryos were transferred onto M-3 medium and incubated for 21 days.

Scanning electron microscopy

Callus and embryo samples were harvested at weekly intervals, fixed in glutaraldehyde (5% w/v in phosphate buffer 0.07 M, pH 7.2) and washed 3 times in buffer (10 minutes each). Then the samples were dehydrated in acetone series (30-50-70-90-100%), dried at the critical point of CO_2 (Balzers CPD 020) and covered with gold (30 nm). The specimens were examined and photographed with either Cambridge Stereoscan 150 (see Fig. 2E), or a TESLA BS-300 electron microscope as previously described /3, 8, 13/.

Results

Primary embryogenesis

The isolated soybean cotyledons became large and pale green after 5 days in culture on callus-inducing M-1 medium containing 20 μM 2,4-D. Callus tissue first became visible at the adaxial surface of cotyledons (Fig. 1A) from the first week in culture. Three different calli were selected: Type I

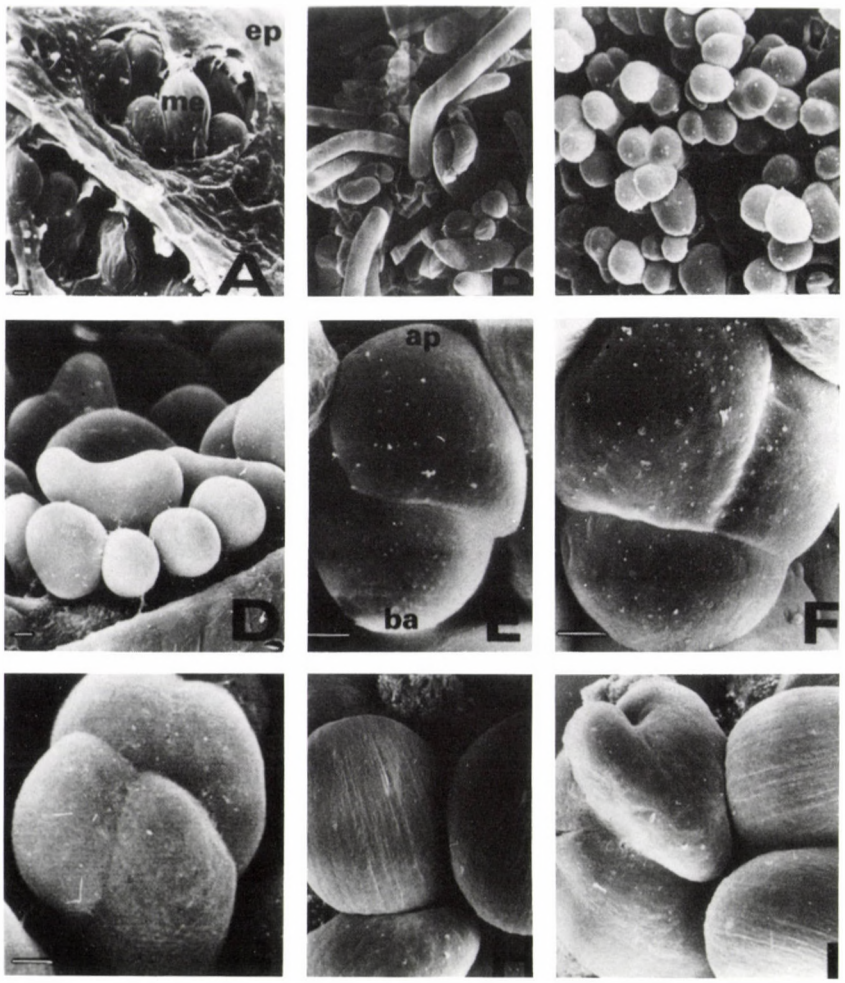

Fig. 1. Somatic embryogenesis and plant regenerations in soybean (Glycine max L., Merr.) tissue culture from cotyledons of immature zygotic embryo (Bar = 10 μ). A: Five days old cultured cotyledons on callus-inducing medium. Mesophyll (me) originated callus cells are breaking out through the epidermis (ep) layer (x155); B: Callus type I, with nonembryonic, elongated cells (x155); C: Callus type II, with nonembryogenic, equally dividing cells (x155); D: Callus type III, with embryogenic, unequally dividing cells (x550); E: The initial event of embryogenesis was the unequal cell division through the transversal cell wall resulting in a polarized bicellular embryo with basal (ba) and apical (ap) cell (x1000); F: The longitudinal division of apical cell resulted a three-cell embryo (x1000). The basal cells growing into callus cells were developing to suspensor; G: The second longitudinal cell division of apical cell resulted a four-cell embryo (x1000); H: Multi-cell somatic embryos at the globular stage (x20); I: Heart-shape somatic embryo (x20)

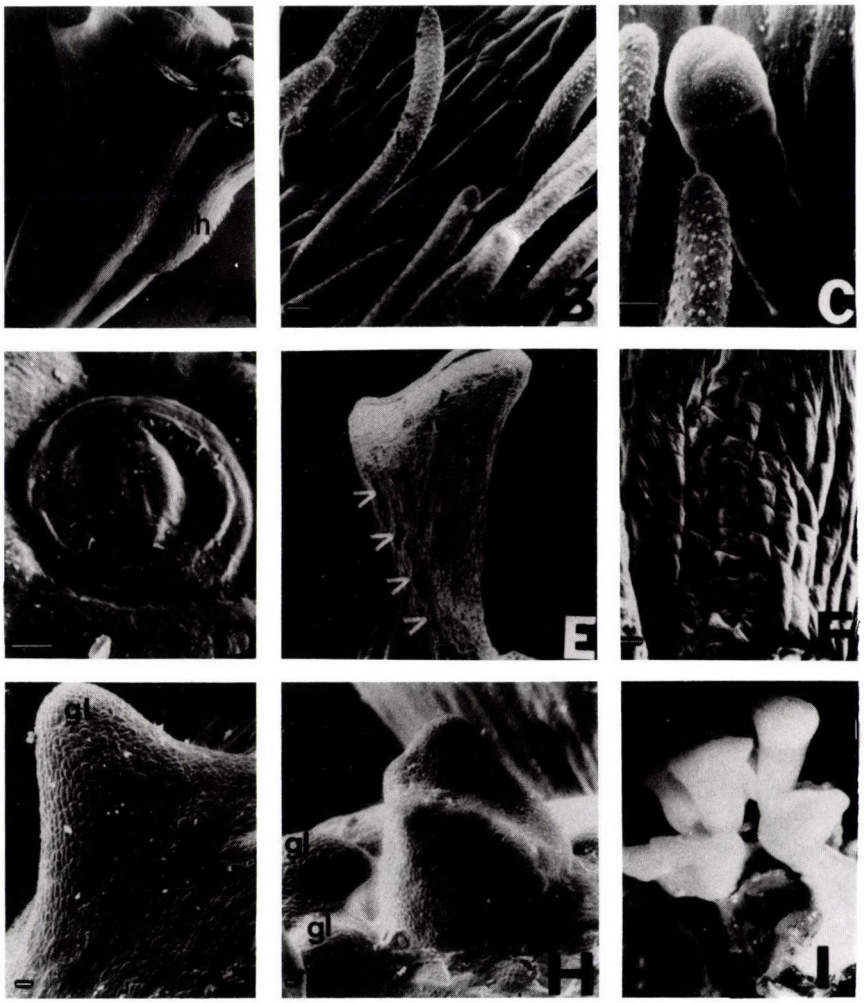

Fig. 2. Secondary somatic embryogenesis and plant regeneration in soybean (Glycine max L., Merr.) tissue culture from primary somatic embryos (Bar = 10 μ). A: Overdeveloped embryo with elongated and fused cotyledons with chimeric zones of fully developed epidermis (lh = leaf hairs) (x20); B: Leaf hairs (lh) on chimeric embryo epidermis (x500); C: Gland hair (gh) on chimeric embryo epidermis (x1000); D: Stoma on chimeric embryo epidermis (x1000); E: On the epidermal surface of overdeveloped embryo meristems were initiated (x20); F: From these meristems (x400) secondary embryos developed; G: Secondary embryo at globular stage (gl) (x200); H: Secondary embryo at heart stage (x100), surrounded by newly developed globular embryos (gl); I: Cluster of secondary embryos at torpedo stage (LM. x10)

a nonembryogenic, soft callus with elongated and translucent cells (Fig. 1B), Type II nonembryogenic friable and pale green callus with equally dividing cells (Fig. 1C), and Type III embryogenic, compact and green callus with unequally dividing cells (Fig. 1D).

Among the calli that show unequal cell divisions numerous somatic embryos at different stages of development (Fig. 1E to I) were found. The responsive explants (43.5% of 721 cotyledons) gave about 10 globular embryos/explant.

The initial event of somatic embryogenesis was a transverse cell division, which produced a basal and an apical cell (Fig. 1E). After a longitudinal division of the apical cell a three-cell stage embryo was formed (Fig. 1F). A subsequent longitudinal division of the apical cells, a four-cell stage embryo (Fig. 1G) emerged (tetrad), while the basal cell growing into the callus cells became suspensor.

The four-cell stage embryo, through numerous cell divisions, developed into a multi-cell stage globular embryo (Fig. 1H). In 3 to 4 weeks after the initiation of cultures, a soft callus tissue with elongated and translucent cells began to overgrow the embryogenic cells. To maintain the embryogenic capability of calli, the nonembryogenic, soft calli were removed monthly.

Cotyledons with embryos at the globular stage were transferred onto M-2 embryo-maturation medium. Embryogenesis from the globular stage (Fig. 1H) progressed through the heart stage (Fig. 1F) to the fully developed mature cotyledonary embryo on this medium. After the elongation of the hypocotyl the embryos were ready to be transferred onto the M-3 plant regeneration medium. Root development was completed and the regenerants (11 plants) were micropropagated on hormone free, half strength MS medium (M-4). After mass propagation, the clones were planted in soil in greenhouse for phenological analysis.

Secondary embryogenesis

When the cotyledons with the induced globular stage embryos were incubated on M-2 medium for 4 to 6 weeks, a secondary embryogenesis was observed. The primary embryo development did not stop: the cotyledons elongated (Fig. 2A) and fused forming an enlarged overdeveloped embryo (Fig. 2B).

Frequently, more than two cotyledons were fused which formed a trumpet-like structure. The epidermis was characterized by usual attributes of

adult leaves in chimeric zones including leaf hairs (Fig. 2C), gland hairs (Fig. 2D) and stoma.

From the fifth week of plating onto M-2 medium, meristematic centers appeared on the epidermal surface of overdeveloped embryos (Fig. 2E). From these meristems (Fig. 2F) numerous secondary embryos developed through globular (Fig. 2G) and heart stages (Fig. 2H) giving rise to a cluster of secondary embryos (Fig. 2I).

After six weeks, embryo clusters were transferred onto M-3 medium for plant regeneration. The overdeveloped primary embryo never, the secondary embryos regenerated to plant with very low (0.08%) frequency.

Discussion

In this work, developmental analyses of primary and secondary somatic embryogenesis of soybean were monitored by scanning electron microscopy.

As in the work of Ranch et al. /21/, the present study found that a concentration of 20 μM 2,4-D was sufficient to induce somatic embryogenesis from immature soybean cotyledons. According to the two proposed systems of classification for zygotic embryogenesis, type I (Crucifer or Onagrad type) embryogenesis was observed in the present study. Both classification systems including either five /22/ or six /18/ basic types, include Type I (Crucifer or Onagrad type) which consists of an initial transverse cell division followed by two longitudinal ones /1, 5, 7, 18/.

The process of embryogenesis from the first unequal cell division to the globular-stage embryo progressed in four weeks from plating confirming the proposed one-cell origin of soybean somatic embryos derived from immature cotyledons /11/. After embryo maturation and plant regeneration the regenerated 11 plants were vigorous for micropropagation.

When the secondary embryogenesis was investigated, the mother primary embryos were found to become similar to the so-called overdeveloped embryo /14, 16, 17, 21, 23/. A unique phenomenon was recognized when the embryo epidermis was characterized with the usual attributes of adult leaves in zones, giving an embryo chimera. The phenomenon is not unique to soybean as it has been described for Eranthis /9/, Oenanthe /24/ and Hemerocallis /15/.

In conclusion, the present study provided basic evidences about soybean somatic embryogenesis, which can help to clarify the differences between the paths of pollen and somatic, the somatic and zygotic, and the

zygotic and adventive embryogenesis /18, 22, 23/. According to our results and the observations of zygotic embryogenesis /18, 20/ it can be concluded that the early cell divisions, to the four-cell embryo stage, in both cases of zygotic and somatic embryogenesis progress in the same way. The secondary somatic embryogenesis induced from the primary soybean embryo followed the steps of primary somatic embryogenesis. The observed secondary embryogenesis was different from the polyembryony /1/ which comprises clusters of embryos originating from the same meristematic centers.

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RESPONSES TO CONTINUOUS AND DISCONTINUOUS NaCl STRESS OF LONG-TERM CULTURED RICE (ORYZA SATIVA L.) CELLS

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Rice (Oryza sativa L.) cells, grown under a continuous stress of 1.5% NaCl, produced a homogeneous mass of dry, compact, nodular callus with high regeneration potential, typical of embryogenic cultures. When transferred and subcultured in conditions without salt, the pre-adapted cells underwent several changes, leading to the formation of heterogeneous populations that comprised different cell types. This newly formed callus, in the 6-month period without salt stress, resembled cell populations of the untreated cultures. These cells are characterized by fast growth, high water content, friable texture, high salt sensitivity and low culture response. They also produce a protein pattern in the SDS-PAGE analysis, that differs from that of cells cultured under the continuous salt stress. This observation indicates that different salt stress regimes induced different responses in the cultured cells in rice.

Keywords: Cell cultures — different NaCl stress — different responses — rice

Introduction

Somaclonal variation, generated in cultured cells, may provide a novel source of materials useful for plant breeding /20/. Attempts have been made to exploit this variability to improve agronomical traits, especially to increase the salt tolerance potential of the present crops /29/.

A number of cell lines tolerant to high salt stress have been developed in different species /1, 12, 13, 24/. Some of these cells appeared to remain tolerant after several subcultures removed from salt stress /7, 32/,

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while others subsequently became sensitive /6/. Although plants could be recovered from tolerant cells /15, 25/ some tolerant lines can also arise from the salt untreated cultures /30/. In certain cases plants regenerated from the stress adapted cells did not exhibit a significant increase of salt tolerance /33/ and some of them even became more sensitive to salt stress /2, 8/. These uncertainties in development of salt tolerant plants through in vitro culture have led to the need for a greater understanding of in vitro induced tolerance mechanisms /14/.

Rice, like other important crops evolved in a glycophytic habitat, are sensitive to salt in the field. Increasing salt tolerance in this species could be partially produced through the conventional breeding methods, however, the mechanism by which tolerance is conferred remains unclear /11/.

To trace the tolerance potential from cell to whole plant in rice, it is important to identify the salt tolerant cell type, and characterize the way cells respond to salt during growth and differentiation. In our previous studies, with long-term cultured rice cells, salt tolerant cells were identified as those cells which are embryogenic. Tolerance was considered as a natural adaptive potential of the embryogenic cells /3/. This potential, however, tends to alter at an early stage of plant development. Variation of the salt tolerance, between growth and differentiation, was observed even in stress regeneration cultures of the salt adapted embryogenic cells /5/. A critical question is therefore whether salt tolerance is dependent on the embryogenic cycle, along which embryogenic cells with high tolerance potential are generated.

The present study reports that embryogenic cells in rice could adapt to grow in conditions with long-term salt stress, but would give rise to other cell types on a salt-free medium. The new nonembryogenic cells, which appeared in the discontinuous stress cultures, were sensitive to salt on stress re-exposure. Similarities, observed in several parameters studied between these cells and those of the untreated cultures, led to the suggestion that "embryogenic cycle dependence" can be the specific character of salt tolerance in long-term cultured rice cells.

Materials and Methods

Cell cultures and salt stress regimes

Salt tolerant cells, selected from long-term suspension cultures of rice (*Oryza sativa* L.) cv. Oryzella were maintained in the presence of 1.5%

NaCl as described previously /3/. Adapted cells, which grew into compact nodular structures, were used in further experiments. The organized cell clusters were transferred to MS /23/ agar basal medium, without inositol, supplemented with 2% sucrose, 300 mg/l casein hydrolysate (CH), 5 mg/l thiamine, 0.5 mg/l pyridoxin, 0.5 mg/l nicotinic acid, 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.5% NaCl. The pH of the medium was adjusted to 5.8 before autoclaving. After 6 months' growth part of the adapted calli were maintained under this continuous stress regime; the other part was transferred to the same medium but without salt for the next 6 months. For the control cultures, cells from the friable, salt untreated suspension were maintained on the same salt-free medium, which was used for the discontinuous stress treatment. The cultures were kept in the dark at 27 ^{o}C, and cells were subcultured to a fresh medium every 4 weeks.

Evaluation of tolerance and culture response

Salt tolerance and the morphogenetic potential of the cultures from the different stress treatments were evaluated by the growth and redifferentiation ability of cells on a medium with or without 1.5% NaCl. For growth potential, 30 cell clusters (4-8 mg each) were transferred to the MS medium with and without salt and after 4 weeks the fresh and dry weights were determined. Plant regeneration was conducted with cell clusters (4-6 mg each) on N6 /10/ agar basal medium, supplemented with 3% sucrose, 1 g/l CH, 2 mg/l 6-benzylaminopurine (BA), 0.5 mg/l 1-naphthaleneacetic acid (NAA), with and without 3% mannitol, and the pH was adjusted to 5.8 before autoclaving. Cultures were kept in the light (140-180 μmol $m^{-2}s^{-1}$) with a 16-h photoperiod at 27-28 ^{o}C. After the first 3 weeks 90 pieces were taken randomly from the derived callus mass of each treatment and plated on the same regeneration medium for further development. Frequency of plant regeneration was calculated after 5 weeks.

Protein analysis

The procedure used for protein analysis was based on previously described protocols /19, 22/. For protein extraction, cells of three cultures were used as following. Samples were taken from 8-10 days old suspensions, 3 weeks old agar cultures for growth, and 2 weeks old regeneration cultures, respectively. The samples were first frozen and then extracted in a full glass potter type of homogenizer with the sample buffer described in /19/. This buffer contained 1 mM phenylmethylsulphonyl fluoride to inhibit protease activities. The homogenates were incubated at 100 ^{o}C for 5 minutes and centrifuged at 12,000 x g for 10 minutes in an Eppendorf microfuge. Protein content of the supernatants was estimated by the method described in /22/ after the aliquots were precipitated and washed with 5% trichloroacetic acid (TCA). Equal amounts of proteins from each sample were loaded on a 10-15% linear gradient polyacrylamide gel containing sodium dodecyl sulphate (SDS), and after electrophoresis the protein bands were stained with Coomassie brillant blue as it was described in /19/. Several standard proteins, having 92.5, 45, 31, 21.5 and 14.4 kD molecular mass, were used for the molecular wight calibration. Proteins, extracted from 2-day seed germinating embryos of non-tissue culture-derived rice plants were used as the control.

The experiment was repeated at least 3 times with similar results.

Results

Cell growth under continuous salt stress

During the 12 months of subculturing under the continuous stress of 1.5% NaCl, the adapted cells continuously generated a homogeneous mass of dry, compact, nodular callus (Fig. 1 A2, B). After 4 weeks in this condition the fresh weight (FW) of cells increased 10.3 times (43.2 mg) with a dry weight (DW) of 17.4% (Table 1). Long-term maintenance in the high saline condition resulted in a better regeneration of the adapted cells on a medium supplemented with 3% mannitol (43.3%) than on the mannitol-free medium (26.7%). All regenerants were green plantlets (Table 2).

Table 1

Effects of NaCl stress on growth of long-term cultured rice cells derived from continuous and discontinuous stress cultures

Derived cell mass	Cell growth with NaCl (%)*					
	FW (mg)		DW (mg)		DW/FW (%)	
	0	1.5	0	1.5	0	1.5
1. Untreated cultures (control)						
Watery &	115.3	9.4	7.4	1.3	6.4	13.8
friable	(± 35.8)	(± 3.7)	(± 2)	(± 0.9)	-	-
	/22.6/	/1.8/	-	-	-	-
2. Continuous 1.5% NaCl stress cultures						
Compact &	-	43.2	-	7.5	-	17.4
nodular	-	(± 8.9)	-	(± 1.7)	-	-
	-	/10.3/	-	-	-	-
3. Discontinuous salt stress cultures						
a) 1 month						
Compact &	131.4	-	10.4	-	7.9	-
nodular	(± 41.8)	-	(± 2.6)	-	-	-
	/25.3/	-	-	-	-	-
b) 6 months						
Watery &	158.9	10.5	8.7	1.1	5.5	10.5
friable	(± 53.5)	(± 2.3)	(± 2.8)	(± 0.8)	-	-
	/21.5/	/1.4/	-	-	-	-

*Mean (± SD) and /increase in times/ of fresh (FW) and dry weight (DW) of 30 callus pieces after 4 weeks grown in the tested media. (-) Not done.

Responses to discontinuous salt stress

When transferred from stress conditions to a salt-free medium and so subcultured for 6 months, several changes were observed in the pre-adapted cells. The first month's growth without stress led to a significant increase in cell mass, with a 25.3 times increase in fresh weight (131.4 mg). However, the water content was higher (DW 7.9% of cell mass) (Table 1). The cells also had a higher regeneration ability (47.8-55.5%) for the two tested media than cells of the continuous stress cultures (Table 2).

Table 2

Regeneration potential of long-term cultured rice cells derived from continuous and discontinuous salt stress cultures

Derived cell population	Mannitol in medium (%)	Regeneration potential of cells transferred*				
		Organized str.**		Plantlets		Green plants
		(No.)	(%)	(No.)	(%)	(%)
1. Untreated cultures (control)						
Watery &	0	3	3.3	0	0	0
friable	3	5	5.5	0	0	0
2. Continuous 1.5% NaCl stress cultures						
Compact &	0	29	32.2	24	26.7	100
nodular	3	27	30	39	43.3	100
3. Discontinuous salt stress cultures						
a) 1 month						
Compact &	0	11	12.2	43	47.8	100
nodular	3	15	16.7	50	55.5	100
b) 3 months						
Compact &	0	12	13.3	6	6.7	100
friable	3	21	23.3	10	11.1	100
c) 6 months						
Watery &	0	1	1.1	0	0	0
friable	3	3	3.3	0	0	0

*Number and percentage of responsive calli, estimated with 90 callus pieces after 5 weeks in the 2nd subculture on the same medium for regeneration.

**Calli producing only organized structures and/or budding somatic embryos.

During the next months, however, the cell population of these cultures became obviously heterogeneous. The previous dry, compact nodular structures first grew in size, accumulated more water, and subsequently turned loose in texture with new, watery and friable cells emerging on the surface of calli. Regeneration ability of the previously high responsive cells, after 3 months subcultured in the discontinuous stress condition, sharply decreased (6.7-11.1% for regeneration media without and with 3% mannitol, respectively) (Table 2).

The frequency of calli which gave rise to new cells at the end of the 6th subculture reached a level of 40-65% of the total. The new, watery and friable cell mass (Fig. 1D) resembled the cell population of the salt untreated cultures (Fig. 1 A1, C) in respect of fast proliferation (21.5-22.6 times increase in FW, that reached 115.3-158.9 mg), high water content (DW ranged in 5.5-6.4% cell mass) and high sensitivity to salt (FW remained at 9.4-10.5 mg under 1.5% NaCl) (Table 1). By the end of 4 weeks growth with this high salt stress, only some fresh, milky-yellow, nodular cell structures were observed among the bleached or brown death cells of these two similar watery and friable cell populations.

Friable cells of the control and of the discontinuous stress cultures did not give rise to plants in both culture conditions with and without mannitol supplementation. At the end of the 2nd subculture for plant regeneration only organized structures were formed at low frequency (1.1-5.5%), in cultures of these cells (Table 2).

Protein synthesis under different stress regimes

Changes in the pre-adapted cells, during subcultures in the discontinuous stress condition, were also observed in the SDS-PAGE analysis (Fig. 2). The most obvious difference in the protein profiles was found at protein bands b-1 and b-2 of high molecular weight (HMW) and protein band b-3 of medium mass (MMW).

When intensities of the selected protein bands of the same sample were compared, there were several patterns of more ($>$), similar ($\approx$), or less ($<$) intensive bands distinguished as following: b-1 $<$ b-2 was observed in samples of the control and discontinuous stress cultures (Fig. 2; 2, 4, 6, 7); b-1 $>$ b-2 in samples of the salt stress cultures and in cultures for regeneration of the stress cultured cells (Fig. 2; 3, 5, 8, 9).

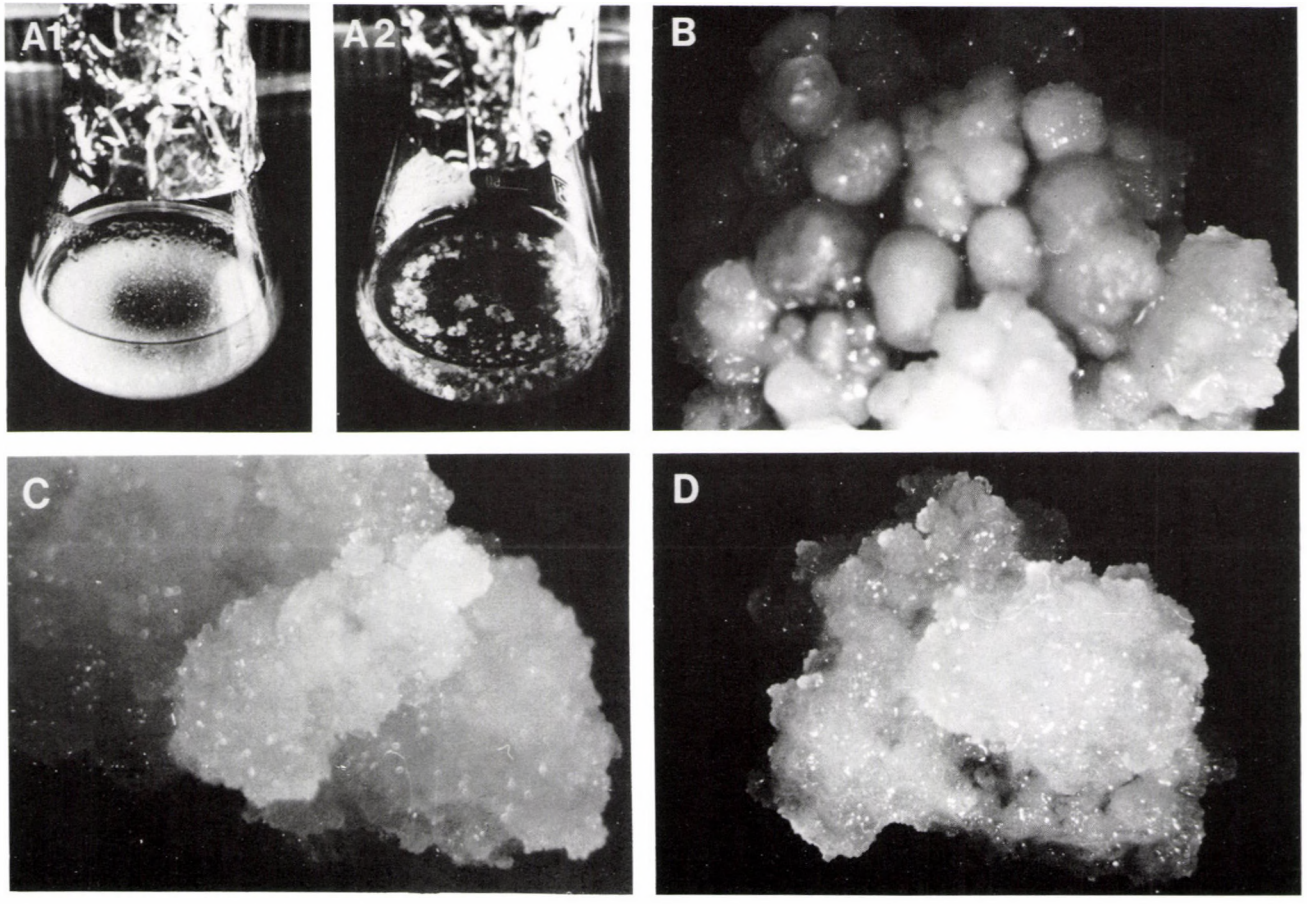

Fig. 1. Appearance of rice cells cultured in different salt stress conditions. Friable cells of the untreated suspension (A1) and untreated agar (C) cultures. Embryogenic cells of the 1.5% NaCl stress suspension (A2) and continuous salt stress agar (B) cultures. Friable cells derived from the 6-month discontinuous stress agar cultures (D)

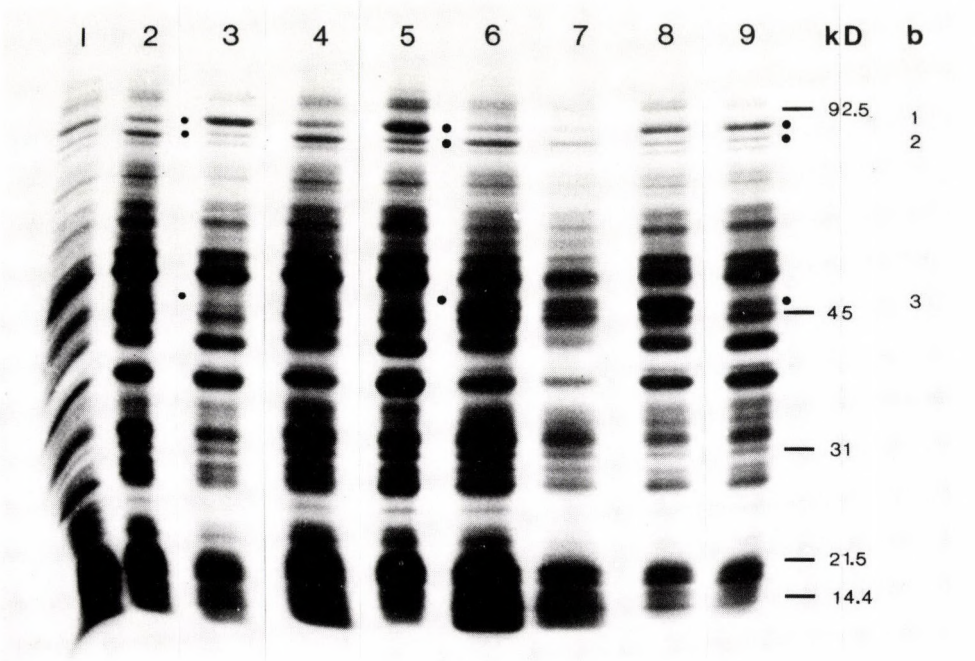

Fig. 2. SDS-PAGE analysis of proteins extracted from rice cells cultured in different salt stress conditions. Protein bands of interest (b-1,2,3) and position of standard proteins are marked by bars and dots. Seed germinating embryos (1). Friable cell population of the untreated suspension (2) and agar (4) salt-free cultures. Embryogenic cells of the 1.5% NaCl stress suspension (3) and 1.5% NaCl continuous stress agar (5) cultures. Mixed population of compact and friable cells derived from 3-month (6) and friable cells from the 6-month (7) discontinuous salt stress cultures. Stress-adapted embryogenic cells on regeneration medium without (8) and with 3% mannitol (9)

In growth cultures, b-3 was visible only in the samples with b-1 < b-2. This band was at low intensity (b-3 < b-2) in samples of the control cultures (Fig. 2; 2, 4) but reached a more or less similar intensity as that of b-2 (b-3 ≈ b-2) in samples of the discontinuous stress cultures (Fig. 2; 6, 7). In cultures for plant regeneration, cells may produce a pattern of b-3 ≈ b-1 or b-3 < b-1, depending on the culture condition without or with mannitol supplementation, respectively (Fig. 2; 8, 9).

In the seed germinating embryos, while b-1 and b-2 were observed and followed the pattern of b-1 > b-2, b-3 seemed not to be present in these explants (Fig. 2; 1).

Discussion

Tolerant cells, transferred from stress suspensions and subcultured on an agar medium with continuous salt stress, continuously generated a typical embryogenic mass of dry, compact, nodular calli with high regeneration capacity. The homogeneity of the cell mass, in the present stress condition, reflects primarily the efficiency of an in vitro selection of embryogenic cells from a mixed population of different cell types in the previous suspension cultures by high salt stress treatment /3/. It also means that continuous salt stress limits growth conditions, to which only the embryogenic cells can adapt. The high regeneration capacity in the long-term culture derived calli indicates the fact that totipotency of cultured rice cells retains still under a long-term stress of NaCl. This observation supports our working hypothesis, suggesting that tolerance is a natural adaptive potential of the embryogenic cells in rice /3/.

Positive effects of salt pretreatment on increase and prolongation of regeneration ability of the cultured rice calli has been reported on a number of occasions. Different laboratories /17, 27, 35/ have found that supplementation of salt to the callus culture medium resulted in selection of high regenerative callus lines. Regeneration of the tolerant callus lines, however, was seriously affected by salt present in the regeneration medium /17, 21/. This observation might be related to the similar phenomenon observed in the salt stress regeneration cultures of the adapted embryogenic cells. Variation of salt tolerance between growth and redifferentiation of the embryogenic cells appeared at an early stage of rice plant development /5/.

When subcultured on the same, but salt-free medium, the stress preadapted embryogenic cells underwent several changes. The first significant increase in growth and higher culture response was folled by the sharp decrease of the regeneration ability and the appearance of new cells on the surface of calli. The new cell mass resembled the cell population of the salt untreated cultures; the fact that they were watery and friable in texture, had low culture response and high sensitivity to salt. The appearance of the new cell mass in the previously homogeneous cultures provides evidence for the possibility that non-embryogenic cells could arise from the embryogenic calli in the in vitro tissue cultures of cereals /26/. This observation also indicates that removing salt stress from the medium extends conditions for growth and differentiation of different cell types in

the in vitro cultures. The rapid decrease of regeneration ability of the discontinuous stress cultured cells might be due to the cell-to-cell interactions /31/ in a successive competition for growth and differentiation of different cell lines /34/ in a heterogeneous cell population. High salt stress prevented embryogenic cells from giving rise to other cell types, resulting in generation of homogeneous embryogenic mass with high regeneration potential retained over a number of subcultures.

The physiological and morphological changes observed above were supported by the biochemical analysis. High intensity of protein band b-1, low intensity of b-2, and the absence of b-3 represent the homogeneous embryogenic mass of the continuous stress cultures. The clear reduction of b-1, intensive increase in intensity of b-2 and the presence of b-3 represent the friable population of both the control, salt γ-untreated cultures and the discontinuous stress regime. The former, control cell population, was derived from mixed populations of different cell types, which were dominated by the non-responsive and high salt sensitive cells, of the long-term salt untreated suspension cultures /3/. While b-1 and b-2 were present in the seed germinating embryos, b-3 was not visible in these explants.

Embryogenic calli of rice were found to synthesize some specific proteins; the non-embryogenic cells produce certain proteins that are not present in the embryogenic mass and the seed embryos /9/. Similar specific responses were also observed in cultured cells of different plant species /16, 28/. In our culture system high intensity of b-1, low intensity of b-2 and the absence of b-3 could be used as indicators for high homogeneity of the adapted embryogenic cultures. Low intensity of b-1 and high intensity both of the b-2 and b-3 could be a sign for increased heterogeneity of a given cultured cell population.

Similar phenotypic changes were also found in cells of several other rice cultivars. Higher salt stress with longer stress treatment did not help the adapted embryogenic cells from giving rise to other cell types in salt-free conditions; some polypeptides specific to the embryogenic cells, which adapted to grow with high salt stress, have been observed on the 2D-gels (data are in preparation).

Results obtained in this study indicate that cultured cells may respond in different ways, depending on the intensity of stress present in the culture environment. High salt stress inhibits differentiation, but embryogenic cells of rice can adapt to grow with totipotency retained over subcultures in the continuous stress condition. The embryogenic cells tend

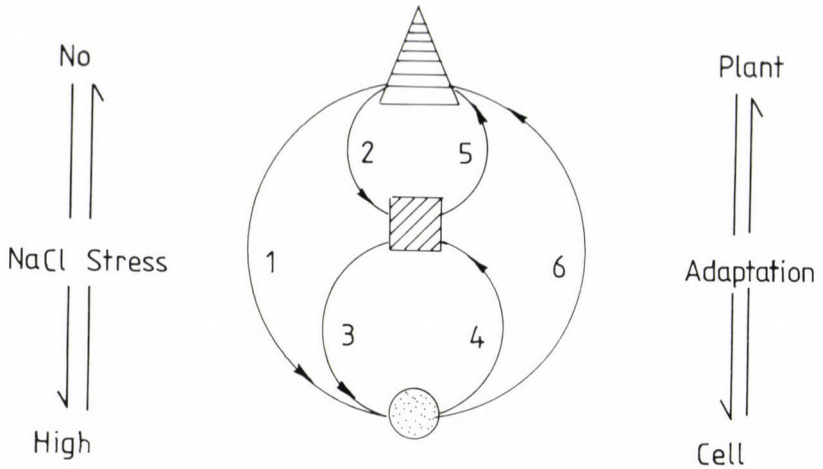

Fig. 3. A general scheme, proposed to illustrate the responses to different salt stress regimes of the long-term cultured cells in rice. Salt stress (left) and the adaptation tendency of the potentially halotolerant embryogenic cells towards the present-day halosensitive behaviour at whole plant level, along the ontogenesis, of the glycophytically evolved plants (right). Symbols: (▲) explants or regenerants, (▨) mixed population of different cell types, (⊛) embryogenic cells. Callogenesis of explants with (1) or without (2) salt. Selection and growth of embryogenic cells under high salt stress (3). Unorganized differentiation of the embryogenic cells in low or no salt stress conditions, resulting in formation of mixed populations of different cell types (4). Organized redifferentiation of cells in the mixed population (through organogenesis or embryogenesis) (5), and plant regeneration (through embryogenesis) from the embryogenic mass (6) in low or no salt stress conditions

to differentiate after removal of salt stress from the culture medium. Cells derived from the tolerant embryogenic mass in salt-free conditions are not necessarily of the same type, responsive to regeneration medium and tolerant to salt at the same high level as the previous embryogenic cells.

Rice plants are known to be sensitive to salt both under in vivo and in vitro conditions. However, the high responsive tissues in certain explants, such as seed scutellum of Japonica rices /18/ and, especially, the young inflorescences of different rice hybrids /4/ were found to be relatively tolerant to salt present in the culture medium. Calli, produced from these explants in high saline conditions, had a higher regeneration potential than those induced in the salt-free medium. While tolerant cell lines are easy to select in a large number of different plant species, stress culture-derived plants that showed an increased salt tolerance, at whole plant level for several generations were reported in a few cases, especially in rice /29/. In some plants studied tolerance potential appeared to be stable at the cell level /33/. Furthermore, in rice, the embryogenic

cells could adapt to grow with high salt stress, a level of NaCl similar to that which inhibited plant development from the stress culture-derived somatic embryos /5/. The above observed cells of the embryogenic cycle are salt tolerant, and cells of the non-embryogenic cycle are salt sensitive. This leads to the suggestion that "embryogenic cycle dependence" can be the specific character of salt tolerance in rice. Further investigations are necessary, especially at whole plant level, to prove this point of view.

In vitro culture system is useful for stress studies, providing possibilities to regenerate conditions with more or less similar intensity and regime of stress, in which plants may have evolved in the past and/or to which they are now exposed along the ontogenesis. Based on the observations made so far, a general scheme, illustrating the effects of salt stress on growth and differentiation of long-term cultured cells in rice, is proposed (Fig. 3). Tolerance potential and the adaptation tendency at cell and whole plant level are also presented.

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CHANGE IN TOXICITY EFFECT OF MERCURY AT STATIC CONCENTRATION TO CHLORELLA VULGARIS WITH ADDITION OF ORGANIC CARBON SOURCES

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The effect of mercury on Chlorella vulgaris is influenced to a large extent by organic carbon sources like glucose, glutamate, and 2-oxoglutarate which is exhibited through changes in growth and metabolic processes of the alga in the presence of static dose (0.200 ± 0.004, p = 0.05) of Hg^{2+}. Addition of the three carbon sources to the Hg^{2+}-amended Chu No 10^{+} medium resulted in an acceleration of pigment biomass, growth, and protein content of the green alga. Glucose at all concentrations was found to be more effective than the other two in reducing the mercury toxicity to C. vulgaris. However, the detoxifying effect is dependent on the type of carbon sources, their concentration and days of incubation. Glucose at 5 mg/l significantly reduced Hg^{2+} toxicity while glutamate and 2-oxoglutarate, at this concentration, did not show such capacity. Similarly the degree of significance of ameliorative effect increased with increase in incubation period which, however, varied with the carbon source. Availability of additional energy and formation of non-toxic or less toxic mercury are the probable causes of reduction in toxicity effects.

Keywords: glucose — glutamate — 2-oxoglutarate — mercury — Chlorella

Introduction

Pollutants of major concern in aquatic ecosystems are: 1) produced and reaching the environment in different amounts, 2) toxic to aquatic organisms, 3) concentrated within the organisms to a level greater than that in the environment, and 4) persistent for long periods of time. Heavy metals fit in each of the above categories. However, some of them are required as

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trace elements by the living organisms, though others like Hg, Cd, Pb, etc. have to react only negatively. Ironically in many of the freshwater bodies, especially in rivers and lakes, the human input of these unwanted elements is many times greater than the natural inflow /20, 27/. Mercury and cadmium are grouped under European Economic Community's (EEC) 'black list' because of their toxicity, persistence or bioaccumulation in the ecosystem at different levels. Mercury is the most important metal which finds its way into aquatic habitat through domestic sewage, industrial effluents, coal mine drainage, pharmaceutical effluents, and agricultural runoff. It enters in the form of vapour into the atmosphere and as sludge into aquatic systems. The toxicological effect of mercury is intensified due to transformation of organic or inorganic compound into a more toxic form by the microorganism which can convert the metallic mercury into methyl and dimethyl mercury. Being cumulative in nature, mercury toxicity in the course of time is likely to get biologically magnified becoming more catastrophic to mankind and man's environment.

The effect of heavy metals on the microbiota, both in aquatic and terrestrial ecosystems, are influenced to a large extent by the physicochemical characteristics of the specific habitat. Numerous studies have demonstrated that the organic and inorganic contituents of a medium affect the toxicity of heavy metals both in controlled and natural conditions /2, 4, 6, 22, 23, 24, 28/. Considerable variation exists among algal toxicity methods despite attempts at standardization. Experimental endpoints in these studies range from percent inhibition to EC_{50}. However, Payne and Hall /21/ suggested that algistatic concentration of a chemical can be used to study its toxicity, for this concentration is environmentally meaningful. But at this dose, no report is available in terms of regulation of the heavy metal toxicity in general and Hg^{2+} toxicity in particular. The present paper describes the effect of three separate carbon sources (glucose, glutamate and 2-oxoglutarate) on the algistatic dose of Hg^{2+} in case of a green alga Chlorella vulgaris.

Materials and Methods

An exponentially growing axenic culture of Chlorella vulgaris Beijerinck was grown in 50 ml of Chu No 10^{+} medium /25/ with A_6 micronutrients /13/ contained in 100-ml borosilicate glass conical flasks. Standard inoculum of mid-log preculture (7 days old) was 0.4 ml per 10 ml (initial cell

density 7.2 x 10^5/ml), contained in 18 x 150 mm borosilicate glass culture tubes for the experiments. The cultures were maintained in a condition similar to that described by Mohapatra and Mohanty /18/.

To study the survival rate, the alga was inoculated separately in nutrient agar plates (initial density 15 cells/cm^2) with varying concentrations of Hg^{2+} (0.0 to 0.3 mg/l) and by counting the colonies after 10 days with the help of a binocular microscope. Glucose, glutamate and 2-oxoglutarate were added separately at different concentrations (0.0 to 20.0 mg/l) to the culture tubes containing the static dose of Hg^{2+} (0.200 $\pm$ 0.004 mg/l at p = 0.05) at log phase to study the effect of these nutrient sources on Hg^{2+} toxicity. The cultures containing only the static dose of Hg^{2+} served as the control. Observations were made by measuring the optical densities (O.D.) of the homogenized cultures, pigment extracts and protein extracts with the help of a Carl Zeiss (Jena) SPEKOL spectrophotometer. Analysis of growth (O.D. at 678 nm), pigment biomass /1/ and protein contents /17/ was made every 4 days till the 12th day. The lowest significant deviations (LSDs), standard errors of the means of triplicates and confidence limits were calculated to find out the degree of significance of the results /26/. The values of LSDs have been included in the text while the standard errors have been presented in Table 1 and Figs 1--3. Only the mean values have been discussed in the text.

Results

Table 1 shows the survival rate of C. vulgaris at different nominal concentrations of Hg^{2+}. From this it was observed that though there was slight decrease in the survival rate up to 0.01 mg/l of Hg^{2+} it was not found significant (LSD = 2.08, p = 0.05). On the other hand, significant reduction of the survival rate of the alga was observed with $\geqslant$0.1 mg/l of Hg^{2+}. In

Table 1

Effect of Hg^{2+} addition on the survival rate of Chlorella vulgaris

Conc. (mg/l)	Survival %	SE
0	100	0.38
0.001	99.2	0.30
0.005	98.3	0.68
0.01	99.1	0.66
0.10	63.8	1.45
0.20	3.8	0.26
0.30	0.0	0.00

SE = Standard error; LC_{50} = 0.128 $\pm$ 0.009 mg/l, p = 0.05; Static concentration = 0.200 $\pm$ 0.004 mg/l, p = 0.05

case of the test alga the LC_{50} of the heavy metal was 0.128 ± 0.009 mg/l, while the population remained static at 0.200 ± 0.004 mg/l and had complete death at 0.3 mg/l.

Addition of glucose, glutamate, and 2-oxoglutarate to the Hg^{2+}-amended Chu No 10^{+} medium, however, resulted in acceleration of growth of C. vulgaris (Fig. 1). The enhancement of growth in glucose, glutamate and 2-oxoglutarate enriched cultures was found dose as well as time dependent. Compared to 0.021 in the control the O.D. at 5, 10, 15 and 20 mg/l of glucose were 0.038, 0.07, 0.075 and 0.088, respectively after 3 days (4th day) of incubation and they increased to 0.10, 0.11, 0.14 and 0.142 after 7 days and to 0.193, 0.248, 0.261 and 0.280 after 11 days of incubation at the respective doses of glucose. However, in case of control the O.D. of the culture decreased from 0.021 on 4th day to 0.018 on 12th day (Fig. 1A). In case of glutamate, though enhancement of growth was observed at all the concentrations of the carbon sources, the O.D. did not increase at 5 and 10 mg/l till the 8th day and there was a slight enhancement of growth thereafter. The respective O.D. values at 5, 10, 15 and 20 mg/l of glutamate were 0.023, 0.025, 0.028 and 0.031 after 3 days, 0.023, 0.025, 0.036 and 0.043 after 7 days, and 0.053, 0.058, 0.062 and 0.072 after 11 days of incubation. In case of control, however, the O.D. (0.015) remained unchanged throughout the incubation period (Fig. 1B).

Interestingly in case of 2-oxoglutarate a different pattern of growth acceleration was observed (Fig. 1C). While the carbon source was found less effective in accelerating growth vis-à-vis reducing the mercury toxicity up to 10 mg/l, it was highly accelerated at the other two (15 and 20 mg/l) concentrations. At 5 mg/l, the carbon source was totally ineffective in accelerating the growth rate. The respective O.D. values at 5, 10, 15 and 20 mg/l of 2-oxoglutarate were 0.015, 0.038, 0.046 and 0.07 after 3 days, 0.018, 0.043, 0.085 and 0.121 after 7 days, and 0.020, 0.053, 0.201 and 0.238 after 11 days of incubation while it was 0.020 in case of control.

The growth enhancement in presence of glucose was found significant at all the tested concentrations even at the very first observation, i.e. on the 4th day (Fig. 1A). On the other hand growth acceleration was not significant with 10 mg/l of glutamate till the 8th day while on the 12th day the acceleration of growth even at the lowest tested concentration (5 mg/l) was found significant (Fig. 1B). But with 2-oxoglutarate, the lowest tested concentration did not show significant enhancement of growth vis-à-vis reduction of Hg^{2+} toxicity till the end of the experiment (Fig. 1C). This carbon source, how-

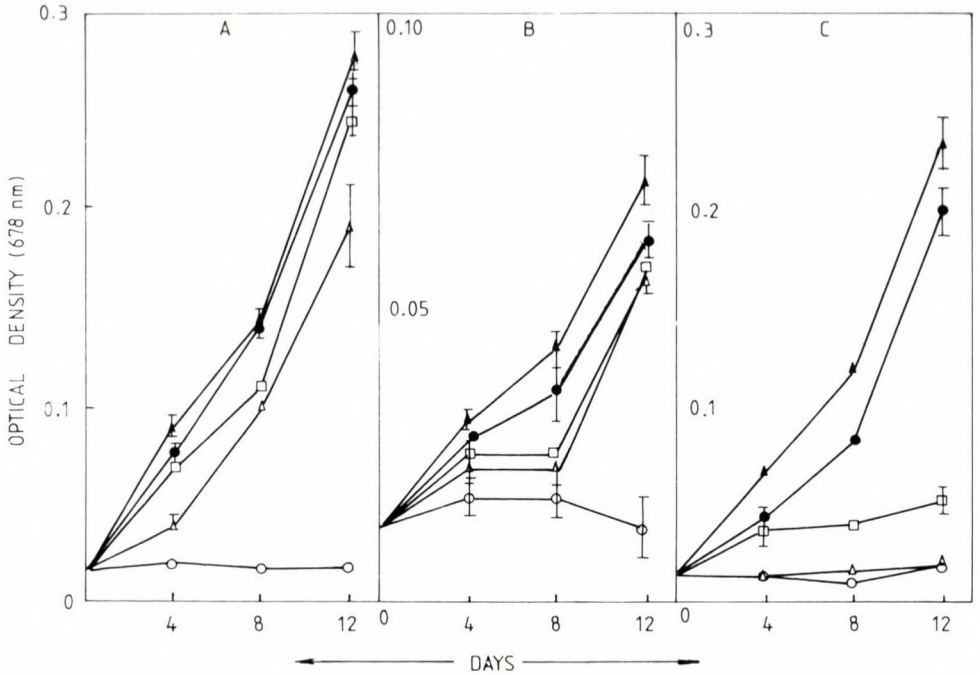

Fig. 1. Effect of algistatic dose of Hg^{2+} supplemented with (A) glucose, (B) glutamate, and (C) 2-oxoglutarate (mg/l) on growth of Chlorella vulgaris (bars represent standard errors of the means). o—o 0, Δ—Δ 5, □—□ 10, ●—● 15, and ▲—▲ 20

ever, with all other tested concentrations caused significant growth acceleration. Among the three carbon sources, growth in the glucose enriched Hg^{2+}-amended Chu No 10^{+} medium was more accelerated whereas enrichment with glutamate showed the minimum.

Figure 2 shows the pigment contents of C. vulgaris in glucose, glutamate and 2-oxoglutarate enriched Hg^{2+}-amended cultures. Compared to control pigment content at 5 mg/l of glucose concentration remained unchanged up to 4th day but acceleration was observed thereafter (Fig. 2A). Pigment biomass at 5, 10, 15 and 20 mg/l of glucose, respectively, were 0.069, 0.319, 0.309 and 0.443 mg/l on 4th day while 1.084, 1.628, 2.814 and 3.948 mg/l on 12th day compared to 0.06 mg/l in the control. Similar trend was also observed in case of glutamate (Fig. 2B). However, in glutamate enriched cultures the rate of acceleration of pigment content was less than that caused by glucose enrichment. For example, at 20 mg/l of glutamate the pigment contents were 0.756, 0.731 and 0.889 mg/l at 4th, 8th and 12th day, respectively, compared to the respective values of 0.443, 1.40 and 2.948

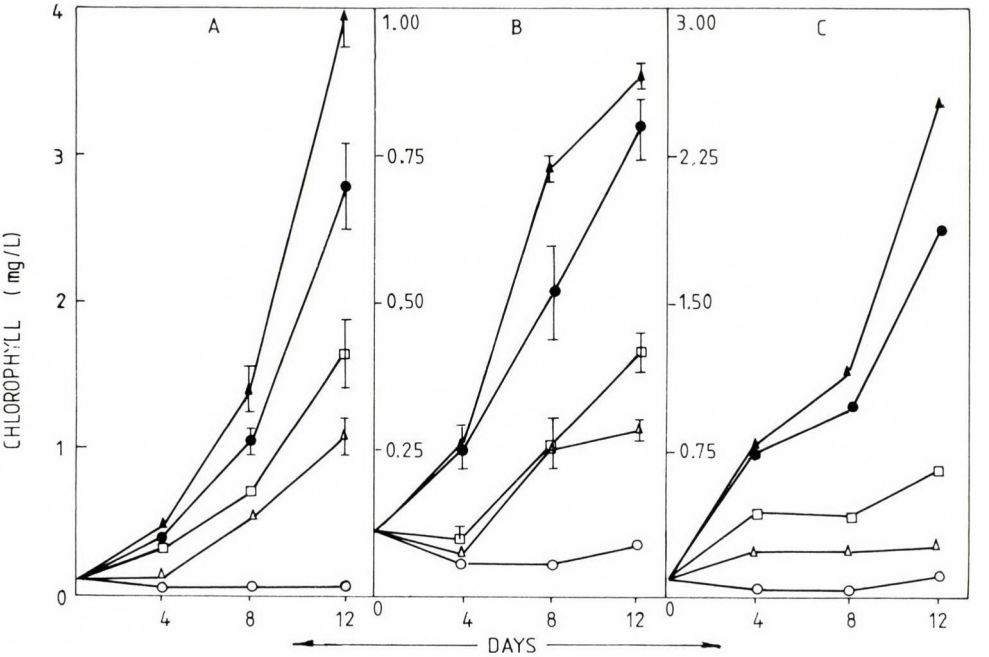

Fig. 2. Effect of algistatic dose of Hg^{2+} supplemented with (A) glucose, (B) glutamate, and (C) 2-oxoglutarate (mg/l) on chlorophyll content of C. vulgaris. o—o 0, Δ—Δ 5, □—□ 10, ●—● 15, and ▲—▲ 20

mg/l in the presence of same concentration of glucose. In case of 2-oxoglutarate enrichment the pattern of pigment biomass followed that of growth (Fig. 2C). There was slow acceleration of pigment contents up to 10 mg/l of the carbon source and it was substantial at the other two higher concentrations. In the presence of 20 mg/l the values were 0.788, 1.146 and 2.525 mg/l on the 4th, 8th and 12th day, respectively, indicating that 2-oxoglutarate was also found less effective, compared to glucose in reducing toxicity effect of Hg^{2+}.

The chlorophyll biomass of C. vulgaris was found to be highly influenced by the carbon sources in Hg^{2+}-amended cultures (Fig. 2), while at the static dose, chlorophyll biomass of the test organism was very low and there was no significant increase in pigment content till the 12th day; more or less acceleration of pigment content was observed at all the tested concentrations of each carbon source. However, the rate of acceleration and the degree of significance (LSD) varied with time, carbon source and dose. In glucose enriched cultures the increase in pigment content was significant

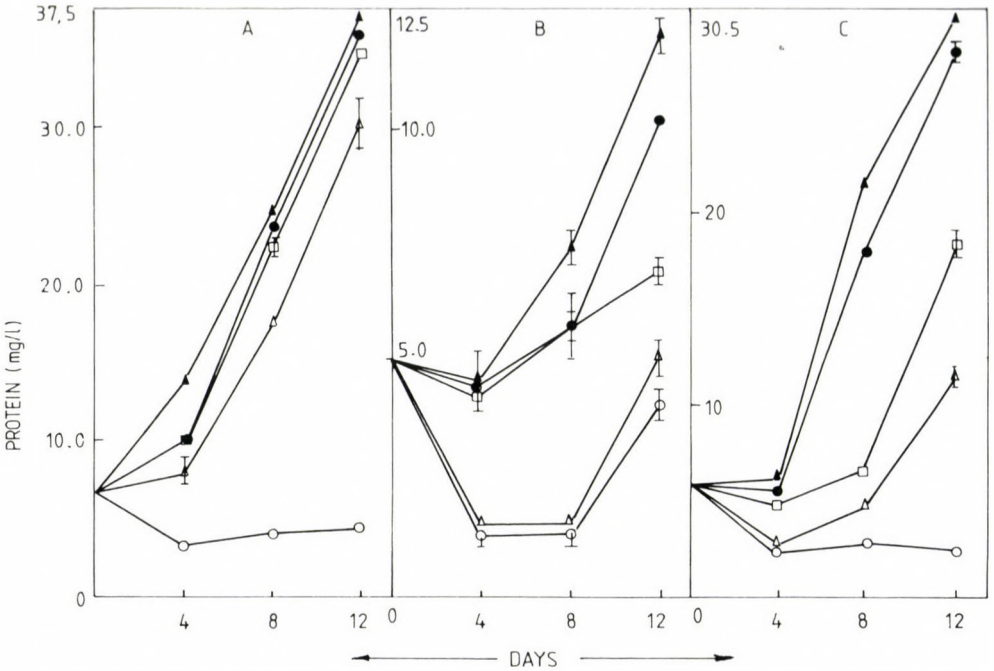

Fig. 3. Effect of algistatic dose of Hg^{2+} supplemented with (A) glucose, (B) glutamate, and (C) 2-oxoglutarate (mg/l) on protein content of C. vulgaris. o—o 0, Δ—Δ 5, □—□ 10, ●—● 15, and ▲—▲ 20

only at concentrations $\geqslant$10 mg/l on the 4th day but at all the tested concentrations on subsequent days. Similarly in glutamate-amended cultures pigment contents varied significantly at all the tested concentrations except with 10 mg/l on the 4th day and between 5 and 10 mg/l on the 8th day (Fig. 2B). On the other hand, the pigment biomass of C. vulgaris supplemented with 2-oxoglutarate was found significant at all the tested concentrations (Fig. 2C).

Protein content of the test alga was more or less accelerated at all the tested concentrations of the added carbon sources (Fig. 3). Glucose was found to be most effective in increasing the protein content despite the presence of static concentration of Hg^{2+} (Fig. 3A). Significant increase in protein content was observed with increase in glucose concentrations in all the three observations, exceptions being at 10 and 15 mg/l of glucose on the 4th day, and at 15 and 20 mg/l on the 8th and 12th day. Compared to the control, there was almost sevenfold increase in the protein content after 11 days of incubation with 5 mg/l, i.e. the lowest tested concentration of glu-

cose. On the other hand, no increase in protein content with different concentrations of glutamate was observed on the 4th day compared to the initial (Fig. 3B). On this day, protein was found to be lower than the initial. However, the content was found increasing at concentrations $\geqslant$10 mg/l of glutamate on the 8th day and significantly at all the tested concentrations on the 12th day. Similarly with 2-oxoglutarate, degradation of protein was observed at $\leqslant$15 mg/l on the 4th day and at 5 mg/l on the 8th day, while the content increased at other concentrations (Fig. 3C). The increase in protein content was only significant at $\geqslant$15 mg/l and at all tested concentrations of the carbon source on the 8th and 12th day, respectively.

Discussion

Free and uncomplexed ionic form of a heavy metal is its most toxic form. Thus, the effects of the environmental variables on the bioavailability and toxicity of Hg^{2+} in case of aquatic microorganisms are usually attributed to their impact on the availability of free ionic form of the toxicant /2, 8, 23/. Studies with copper, however, have demonstrated that addition of inorganic (ammonia and hydroxyl) and low molecular weight organic (alanine, glycine, glutamate and citrate) ligands make Cu^{2+} ions less toxic /7, 9, 10, 14/. It is quite likely that the same holds true for Hg^{2+} which resulted in growth acceleration of C. vulgaris in glucose, glutamate, and 2-oxoglutarate enriched cultures as observed from the present experiment. It is also established that increase in the organic carbon content, both in nature and culture, accelerates growth and metabolic processes of phytoplankton by reducing the cellular uptake of the heavy metals /6, 24, 28/.

At sublethal concentration heavy metals displace Mg^{2+} ion of the tetrapyrol ring of chlorophyll molecules causing imbalances in protochlorophyll formation as well as affecting chlorophyll molecules in the pigment systems and causes malfunctioning of photoelectrotransport machinery /29/. There are also reports that increase in mercury concentration inhibits chlorophyll biogenesis causing accumulation of precursors /11, 29/. Under culture conditions glucose, amino acids and other reducing sugars produce less toxic complexes with heavy metals /5, 16, 22/. Cationic metal-ligand species are generally considered to be more toxic than anionic or neutral species because they are more able to compete for sites on cell surface. There

exists a negative correlation between the mercury-ligand species (Hg^{2-zL}) and the toxicity of the Hg salts /2/. It is, therefore, to be expected that the relative toxicity of these complexes would be less than that of the free Hg^{2+} because of the reduced charge (net charge 2-zL) and the effects of steric hindrance. Certain carbon sources like glutamate, glutamine, aspartate, alanine, and proline form the respective neutral complexes with Hg^{2+} (i.e. the Hg^{2+} ion is bound through the α-amino and α-carboxyl groups and carries a net charge of +1 while the terminal carboxyl group is negatively charged resulting a net charge zero). Being neutral such complexes have a low affinity for the binding sites on the cell surfaces /12/. The acceleration of pigment content with addition of different carbon sources in the present experiment is certainly due to decrease in the free Hg^{2+} ion in the medium. Availability of glucose, glutamate and 2-oxoglutarate seems to provide favourable conditions for formation of such low toxic ligand complexes.

Measurement of chlorophyll biomass is a means to assess the phytosynthetic efficiency of algae /19/. Increase in pigment biomass enhances the photosynthetic activity of algae while decrease in their concentration retards photosynthesis. The reduction of photosynthesis and chlorophyll content was reported when Hg^{2+} was added with humic acid instead of other organic nutrients /16/. This was attributed to the acidic nature of the carbon source by Hongve et al. /16/. In the present study the low ameliorative action of glutamate in the Hg^{2+}-amended system was probably due to the above facts. The increase in growth rate with increased concentration of carbon sources might also be due to acceleration of rate of photosynthetic carbon fixation by the alga.

Mercury affects protein content of algae by influencing both synthesis and degradation processes /11, 15/. The metal ion has a greater affinity towards sulphur containing amino acids. It attacks the -SH bonds causing the reduction of synthesis and acceleration of protein degradation process /22/. The binding preference of mercury for sulphohydryl thioethers and imidazole groups at catalytically active centres of enzymes provides the biochemical basis for much of its toxicity. Consequently, the molecular arrangements which inhibit the ability of Hg^{2+} to combine with these enzymes will result in reduced toxicity. The ameliorative effects of the carbon sources in the present experiment vis-à-vis the direct relation between the increase in protein content and organic carbon addition might be due to such molecular arrangement of the complexes. Moreover, because the Hg^{2+} ion is bound to the centre of such complexes /2, 12/, it is presumably shielded from direct

interaction with the cells. This probably protected the protein synthesizing enzymes from direct toxic action of Hg^{2+} and resulted in synthesis of protein.

Further, Chlorella has been found to grow well with addition of organic carbon sources. It can grow in dark on added glucose maintaining its pigment systems intact /19/. One possible generalization is that the monosaccharides are most effectively taken by the algal cells compared to other carbon sources. Addition of such carbon sources provides additional energies which suppress the inhibitory action of heavy metals on metabolic acitivities of phytoplankton /29/. In the present observation glucose was found most effective in toxicity reduction of Hg^{2+} drawing the conclusion that the ameliorative action of a carbon source in Hg^{2+}-amended system is directly related to its energy content and its ready utilization by the cell.

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EFFECTS OF LOW ENVIRONMENTAL pH IN THE PRESENCE OF ALUMINIUM ON FILTRATION PROPERTIES OF THE BLOOD OF ATLANTIC SALMON (SALMO SALAR L.)

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The time for filtration of whole blood samples through 8 μm pores of polycarbonate membranes provides an index of the deformability of fish red blood cells. Comparison of these and other haematological measurements on salmon before and after exposure to pH 5.0 and 20 μM aluminium for 2 hours shows changes which are indicative of hypoxia. The increase in deformability of the red cells will assist transfer of oxygen from the gills to the tissue cells and hence compensate environmental hazards of low pH and aluminium.

Keywords: Environmental effects — blood — Atlantic salmon

Introduction

The extent to which the red blood cells of vertebrates are able to deform during their passage through the microcirculation is an important property influencing the supply of oxygen to the tissues and its loading in the respiratory organ /1, 5/. Many studies of this property have been made on mammalian blood and of several techniques available that utilizing filtration through membranes containing pores of known dimensions is relatively simple. This technique has revealed changes in filtration time which provide an index of red cell deformability which is affected by a number of conditions /20/ including human diseases such as diabetes and renal failure /16/ and is also affected by heavy metals such as lead /19/. Among fish the

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method has shown effects of temperature /10/, oxygen tension /8/, and exercise /15/. In the Pacific salmon (Oncorhynchus keta) changes have also been observed during transfer from seawater to freshwater /17/.

Acidification of surface waters in many countries is known to have led to the loss of salmon and brown trout /18, 22, 27/. The lowered pH of these natural waters increases the mobility and bioavailability of many metals of which aluminium is of particular interest in relation to its toxic effects on fish. A number of studies have suggested that metals alone or in association with increased acidity have lethal effects on fish by reducing the availability of oxygen and salts /2/. Several sites in the respiratory chain from water to mitochondria have been implicated, notably the gill water/blood barrier /13/ and blood oxygen transport /6, 23/. Red blood cells form a vital part of this mechanism and as indicated above their mechanical properties are influenced by environmental conditions. Advantage was taken in the present study of the availability of blood samples from salmon used in studies on other, non-respiratory effects of lowered pH and aluminium /25/.

Materials and Methods

The basic methodology follows that of Kikuchi, Arai and Koyama /14/ who developed the apparatus used in the present study but it has been significantly modified by the use of a photoelectric method for recording the time course of the blood flow through the nuclepore filters /12/. Blood samples are taken with a known dilution of heparin (500 IU/ml blood), and from the time course a value for filtration rate during the initial part of the flow was determined. From this filtration time for the passage of 0.3 ml whole blood a calculation is made of the time for a single red cell to pass through an 8 μm pore in the filter using the equation of Kikuchi et al. /14/. For this calculation, haematocrit value (microhaematocrit centrifuge), and mean cell volume (using red cell count measured with a haemocytometer) were also required. In addition estimates of total plasma protein were obtained with a hand-held refractometer using plasma remaining in the haematocrit tube after removal of the centrifuged red cells.

Comparisons were made between blood obtained from fish in neutral (pH 6.5-7.0) aerated river water (12 ^{o}C) and those that had been exposed to low pH (5.0) by the addition of dilute H_2SO_4 and aluminium (20 μM Al/l) as $KAl(SO_4)_2$ or $AlCl_3$ for 2 hours; monomeric aluminium was assayed by the catechol violet method /9/. In the first series of experiments blood was obtained directly from caudal vessels of large 4^{+} parr (300-500 g reared at Almondbank in freshwater) after stunning. The second made use of samples from adult Atlantic salmon (3-4 kg) having the dorsal aorta cannulated and allowed to recover for at least 24 hours. Each fish was used as its own control and samples were taken when it was in freshwater, after 2 hours in freshwater at pH 5.0, and/or 2 hours in freshwater at pH 5.0 with added

aluminium. Blood from these fish was also used for other measurements by Potts et al. /25/ which should be consulted for further details of the experimental procedure.

For some of the blood samples from cannulated fish a comparison was made between the filtration time of the original sample and part of it which had been mixed with a heparinized saline containing adrenaline so that the final adrenaline concentration in the blood used for the filtration measurement was 10^{-6} molar.

Mean values and standard errors were determined together with probabilities that any differences were significant using Students t test. Where the comparison was between the blood from the same fish, a paired t test was also used.

Results

The main results of these experiments are summarized in Tables 1 and 2. Comparison of the results using the different sampling procedures shows the same general direction of changes between controls and exposed fish with respect to haematocrit, mean cell volume and total protein. All three parameters increased following 2 hours exposure to the acid/aluminium regime.

Table 1

Summary of results obtained with blood samples from caudal puncture (n = 16) and dorsal aorta cannulated fish (n = 6). In each group controls are untreated fish and are compared with samples following treatment with acid (pH 5) and aluminium. Probabilities are based on Student's t-test (underlined for paired test)

	Caudal samples		
	Control (SEM)	t-test probability	Acid/Al (SEM)
Haematocrit (%)	38.62 (1.74)	0.3 —0.2	40.75 (0.73)
Mean cell volume (μm^3)	295.6 (16.3)	0.2 —0.1	341 (25.6)
Total protein (g/dl)	5.2 (0.25)	0.01—0.001	6.93 (0.458)
Filtration time (s)	8.47 (1.03)	0.4 —0.3	9.274 (0.6)
RBC pore passage time (ms)	1.099 (0.093)	0.2 —0.1	1.277 (0.076)
	Dorsal aorta samples		
	Control (SEM)	t-test (paired)	Acid/Al (SEM)
Haematocrit (%)	22.96 (2.61)	0.1 —0.05 (0.05—0.02)	29.21 (1.99)
Mean cell volume (μm^3)	316.7 (27.38)	0.4 —0.3 (0.3 —0.2)	349.8 (22.03)
Total protein (g/dl)	2.5 (0.184)	0.05—0.02 (0.01—0.001)	3.28 (0.222)
Filtration time (s)	12.62 (4.03)	0.4 —0.3 (0.2)	8.1 (1.3)
RBC pore passage time (ms)	2.95 (0.387)	0.1 —0.05 (0.02—0.01)	1.38 (0.181)

Table 2

Comparison of blood parameters from cannulated adult salmon (n = 8) using samples before and after treatment in vitro with adrenaline (10^{-6} M). Probabilities are given for results of Student's t-test (underlined for paired test)

	Control (SEM)	t-test (paired)	Adrenaline (SEM)
Haematocrit (%)	26.88 (1.46)	0.9 0.9	26.81 (1.4)
Mean cell volume (μm^3)	295.38(16.39)	0.4—0.3 0.2—0.1	321.63 (20.8)
Total protein (g/dl)	2.08 (0.25)	0.9—0.8 0.2—0.1	2.16 (0.22)
Filtration time (s)	9.48 (0.87)	0.6—0.5 0.6—0.5	10.35 (1.6)
RBC pore passage time (ms)	1.51 (0.12)	0.4—0.3 0.4—0.3	1.778 (0.232)

However the significance of these changes was greater for the samples obtained via the dorsal aorta cannulae and this was especially true of the paired t test results as this procedure was not applicable to the data for caudal samples as each of the latter came from a different fish.

Statistically the changes in plasma total protein are very significant for both series of experiments. It is also apparent that mean levels of total protein of caudal samples are about double those obtained from cannulated fish.

Results of the filtration experiments using caudal samples showed a slight increase in filtration time whereas blood from cannulated fish showed an opposite tendency, moreover the results were much more significant. In particular the measurements of pore passage time, which take into account differences in haematocrit and MCV of different samples, were highly significant especially when a paired t test was applied.

Discussion

In general the results obtained from cannulated fish are probably more reliable especially as some sham tests were made in which no treatment was given although blood sampling was repeated as in the experimental fish. These controls showed a slight increase in pore passage time in spite of a

slight decrease in haematocrit and MCV over a period of five days. There was also a slight decrease in total protein. Thus it seems reasonable to conclude that the changes observed following the experimental treatments are due to the treatment itself, rather than the repeated blood sampling. Some variability in the results can be attributed to slight differences in duration and concentration of pollutant, although this was probably more consistent in the experiments with caudal sampling. Such studies confirm the suggestion /3, 4/ that fish blood, especially when sampled from chronic catheters, can provide valuable data as an indicator of environmental pollution. The results from the caudal-sampled fish may be complicated by stress, which might account for shorter filtration times for controls, and contamination of the blood during sampling. Blood filtrability through the 8 μm pores is especially sensitive to such contamination although the sample seems perfectly normal from measurements of many other parameters.

The general conclusion from the present experiments is that increased acid/aluminium in water produces an increase in haematocrit, and MCV as found in a number of previous studies /21, 23/. In spite of this swelling of the erythrocytes, there is a significant decrease in filtration time, i.e. the red blood cells show an increase in deformability. Similar increases in deformability following 'stressful' situations have been found in rainbow trout particularly following hypoxia and exercise /8, 15/. The low pH and aluminium treatments used in the present experiments will have made the fish hypoxic and hence affected the red blood cells in a way similar to experiments /8/ which showed enhanced RBC deformability, in spite of increased red cell volume, following environmental hypoxia. In the latter study it was also found that when blood from normoxic trout was equilibrated with low oxygen mixtures it showed no significant change in RBC deformability. Hence it was concluded that some blood constituent produced in vivo during hypoxia was suggested and catecholamines are an obvious possibility. This supposition is further supported because hypoxia and exercise tend to be associated with increases in catecholamine content of the blood /11, 26/. The hypothesis was suggested that increases in plasma catecholamines affect red cell membrane properties including greater deformability. However, in the present preliminary series of experiments in which blood samples were treated with adrenaline in vitro no significant change in RBC deformability was observed (Table 2). The concentrations used during these experiments were low (10^{-6} M) for such conditions as measurements were made soon after mixing and the latter was not ideal. It is evident that a further series of adrenaline

treatment experiments needs to be carried out under more controlled conditions. Such experiments with blood from carp and eel have shown significant effects of adrenaline on red cell deformability (Hughes, in preparation). The presence of an increase in total protein following exposure to low pH and aluminium is a consistent finding the precise meaning of which requires more detailed analysis of plasma constituents. However, its extent may simply reflect the increase in haematocrit values.

Conclusions

The functional significance of the increase in red cell deformability during hypoxia and hypoxia-related conditions would seem to be a combination of its rheological and gas-exchange consequences. When a possible role of this property was suggested /1/ a reduction in deformability with erythrocyte swelling was not envisaged. The reduction in resistance to blood flow through the microcirculation would assist perfusion of both the gills and tissues. Being combined with an increase in mean cell volume this would also tend to decrease diffusion distances from the capillary wall to the erythrocytes. Swelling of the red cells also results in modifications of the oxygen-combining properties of the haemoglobin which are further adaptations to hypoxia /24/. As a consequence of these responses salmon would be more able to survive the adverse effects of decreases in pH and heightened aluminium in their environment.

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SWIMMING PERFORMANCE OF RAINBOW TROUT FOLLOWING EXPOSURE AND RECOVERY FROM THE PYRETHROID S-DELTAMETHRIN

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Oxygen consumption and swimming performance of rainbow trout exposed to sublethal concentrations (10-50 µl/20 L) of pyrethroid (S-deltamethrin) pesticide was studied in a Brett-type respirometer during recovery. Results showed wide variations reflecting the varied responses of different individual fish to the test regime. In most cases values of oxygen consumption for the controls were higher at each swimming speed and this became significantly reduced following treatment with pesticide. Oxygen consumption soon after the treatment gave the highest values but these fish were unable to sustain swimming speeds as high as the controls or following recovery. The comparisons of swimming performance before and after treatments with deltamethrin have shown a significant effect on locomotory ability of rainbow trout which at the end of strong exposure (e.g. 50 min of 40 µl/20 L) are almost incapable of swimming.

Keywords: Rainbow trout — S-deltamethrin treatment — oxygen consumption — swimming performance

Introduction

Standing and running waters of the natural environment often become loaded with communal and industrial sewage, chemical fertilizers, herbicides, pesticides, heavy metals and detergents. All of them affect aquatic organisms causing structural and ultrastructural damage to vital organs and alterations in biochemical and physiological functions /1, 6, 7/. The syn-

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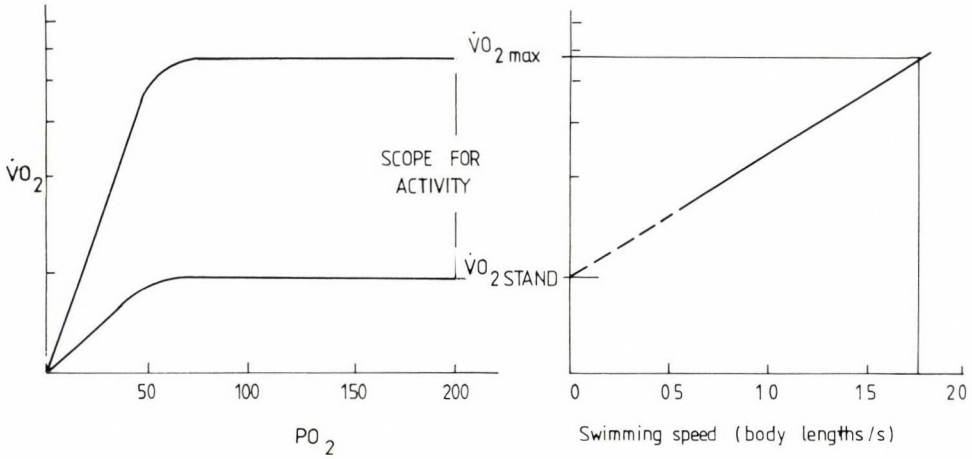

Fig. 1. Diagram illustrating data plotted to show swimming performance of a trout in a Brett-type respirometer /8/

thetic pyrethroids are one of the most widely used insecticides for pest and mosquito control and their different formulas are highly toxic to fish /13/.

Physiological effects and action mechanisms of various toxicants have been widely studied in acute and sub-acute tests /10, 11, 13/. However, there are few studies on the prolonged effects of chemicals on fish, especially during their recovery period. Short term, sublethal changes in coughing rate, cardiac and ventilatory frequencies of hatchery-reared rainbow trout exposed to menazon and gamma BHC-containing pesticides were monitored during recovery /2/. The coughing response was sensitive to water pollutants. Average frequency of ventilation was higher during the exposure and decreased parallel with the time of recovery. Average heart rate was also higher in treated trout as compared to controls. These changes resembled those produced by hypoxic conditions and provided useful sublethal physiological indications for rapid environmental stress (pollution) often occurring in natural waters.

The effects of reduced gill area and hyperoxia on the oxygen consumption and swimming speed of rainbow trout were studied by Duthie and Hughes /3, 4/. It was shown that reduced gill area or damage to the filaments of the first or second gill arches limited the active oxygen uptake (see also /9/). Critical swimming speed values were significantly reduced as a consequence of gill area reduction.

Under these conditions, however, standard oxygen consumption, determined by extrapolation to zero swimming speed (Fig. 1), remained constant

and hence the gill damage inflicted had reduced the so-called scope for activity, i.e. the difference between active and standard metabolism.

Testing of fish subjected to toxicants using these procedures has received little attention and was used in the present series of experiments with rainbow trout following exposure to a pyrethroid insecticide deltamethrin. Particular attention was paid to recovery from brief exposures to supra-lethal concentrations and it was found that fish allowed to recover in a tunnel respirometer did so more readily than in static waters. In some cases subsequent testing of their performance in the respirometer showed an improved swimming ability.

Material and Methods

Rainbow trout were obtained from the Midland Trout Farm, Nailsworth, Gloucestershire and kept in the closed water circulation of RUCAR for at least one week before experimentation. The specimens used had a mean body length of 28.32 cm $\pm$ 0.387 (SE) and a body weight of 285.55 g $\pm$ 11.27 (SE). They were fed daily on standard trout pellets but remained unfed once the experimental period had begun. Before determinations of swimming performance they were usually left in the Brett-type respirometer overnight with a water speed of 0.5 body lengths/second. The next morning the speed was increased progressively and oxygen consumption measured when it had reached a plateau -- usually after 30 minutes. Such control runs were carried out for all fish and gave results comparable to those obtained in previous experiments using this procedure /12/. Individual fish were then exposed to the pyrethroid (Decis 2.5 EC) containing 25 g/litre S-deltamethrin. Small quantities (10-50 µl) were dissolved in a few millilitres of acetone and then diluted in 20 litres of well-aerated water contained in plastic bins at about 16 ^{o}C. Exposure times varied from 10 to 60 minutes. Following exposure, fish were returned to clean water in a bin for at least five minutes to allow washing off of the pesticide. They were then placed in the respirometer once again to test the extent to which their swimming had been affected by the exposure. In other cases they remained in the clean water for a more prolonged recovery in a bin or respirometer before their swimming performance was tested at various stages of recovery. It was necessary to vary some details of these procedures because of differences in the apparent recovery of individual fish from the pesticide.

Results

A) General observations on locomotory and respiratory behaviour during exposure to pesticide

Individual responses of fish varied following treatment with different concentrations (10-50 µl/20 l) of S-deltamethrin during different exposure times (10-60 min). All the five fish exposed to 10-40 µl/20 l doses for 10-20 min survived. Twenty-one fish were exposed to a 50 µl/20 l solution. Fourteen of them survived all exposure times and seven died: two of the latter had been exposed for 30 min, and five individuals died after 45 min of exposure.

Some fish responded slightly, some of them violently. The appearance of reactions varied widely. During the first few minutes, the body became curved, the swimming ability decreased, the number of coughs tended to increase rapidly and following a peak they decreased, the fish lost their balance and rolled for shorter or longer periods, shook violently, varied their position between the bottom and the surface swallowing air and trying to escape.

Pronounced signs of reactions to the effects of pesticide treatment were not uniform in various individuals. Those fish which showed deep effects of pesticides (i.e. paralysis), in the advanced stage did not show any locomotion, their arrhythmic ventilation stopped and they died with an open mouth.

Following exposure to the pesticide, when recovering in a bin containing well-aerated tap water, the same signs still persisted. However, no consistent overall picture can be drawn from the individual responses. Symptoms of pyrethroid effects resembling hypoxia still existed after 1-3 hours of recovery in fresh water.

B) Swimming performance and the effect of deltamethrin

1) Overall analysis

In order to give a general impression of the overall nature of the effects of deltamethrin on swimming performance, data from all swimming tests were grouped in two sets:

a) those which had not been exposed to the pesticide;

b) following exposure to various concentrations and the swimming tests carried out between 15 min and 24 h after return to clean water.

As might be expected the results showed wide variations reflecting the varied responses of different individual fish to the test regimes.

The results can be summarized in the following two relationships:

No pesticide $VO_2 = 68.755 + 67.993\ BL \qquad R^2 = 0.606$

Pesticide $VO_2 = 62.495 + 62.03\ BL \qquad R^2 = 0.711,$

where VO_2 = ml O_2/kg/h and BL = Body lengths/second

Thus the way in which the pesticide reduced oxygen consumption reflects the reduced activity of trout during treatment with deltamethrin. This effect is even more drastic as at the end of the treatment period fish

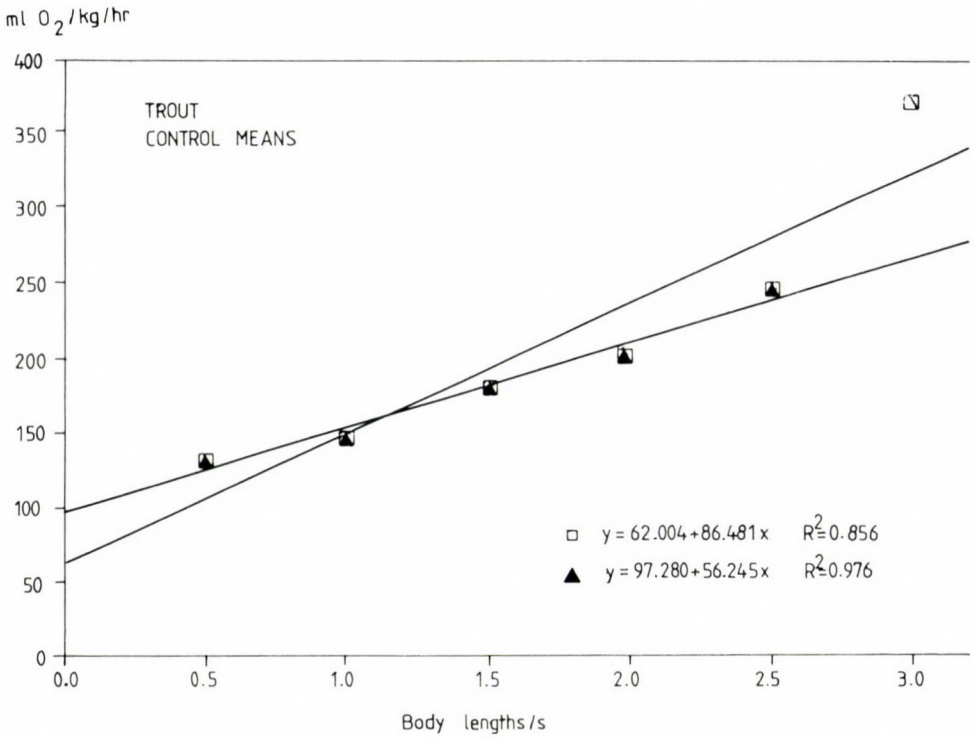

Fig. 2. Plots of oxygen consumption at different swimming speeds (body lengths/s). Mean values for six fishes are plotted under control conditions. In one case the regression line for all the data is shown (□) and in the other case (▲) the point for 3.0 BL/s is omitted from the analysis

usually showed no locomotory activity and data used in this analysis were only those at stages of recovery when they were able to swim.

The relationship for the untreated fish shows a higher correlation when mean values at each swimming speed are used (Fig. 2)

$$VO_2 = 62.004 + 86.481\ BL \qquad R^2 = 0.856$$

and if the final values at 3 BL/second were omitted, as these can only be maintained for short periods, the relationship was

$$VO_2 = 97.28 + 56.245\ BL \qquad R^2 = 0.976$$

In many of the tests untreated fish were unable to swim at speeds exceeding 2.5 BL/second whereas some of the pesticide-treated specimens showed sustained swimming at 3 BL/second sometimes at higher speeds for short periods.

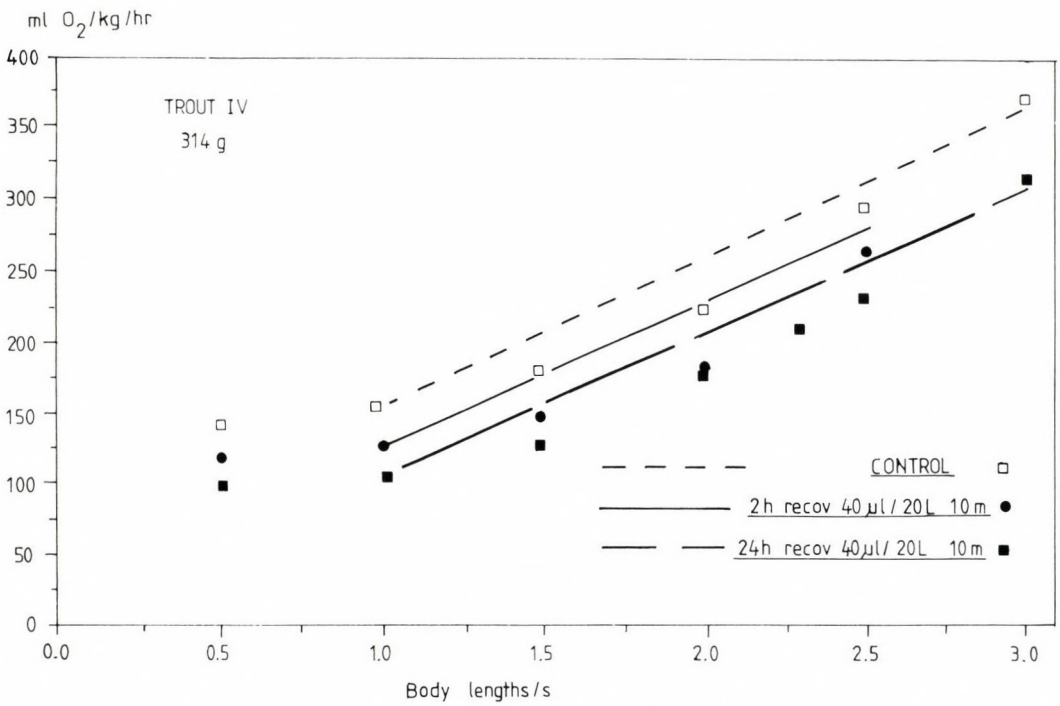

Fig. 3. Plot of oxygen consumption at different swimming speeds (body lengths/s) for an individual rainbow trout under three conditions: a) control (- - -), b) shortly after exposure to deltamethrin (——), c) 24 h after return to clean water (— — —)

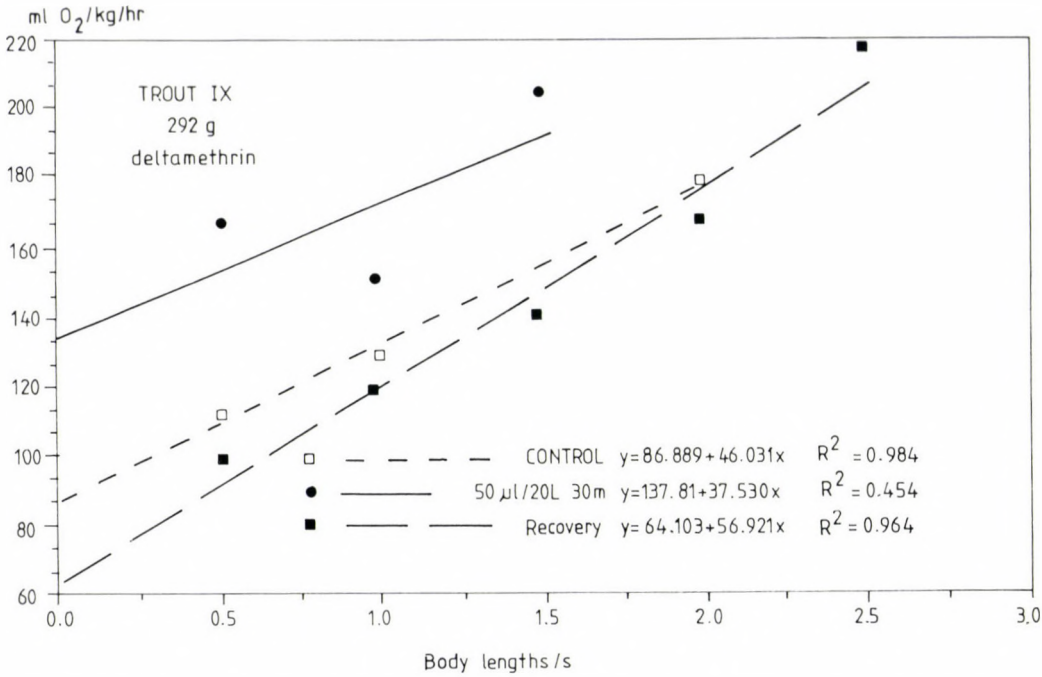

Fig. 4. Similar plot to Fig. 3, but in this case the fish is able to swim at a higher speed following the treatment than before exposure to deltamethrin

2) Performance of individual fish (Figs 3, 4)

Whilst the general analysis gives a useful indication of the overall effects of deltamethrin on rainbow trout swimming, differences between individuals tend to obscure some of the more detailed effects. Thus data obtained for individual specimens swimming under control conditions followed by testing shortly after treatment and for recovery gave the following results. In most cases values of oxygen consumption for the controls were higher at each swimming speed and this became significantly reduced following treatment with pesticide. Recovery for periods exceeding 20 hours usually led to values of oxygen consumption which more closely approximated the control values. In some cases, however, oxygen consumption soon after the treatment gave the highest values but these fish were unable to sustain swimming speeds as high as the controls or following recovery. In this and other cases it was quite often found that the highest sustained swimming speed following 20-24 h recovery period exceeded even that of the control by 0.5 BL/s, i.e. it increased from 2.0 to 2.5 or 2.5 to 3.0 BL/s.

In all cases fish tested in the respirometer within a few hours after removal from the pesticide showed the minimum sustainable swimming speed.

Discussion

The swimming ability of fish overcome by sub-acute toxic stresses usually decreases to such an extent that they are unable to move and cannot escape from predators in the wild. Various compounds initiate different physiological responses or cause cell and tissue damage. These responses, however, vary widely from species to species, and even are variable in the same taxa, according to their individual sensitivity. The observed responses are usually striking, but the mechanisms of action of most toxic compounds still remain obscure. In this context variations in individual responses are difficult to understand and may be connected to the mechanism of infiltration.

L'Hotellier and Vincent /14/ summarized experimental results on the toxicity of deltamethrin for 20 fish species showing variations of LC_{50} ($\mu g\ L^{-1}$) (96 h) for technical or formulated products. Among the fish species studied by several authors, rainbow trout proved to be one of the most sensitive species. Effects of pyrethroids may be modulated by temperature, water chemistry, leaching and absorption and this can give rise to differences in measured levels of LC_{50}, etc.

Little evidence can be found in the literature for physiological responses during recovery of fish which survive sub-acute pollution, although this type of pollution regularly occurs in many running waters. Symptoms, such as loss of balance, instability, convulsion suggest that chlorinated hydrocarbons (and possibly other formulae) affect the central and peripheral nervous system in unknown ways, organochlorines may uncouple oxidative phosphorylation causing increased oxygen requirements, and carbamate pesticides have an anticholinesterase action /15, 16/.

An important feature of deltamethrin which is relevant to its use as a pesticide is the contrast in sensitivity of man and other mammals to that of fish and aquatic invertebrates. Laboratory tests on rats, mice, farm animals and poultry have shown a relative insensitivity. The literature gives values for the 96-hour LC_{50} of fish in the range of 0.4 to 2.0 μg/litre and this high toxicity has been confirmed in the present studies; slightly higher values were also found in studies using crustaceans. The pyrethroid appears to enter animals via the oral membranes. The very large surface area of the

gills in fish must increase the ease with which this can take place in the aquatic environment. Tests with deltamethrin indicate a relatively rapid degradation in the natural environment, for example it has a half life of 1.1 weeks and the time needed for 90% loss was 4.6 weeks when degraded in cotton plants under glasshouse conditions. Variations in values obtained in some tests using the same fish species were partly accountable by local differences in population and in the water quality and detailed procedures used. There are considerable difficulties in tests where a constant concentration of pesticide needs to be maintained. Such problems were reduced in the present experiments by the shortness of the exposure period.

The experiments described here have shown a remarkable ability of rainbow trout to recover from lethal doses and in some cases their swimming performance appears to be enhanced following the treatment. In view of the varied nature of the response during exposure to pesticide it is difficult to pinpoint a primary effect on the fish. Entry via the gills seems very probable although the skin may also be involved. No damage to the gills was observed in the present studies although some authors have observed such effects. However, the increased frequencies of ventilation and coughing are general indications of irritant action on the gills and possible hypoxia due to impaired gas transfer. Good recovery of rainbow trout from exposure to deltamethrin has also been observed by other workers (unpublished, see /5/). The much greater sensitivity of fish may be the result of an inability to degrade the pesticide in addition to the greater surface area through which it may enter the organism. Some metabolic studies have suggested that extracts of a number of tissues can hydrolyse pyrethroids about 100 times faster in mammals than in fish. As a consequence the main toxic molecules will persist in fish whereas in mammals they will be broken down to less toxic components in a shorter time. There is evidence that one of the main effects of deltamethrin is to keep open the sodium channels of cell membranes with consequent disruption of many processes particularly in nervous, muscular, and secretory tissues. Perhaps some of the characteristic tremor, salivation and convulsions of mammals and the comparable effects observed in rainbow trout are related to this action on the sodium channels. There is also evidence for a secondary effect as an anticholinesterase which again would affect the nervous and muscular systems.

The comparisons of swimming performance before and after treatment with deltamethrin have shown a significant effect on locomotory ability of rainbow trout which at the end of strong exposures (e.g. 50 min of 40 µl/

20 L) are almost incapable of swimming. Nevertheless they have a surprising ability to recover and after 24 hours most fish were swimming at least as well as the controls, although quite often their oxygen consumption for the same swimming speeds was reduced. Most strikingly, however, many specimens showed an ability to swim at a faster speed (usually 0.5 BL/s) after 24 hours' recovery. The interpretation of any enhancement in swimming performance is difficult but seems to be real when the performance of individual fish is analysed. Caution must be given to any suggestion that this represents a real increase in swimming capability because of the known improvement in swimming of some fish following repeated trials in such respirometers. Nevertheless the specimens tested here had experienced the respirometer on several occasions before the final recovery trial and any improvement is likely to have occurred before this particular test. Thus the enhanced effect following recovery from deltamethrin exposure might be due to behavioural effects rather than strictly physiological effects such as improved gas exchange capability, muscle or swimming efficiency.

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VOLUME DENSITIES AND ABSOLUTE VOLUMES OF MITOCHONDRIA IN BODY TRUNK RED MUSCLE OF A TILAPIA, OREOCHROMIS NILOTICUS (TREWAVAS)

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The volume densities /V_V(mt,f)/ and absolute volumes of mitochondria /v(mt)/ were determined in body trunk red muscle of 15 specimens of Oreochromis niloticus weighing 0.65—812.3 g. V_V(mt,f) had a volume of 0.284 ± 0.012 (S.E.) and V(mt) a value of 0.551 ± 0.202 (cm^3) (S.E.). Both parameters had scaling values of -0.028 and 1.13, respectively, when related to body weight. These results show that there might not be much change in the oxidative metabolism of red muscle with development. The greater than unity value for the scaling value of V(mt) in relation to body weight is due to the high scaling value of body trunk red muscle (1.157) in relation to fish body weight /11/.

Keywords: Red muscle — mitochondria — weight — tilapia

Introduction

The volume density of mitochondria has been used to estimate the oxidative metabolism in muscle fibres of some mammals /3, 12/. Good correlation has also been shown between muscle mitochondrial and capillary volume densities in some fish /9, 15/. Mitochondrial distribution in red muscle of fish has been shown to be uneven with most of the mitochondria being subsarcolemmal /2, 5, 6, 8, 9/ which should affect oxygen diffusion distances in the muscle.

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In the present study, the volume densities and absolute volumes of mitochondria were determined in body trunk red muscle of different sizes of Oreochromis niloticus to assess any changes in the muscle's oxygen metabolism with development.

Materials and Methods

Fifteen specimens of O. niloticus weighing 0.65-812.3 g were used in determination of mitochondrial volumes. The fish had been kept in aquaria at 25 °C at the Research Unit for Comparative Animal Respiration, University of Bristol (U.K.) where they were fed about twice per day on Mainstream Expanded Trout diet pellets (BP nutrition). The fish were stunned by a blow to the head and strips of red muscle from the body trunk, at the lateral line region, 1/3 of the fish length from the tail region dissected out and pinned to cork strips at their resting length. The muscle was fixed in 2.5% glutaraldehyde in 0.1 m sodium cacodylate buffer (pH 7.4) for several hours. The muscle was subsequently processed for electron microscopy by washing in 0.1 m sodium cacodylate buffer, post-fixing for 1 h in 1% osmium tetroxide in 0.2 m sodium cacodylate buffer, dehydrating in a series of ethyl alcohols up to 100%, clearing in epoxypropane and embedding in epon resin.

Ultra-thin sections (60-90 nm) were cut on a Reichert ultramicrotome. The sections were mounted on Formvar-coated 150 or 200 mesh copper grids, double stained with uranyl acetate and lead citrate and viewed with a Phillips 201 electron microscope. Five blocks were sectioned per fish.

The volume density of mitochondria /V_V(mt, f)/ was determined on negatives of cross sections of red muscle fibres which were projected at a magnification of x5150 onto a 0.8 cm square lattice counting grid. The number of intersections of test points with mitochondria was divided by the number test points falling on the whole fibre to determine V_V(mt, f). Mitochondria were also scored as being sub-sarcolemmal (immediately beneath the sarcolemma) and intermyofibrillar. 20-22 fibres, photographed randomly, were used in determining V_V(mt, f) per fish.

The total (absolute) volume of mitochondria for body trunk red muscle /V(mt)/ was calculated from the equation /3/:

$$V(mt) = m_H \, V_V(mt, f) \cdot V_V(f, m) \cdot \delta^{-1},$$

where m_H is the muscle weight (g), V_V(mt, f) is the volume density of mitochondria per fibre (without units), V_V(f, m) is the volume density of muscle fibres (assumed to be 1) and δ^{-1} represents the density of muscle tissue (constant, 1.06 g·cm^{-3} /13/). Body trunk red muscle weight had been determined in a previous study /11/.

Results

Mitochondria were tightly packed in the sub-sarcolemmal region and were circular or ovoid in shape with long closely packed cristae. Most mitochondria were sub-sarcolemmal with the rest being intermyofibrillar (Table 1). The volume density of mitochondria /V_V(mt, f)/ decreased with fish

Table 1

Volume densisites of mitochondria /V_V(mt, f)/ (± S.D.) in red muscle of different sizes of O. niloticus in the present study

Fish weight (g)	/V_V(mt, f)/	s	i	sm
0.65	0.311 ± 0.169	0.271	0.04	87.1
3.9	0.308 ± 0.127	0.270	0.038	87.6
6.0	0.305 ± 0.142	2.245	0.06	80.3
7.7	0.265 ± 0.0871	0.238	0.027	89.8
19.1	0.246 ± 0.0439	0.224	0.022	91.1
31.0	0.257 ± 0.0812	0.216	0.041	84.0
44.8	0.284 ± 0.09	0.230	0.054	80.9
63.9	0.315 ± 0.115	0.249	0.065	79.0
82.7	0.365 ± 0.118	0.269	0.096	73.7
138.0	0.326 ± 0.114	0.251	0.075	76.9
223.5	0.277 ± 0.0962	0.225	0.052	81.2
300.0	0.245 ± 0.130	0.186	0.060	75.6
527.5	0.170 ± 0.0414	0.140	0.030	82.3
624.5	0.294 ± 0.115	0.230	0.064	78.2
812.3	0.237 ± 0.054	0.198	0.039	83.5

s = sub-sarcolemmal mitochondrial volume density, i = intermyofibrillar mitochondrial volume density and sm = % of mitochondria scored as sub-sarcolemmal

weight by a scaling factor of -0.028 (Fig. 1) which shows a slight change in this parameter with fish weight. The total volume of mitochondria /V(mt)/ for body trunk red muscle increased with fish weight by a factor of 1.13 due to an increase in body trunk red muscle per unit weight of fish by a factor of 0.16 /11/.

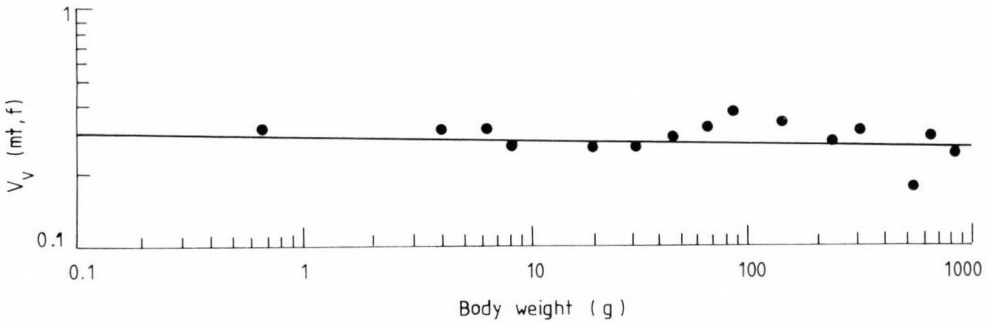

Fig. 1. Bilogarithmic plot of volume density of mitochondria /V_V(mt, f)/ against body weight. $V_V(mt, f) = 0.313\ W^{-0.028}$

Discussion

The volume density of mitochondria has been used as an approximate valid estimate of the concentration of respiratory chain enzymes in muscle fibres /14/ as the concentration of cristae in mitochondria appears to be constant and fully active mitochondria in various types of mammalian skeletal muscle operate at a constant rate /3/. If these observations are true for red muscle in fish, then mitochondrial density should be a valid estimate of the aerobic capacity of fish red muscle.

The function of mitochondria depends on the capillary density and rate of blood flow which affect the volume of blood (erythrocytes) and thus the oxygen supply to muscle. The good correlation between total capillary length per unit volume of muscle and mitochondrial density in some mammals /1/ shows that a close correlation exists between the capacity of capillaries to deliver oxygen to muscle fibres and the capacity of mitochondria to consume the oxygen /14/. In another study of mammals there was great variability in the number of capillaries per mm^2 of fibre and volume density of mitochondria /4/. Factors such as feeding habits, activity of fish, proportion of red muscle in relation to other muscle types and surface area of capillaries in muscle should be known before the capillary/mitochondrial relationship is fully understood.

A mitochondrial volume density of 0.20 for red muscle in O. niloticus (which is slightly lower than the values in the present study) has been demonstrated /7/. This could be due to the higher temperature at which fish in

the study were kept (28 ^{O}C) as compared to 25 ^{O}C in the present study. Fish in colder regions of the world tend to have higher mitochondrial densities in their red muscles than in warmer regions /7/ and acclimation to lower temperatures tends to increase mitochondrial volume density /10/. The age (size) of O. niloticus kept at 28 ^{O}C /7/ is also important as more mature O. niloticus have lower mitochondrial densities than the younger ones as is evident in the present study.

The slight decrease in mitochondrial volume density with age in the present study could be due to a decrease in energetic demand per unit volume of red muscle fibre or a higher turnover rate per unit volume of mitochondria. The greater than unity value for total mitochondrial volume density in body trunk red muscle for different sizes of O. niloticus in the study could be due to the overall increase in demand placed on the red muscle per unit fish weight with increase in fish size. Red muscle plays a greater role in sustained swimming in larger tilapia than small ones.

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AUTORADIOGRAPHY OF PITUITARY CELLS STIMULATED BY CASTRATION OR METHIMAZOLE FEEDING*

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It has been argued that the increase in size of the pituitary of castrated cockerels is due to hypertrophy of gonadotropic cells, some of which have been converted from other pituitary basophils. A counter argument could maintain that new gonadotropes are recruited from chromophobes that are replaced by mitotic division of undifferentiated cells. Since mitosis requires thymine for DNA replication, labelled thymine (H^3-thymidine) incorporated in DNA identifies new cell formation. On this basis, the chicken pituitary does not produce enough new cells to account for the size differences associated with castration or methimazole treatment.

Keywords: Pituitary — castration — autoradiography — cocks

Introduction

It has been suggested /1/ that castrated cockerels increase the size of their pituitaries by converting other pituitary basophils to gonadotropic cells and the subsequent hypertrophy of gonadotropes. This speculation was based partly on the finding that mitotic activity occurs at a very low rate in the post hatch avian pituitary /2/ and thus cell division could not account for the increase in gonadotropes. The possibility exists that

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new gonadotropes are recruited from chromophobes rather than from differentiated basophils and exhausted cells are replaced by mitotic division of undifferentiated cells at the same rate that old cells are lost. Since the assimilation of thymidine is essential to mitosis, cells containing administered thymidine (H^3-thymidine) are those that have recently undergone mitosis. Tritium in the nucleus of cells thus identifies those cells that have recently undergone mitosis.

In the current study H^3-thymidine was provided to untreated, castrated and methimazole fed birds in order to determine the role of mitosis in pituitary hypertrophy.

Materials and Methods

Nine 5-week-old White Leghorn cockerels were separated into 3 groups of 3 birds each. One group served as untreated controls, a second group was fed 0.1% methimazole in the standard University of Arizona growing mash, and the third group was castrated at 4 weeks of age and allowed one week to recuperate from the castration surgery. All birds were injected intraperitoneally with 0.1 ml solution of 1 Ci/ml H^3-thymidine /Methyl-H^3/ (ICN Radiochemicals, Costa Mesa, CA) daily for 2 weeks. The cockerels were killed by cervical dislocation, blood samples were taken and their pituitaries and thyroids were removed and fixed in Bouin-Hollande for light microscopy. After dehydration in ethanol, the excised tissues were embedded in paraffin and serial sections were prepared for immunocytochemistry and autoradiography /3/.

Coronal sections were cut at 4 microns and mounted on albuminized glass slides and stained using an avidin-biotin-peroxidase (ABC) kit (purchased from Vectostain, Vector Laboratories, Burlingame, CA). All sections were incubated with biotinylated goat anti-rabbit IgG for 30 min at 37 ^{o}C, washed in phosphate buffer saline (PBS), and incubated with avidin-biotin (prepared from 10 µl avidin and 10 µl biotin). The labelled complex was incubated with 3,3'-diaminobenzidine (DAB) and 0.002% hydrogen peroxide in 0.05 M tris buffer, pH 7.6.

Immuno-stained slides were dipped in Kodak nuclear track emulsion (Eastman Kodak Company, Rochester, NY). The Kodak emulsion was prepared according to instructions provided by Eastman Kodak Company.

After applying emulsion, slides were air dried at darkroom temperature and placed in light-tight black boxes with a desiccant (Drierite). Slides were exposed to the radioactive tritium at 4 ^{o}C for 4 weeks prior to development in Kodak D-19 (10 min, 20 ^{o}C). Developed slides were washed in water, fixed in two changes of Kodak fixer (5 min each) and washed in tap water before staining with hematoxylin and eosin.

All slides were examined at x1000. The cells containing nuclear tritium in a 1 mm^2 area of each slide were counted and expressed as the mean number of labelled cells/mm^2 in tissue.

Results and Discussion

The average weights of pituitary glands from cockerels used in this study are presented in Table 1.

Table 1

Pituitary weights (in milligrams) of 7-week old cockerels (mg; $\bar{x} \pm SD$)

Untreated	Methimazole-treated	Castrated
6.88 ± 0.23^{b}	5.01 ± 0.31^{c}	11.29 ± 0.65^{a}

$^{a-c}$Treatment means, with no common superscript are significantly different from each other ($P < 0.05$)

The concentrations (cells/mm^2) of H^3-thymidine labelled pituitary and thyroid cells are presented in Table 2. Concentrations of pituitary cells labelled with H^3-thymidine and stained for immunoreactivity from untreated, MMI treated and castrated cockerels are presented in Table 3. Figures 1 and 2 contrast the concentration of cells with H^3-thymidine in the pituitary (Fig. 1) and in the thyroid gland (Fig. 2). Figure 1A is an area of the cephalic lobe of the pituitary and Fig. 1B is of the caudal pituitary lobe. The areas of the pituitary depicted in Fig. 1 were selected to show nuclei with tritium. Three cephalic lobe cells have exposed granules over their nuclei (Fig. 1A) but only one caudal lobe cell (Fig. 1B) has exposed granules over its nuclei. In contrast, the thyroid (Fig. 2) has 18 cells with evidence of tritium in their nuclei. The area of the thyroid gland seen in Fig. 2 is a little less than the area of the combined cephalic and caudal lobes seen in Fig. 1. Furthermore the thyroid section includes a considerable amount of colloid while the pituitary is composed of closely packed cells. Each figure represents less than a mm^2 of slide area.

The pituitaries of untreated birds have approximately 2 labelled corticotropic cells (ACTH) per mm^2 in the cephalic and one labelled somatotrope (GH) cell per mm^2 in the caudal lobe (Tables 2 and 3). Castrated birds have 1.73 labelled lactotropes (PRL) and/or corticotropes per mm^2 in the cephalic and 1.26 somatotropes per mm^2 in the caudal lobe. Methimazole treated birds have only 0.66 labelled PRL cells in the cephalic lobe and less than one GH cell per mm^2 in the caudal lobe.

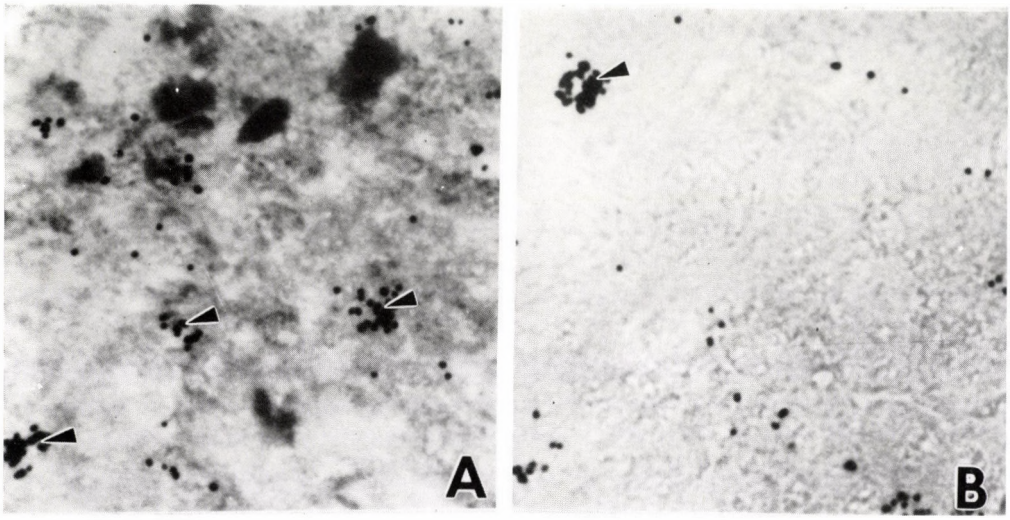

Fig. 1. Autoradiograph of the pituitary cephalic (A) and caudal (B) lobes of a normal White Leghorn cockerel receiving H^3-thymidine injections. Arrows indicate radiation exposed granules over cell nuclei. See size bar in Fig. 2

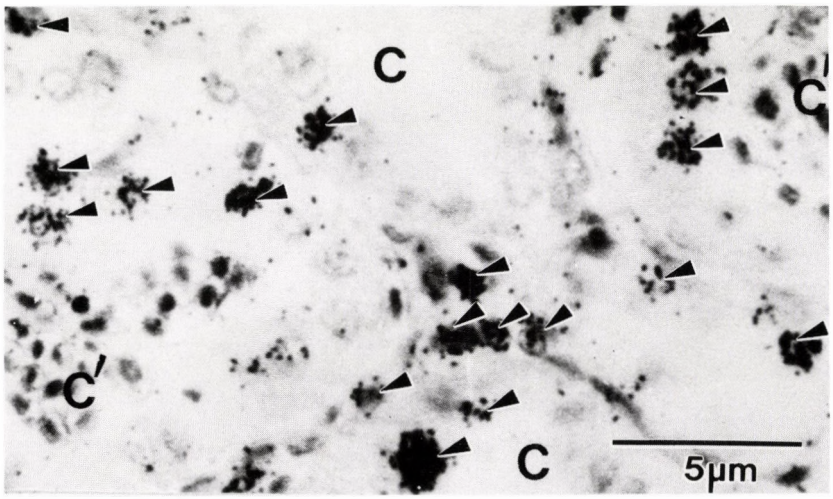

Fig. 2. Autoradiograph of the thyroid gland of a methimazole treated White Leghorn cockerel receiving H^3-thymidine injections. Arrows indicate radiation exposed granules over cell nuclei. C = colloid area; C' = colloid with red blood cells

Table 2

H^3-thymidine labelled cells/mm^2 in the chicken pituitary and thyroid glands (Mean ± SD)

Pituitary	Normal	MMI-treated	Castrated
Cephalic	1.86 ± 0.28^a	0.66 ± 0.15^b	1.73 ± 0.27^a
Caudal	0.93 ± 0.13^a	0.96 ± 0.18^a	1.26 ± 0.20^b
Thyroid	2.06 ± 0.26^a	4.33 ± 0.28^b	2.40 ± 0.21^a

^{a,b,c} Treatment means, with the same superscripts are not significantly different from each other ($P < 0.05$)

Table 3

H^3-thymidine labelled cells stained for immunoreactivity to GH, PRL or ACTH in the chicken adenohypophysis (number of cells/mm^2, mean ± SD)

Cell type	Untreated	MMI-treated	Castrated
GH	1.00 ± 0.16^a	2.00 ± 0.15^b	0.73 ± 0.18^a
PRL	0.00	1.06 ± 0.06^a	1.26 ± 0.11^a
ACTH	1.26 ± 0.11^a	0.00	1.13 ± 0.09^a

None of the immunostained LH or TSH cells contained radioactive tritium.

^{a,b,c} Treatment means, with the same superscripts are not significantly different from each other ($P < 0.05$)

The thyroid gland has actively dividing cells that assimilate H^3-thymidine (see Fig. 2) but there is a considerable amount of acellular follicular colloid that is also present. Consequently, although Table 2 indicates that the thyroid has twice the number of cells with H^3-thymidine as in pituitary tissue, this value is greatly underestimated since half of the area is colloid (Fig. 2).

The size of the pituitary glands of castrated birds is twice that of normal birds and the pituitary of MMI-treated birds is only three-fourths that of a normal bird (Table 1). If these size differences are taken into account the area of tissue that is measured is a greater percentage of the total gland in MMI-fed birds and a lesser percentage of the castrated bird

pituitary. Since virtually all the labelled cells occur in the cephalic lobe, those cells normally limited to the cephalic lobe (TSH, PRL and ACTH) may be expected to represent the reduced population of labelled cells. In this study none of the cells with H^3-thymidine were immunoreactive to TSH or LH.

In any event the number of labelled pituitary cells is so low in all birds (1 to 2 cells per mm^2) that it must be concluded that hyperplasia plays an insignificant role at best in pituitary size differences and the pituitary size increase in castrated birds is due to hypertrophy and perhaps the conversion of other basophils to gonadotropes.

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SEASONAL AND CIRCADIAN FLUCTUATION OF PLASMA LH LEVEL AND ITS CHANGE IN THE DOMESTIC GOOSE AS AN EFFECT OF GnRH TREATMENT

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We have defined the seasonal and circadian changes of the LH level of plasma of 2 years old male and female Hungarian domestic geese by means of the LH antigen-antibody of chickens in the heterologous RIA system. The LH level of plasma in geese living in conditions of natural light does not show any variation resulting from sexual differences, but the application of GnRH analogue (Ovurelin) causes higher peaks in ganders than in females. Since the LH level of plasma at the end of November in sexually inactive birds is high at night and lower during the day; the fluctuation in females can be considered as having a circadian rhythm. Five minutes after the intravenous application of GnRH analogue the LH level reaches its peak in the plasma and this high LH level returns to pre-injection values in 3 hours. The LH level shows a characteristic seasonal cycle in both sexes. The spring peak (reproduction cycle) is followed by a definite decrease at the beginning of the photorefractoriness (mid-June) with a slight autumnal increase in female geese.

Keywords: LH — GnRH treatment — plasma — domestic goose — sexual activity

Introduction

We have relatively abundant data on the LH plasma level of birds. In chickens in the course of embryogenesis the LH can first be detected in the plasma after 10.5 days /46/. 13.5 days after starting incubation, the production of oestrogen in the ovary and testosterone in the testicles grows

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significantly. This is linked to the sudden increase of the secretion of gonadotropic hormones (LH) /45, 47/. However, according to Weniger et al. /36/ and in contradiction to these data, on the 19th day of incubation the secretion of oestradiol is not yet under hypophyseal control. Immediately before hatching (on the 18th and 19th days of incubation) the plasma level of LH significantly decreases /46/.

A slow and gradual growth of the LH concentration of infantile birds can be detected /37, 38/ which, in the premature state (between the 11th and 13th weeks in chickens and between the 17th and 19th in ducks), reaches a relatively high level. However, one or two weeks before egg-laying, the LH level decreases drastically again /16, 39/.

The relatively low LH concentration occurring in this way actually amounts to half of the interovulational basic value. Following the progesterone peak which times the ovulation, or concurrently with it, a high preovulatory LH peak emerges in the plasma, which results in the rupture of F1 follicle /1, 10, 25/.

During clutch /7, 14/, and in relation with age, the heights of the preovulatory LH peaks decrease /29/, which might be a result of feedback or desensitization effects /11/.

Concurrently with the decrease of the plasma LH level in broody birds and the stopping of preovulatory peaks, the growth of the prolactin concentration can be detected /5, 32/.

According to the results of experiments carried out in different species, the LH level shows definite seasonality and there is strong correlation between the ethological phases of the reproduction cycle and the changes of LH plasma concentrations /24, 28, 42, 44/.

The LH concentration is low in the photorefractory period following brooding /15, 17, 33/.

The LH level in photosensitive birds grows rapidly after photostimulation /12, 19/. The LH concentration increases significantly in Japanese quail and Zoonotrichia which are moved from short photoperiod into long photoperiod after 19-20 hours. The hormones remain at this high level for a couple of days.

We have relatively few data on the circadian rhythm of LH secretion and plasma concentration. Some authors give an account of the existence of the circadian rhythm of LH level /2, 23, 26, 27, 39, 43/. Conversely, other authors were unable to find any regular rhythm in LH fluctuation /3, 13, 18, 42/. Very few experiments deal with the changes of the plasma LH level

of goose species with respect to the season and the ethological characteristic phase of the reproduction cycle (Barheaded geese /8/, Migratory Canada goose /15/).

We know little about the hormonal background of the reproduction cycle of domestic geese and have no data at all about gonadotropic hormone levels. In the present experiment we studied the seasonal and circadian changes of the LH plasma level of Hungarian male and female domestic geese kept in natural photoperiod and defined the acute changes in LH concentration in consequence of the applied superactive GnRH analogue (Ovurelin).

Materials and Methods

1st experiment

The seasonal study of plasma LH level

Experimental animals: 2 years old Hungarian domestic geese (10 ganders and 10 geese) were kept in natural circumstances all year round. The animals had 16% protein feed and water ad libitum. Blood was taken from the brachial vein on the following days: 1st February, 16th March, 17th May, 14th June, 14th August, 2nd October, 8th November. It was taken in the morning between 9 and 11 o'clock and was put into heparinized plastic tubes.

2nd experiment

The circadian study of plasma LH level

Experimental animals: Hungarian domestic geese (4 ganders and 5 females) were placed in individual cages in natural photoperiod (9L-15D). The date of examination: 27-28 November 1989. The blood was taken through the jugular vein with silicon caoutchouc cannula leading into the right atrium. The implantation of the cannula was carried out in narcosis (Equithesin) supplemented with local anaesthesia (Lidocain). After exposure of the right jugular vein a 0.20 x 0.37 Silastic cannula (Dow Corning, Medical - Grade Tubing) was led into the right atrium. A polyethylene tube was attached to the distal end of the silicon caoutchouc cannula which was led into the top of the head under the skin of the neck. Two days after the operation blood was taken at the following times: 16.00, 20.00, 24.00, 04.00, 08.00, 12.00, 16.00, 20.00. In each case 2 ml of blood was taken with a syringe from a polyethylene tube from the top of the head (which was subsequently closed each time).

3rd experiment

The effect of a single GnRH treatment on the plasma LH level

Experimental animals: Hungarian domestic geese (5 ganders and 5 females) were placed in individual cages in natural photoperiod (8L-16D). The date of examination: 14-15 December 1989. The blood was taken through the jugular vein with a cannula led into the right atrium (see 2nd experiment). The animals were treated with a fixed cannula. For the treatment we applied D-Phe6-GnRH-EA analogue in a dosage of 5 µg/animal (Ovurlein, Reanal, Budapest). Times of blood taking: -15, 0, 5, 10, 15, 30, 60, 120, 240, 360 minutes. The injection of GnRH was given at "0" minute.

LH RIA

Determination of LH was done by ^{125}I RIA. We used Sharp and Talbot's method (AFRC Poultry Research Centre, Roslin, Midlothianm 1985), and adopted it to goose plasma.

Materials

-- Antigen -- chicken LH (PRC-AEI-S-I)*
-- Antiserum -- anti rabbit cLH (dil. 1:20,000)*
-- Tracer -- ^{125}I -- cLH (10,000 cpm/tube) spec. act.: 6.6 MBq/µg.
-- Diluent -- 0.5 M BPS (pH = 7.5) cont. 0.3 M NaCl, 0.017 M NA_2 EDTA, 0.03 M NaN_3, 2% (v/v) horse serum.
-- 2nd antibody -- anti rabbit goat IgG
-- Normal rabbit serum (1:200)
-- Separation -- Peg 6000 7% (w/v)

Intra-assay (CV%): 5.18
Inter-assay (CCV%): 11.78.

RIA

The procedure of RIA takes 4 days. On the first day the samples and the antiserum were placed in polystyrene test tubes. The cockerel pool (internal standard) contained 20 µl blood serum diluted to 200 µl in RIA diluent as required. The hen and goose pool contained 40 µl serum diluted to 200 µl diluent. After vortex on whirlimixer, the tubes were left overnight at 4 °C.

On the second day the tracer was added to the test tubes, giving approximately 10,000 cpm/tube. Whirlimixer, overnight at 4 °C.

On the third day the normal rabbit serum and goat anti rabbit IgG were added to the tubes. Whirlimixer, overnight at 4 °C.

On the fourth day PEG was added to the tubes to separate the bound and unbound molecules. After vortex and 15 minutes storage at 4 °C the tubes were spun for 20 minutes at 2500 g. After centrifugation the supernatant was aspirated with a Pasteur pipette attached to a water pump. The tubes were counted for 60 s.

The quantitative changes of LH were evaluated with Halbergh's "Cosinor" analysis in order to set up the circadian rhythm. We stated the divergences between the average of the different groups with Student t cluster and "F" probe.

*Donated by Dr. P. J. Sharp (AFRC PRC, Roslin, U. K.).

Results

1st experiment (Seasonal examination)

During the spring reproduction cycle the LH plasma level grows in ganders from the beginning of February to the middle of March and gives a high value until the middle of May. In the middle of June (at the beginning of the photorefractory period) there is a slight decrease which is followed by a minor second peak in August at the end of moulting. In October and November (during the autumnal sexual reactivation) the values decrease slightly but constantly. The differences are only significant in March because of the great deviation. At the beginning of the photorefractoriness (14th June), we observed an especially large deviation. This is obviously linked to the fact that some of the animals are already photorefractory and some of them still have a high LH level and are in sexually active state.

The seasonal fluctuation of LH is more definite in female geese than in males but the differences are not significant in most cases (Table 1). Between the beginning of February and March we can observe a significant increase. A decrease starting in May leads to a very low LH level by the beginning of the photorefractory period (14th June). There is another peak in August (at the end of moulting) which, after a marked decrease in October, is followed by the autumnal peak in November during a partial sexual reactivation (Fig. 1a, b).

Table 1

A comparison of the changes of plasma LH levels (ng/ml) (± SD) between the groups of ganders and laying geese. (Gander: n=10; female: n=10)

Time	Female $\bar{X}$ +/- s		Male $\bar{X}$ +/- s	
February 01.	2.77	1.66	4.65	2.85
March 16.	10.44	5.44	7.59	1.31
May 17.	7.28	3.91	7.60	4.67
June 14.^{x}	0.84	0.58	4.22	3.79
August 14.	7.98	6.36	7.59	3.57
October 02.^{xxx}	1.66	1.39	5.86	2.11
November 08.	4.59	3.06	4.13	2.63

^{x} P=0.05, ^{xxx} P=0.001.

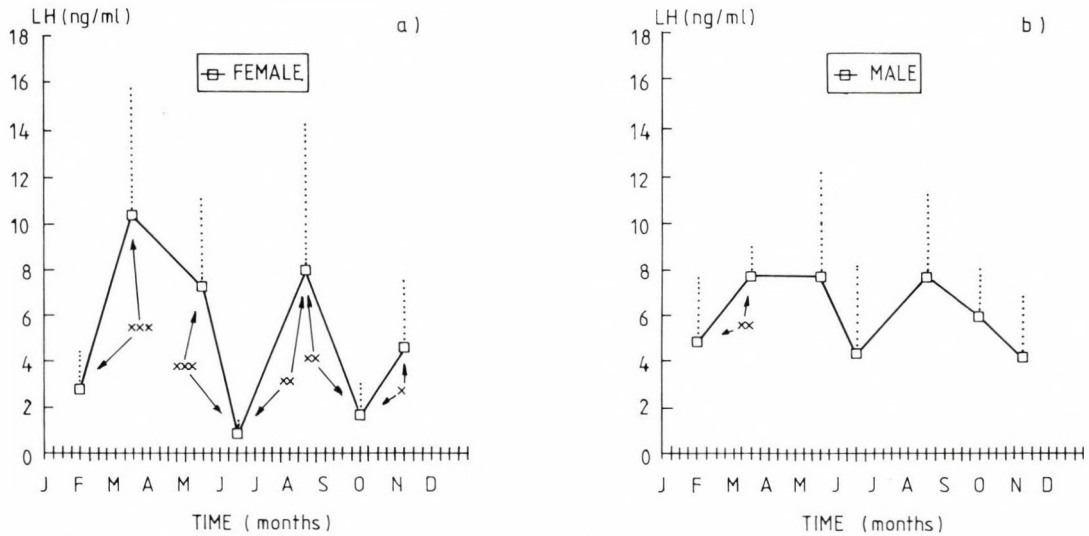

Fig. 1. a) Seasonal fluctuation of plasma LH level (± SD) of domestic geese (female: n = 10), x (P=0.05), xx (P=0.01), xxx (P=001); b) Seasonal fluctuation of plasma LH level (± SD) of domestic geese (gander: n=10), xx (P=0.01)

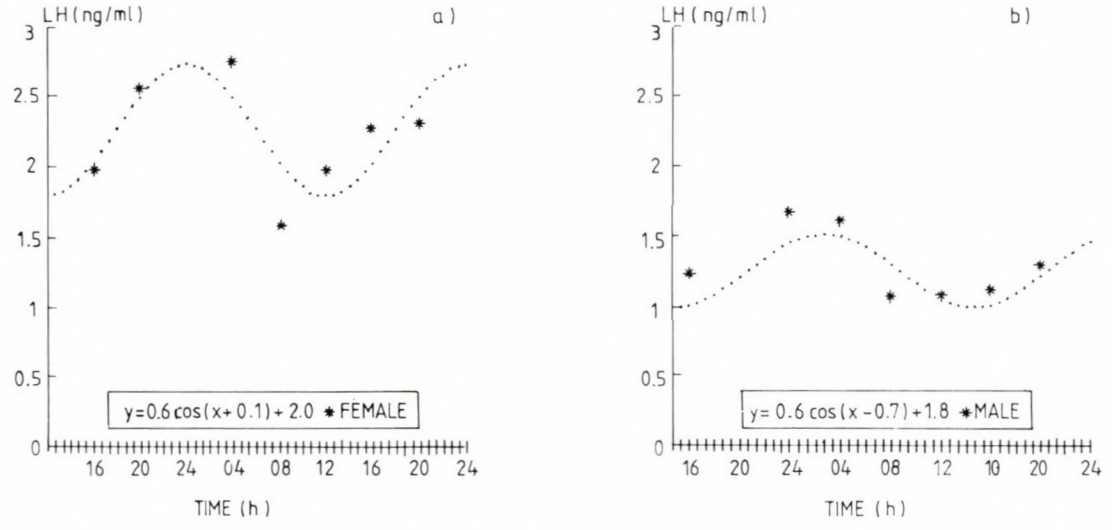

Fig. 2. a) "Cosinor" analysis of the circadian fluctuation of plasma LH level in domestic geese (female: n=5; standard deviation = 0.27, F=1.6, correlation index = 0.77); b) "Cosinor" analysis of the circadian fluctuation of plasma LH level in domestic geese (gander: n=4, standard deviation = 0.28, F=1.1, correlation index = 0.59)

2nd experiment (Circadian study)

On the basis of the analysis of blood samples taken every 4 hours it can be stated that the LH level fluctuates in a pattern close to the sinus curve — to a larger extent in females than in males. In females the average LH is slightly higher than in ganders but because of the great deviation the difference between sexes is not significant. Neither do we find in most cases a significant difference between males and females if we compare daily averages.

Measurement results show that in the case of both sexes the LH level is higher in the dark period (from 20 to 04) than in the light period (from 08 to 12).

The results of cosinor analysis prove that in the case of females there is a definite circadian rhythm with measurement points closely approaching the calculated sinus curve. In the case of ganders the deviation between measured and calculated values is greater and the correlation index is smaller. On the basis of the "F" test there is no significant circadian rhythm in ganders (Fig. 2a, b).

Table 2

A comparison of the changes of plasma LH level (± SD) in groups of synthetic GnRH analogue treated domestic geese. (Gander: n=5; female: n=5)

Time	Female $\bar{X}$ +/- s		Male $\bar{X}$ +/- s	
-15'	2.14	0.71	3.01	1.19
0'	1.61	0.44	2.57	1.30
5'	7.38	1.74	6.09	0.67
10'	5.40	1.50	4.91	0.36
15'	7.10	0.96	6.72	0.82
30'	5.54	1.98	5.22	0.60
60'^{xxx}	2.17	0.62	4.10	0.45
2 h	1.45	0.86	3.45	2.37
4 h	1.40	0.73	3.13	1.70
6 h^{x}	1.05	0.44	3.95	2.30
24 h	2.27	1.91	2.81	1.62
24 h 30'^{x}	0.87	0.92	2.67	1.47
25 h	2.55	1.72	1.03	0.48

^{x}P=0.05, ^{xxx}P=0.0001.

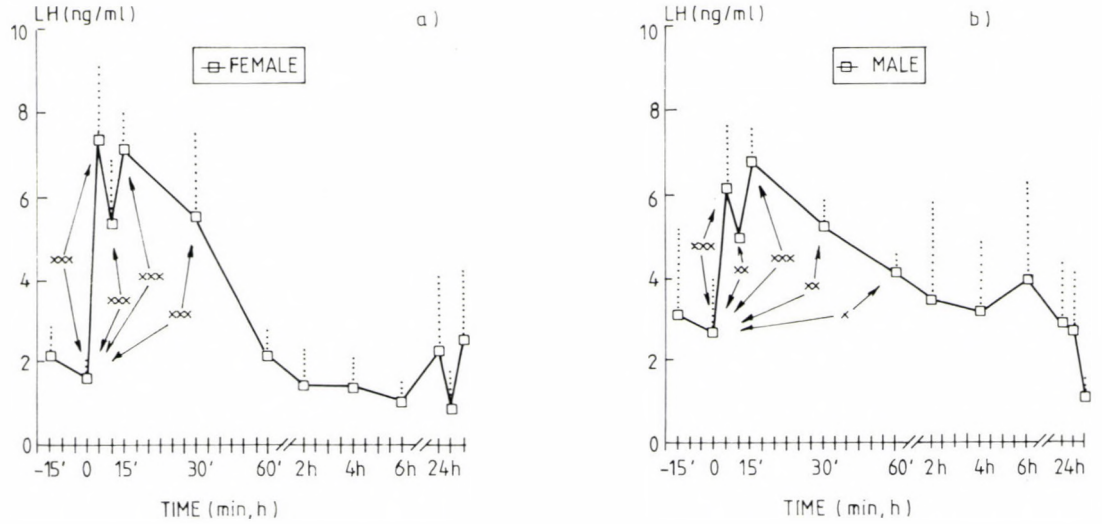

Fig. 3. a) Effect of a single GnRH analogue injection on plasma LH levels in groups of five domestic geese. The dotted lines represent the SD. x (P=0.05), xx (P=0.01), xxx (P=0.001); b) Effect of a single GnRH analogue injection on plasma LH levels in groups of five ganders. The dotted lines represent the SD. x (P=0.05), xx (P=0.01), xxx (P=0.001)

3rd experiment (GnRH treatment)

The basic values of LH plasma do not differ significantly in the cases of ganders and females. After the intravenous injection of GnRH a sharp LH peak occurs which shows a 2.5-fold increase in males and a 5-fold increase in females in 5 minutes. Atfer 10 minutes we see a decrease in both sexes which is followed by a second peak again in both sexes in 15 minutes. Thirty minutes after the injection there is a constant decrease both in males and females, reaching the value measured after 60 minutes. In the next 2 hours this decrease lessens, but in the 3rd hour after injection the original values can be found again (Fig. 3a, b). We found in most cases no significant difference between sexes (Table 2).

Discussion

Domestic geese are characterized by a prolonged spring reproduction cycle (from end of January to beginning of June) which, after the long day photorefractoriness in summer, is followed by a lesser or greater reactivation of gonads in October and November. The low light intensity applied during photorefractoriness results in the complete reactivation of gonads in October with a full reproduction cycle coming between the middle of October and end of December /4, 6, 20/. The results of this present study show that in geese living in natural light conditions, the LH level of plasma reacts differently in male and female geese during the spring and the partial autumnal cycle. In ganders the level of LH like testosterone /20/ is high between the beginning of February and the end of May. However, in October and November, when the level of testosterone gives another -- albeit lower -- peak, no further growth of LH can be detected. The testicle reactivates despite the continuous decrease of the moderately high LH level. In female geese, however, the high spring and very low summer LH concentrations are followed by another gonadotropic peak in November. Therefore a definite parallelism can be detected between the sexual steroids /6/ and the LH level of plasma in autumn.

There is also a difference between males and females in the rate of decrease of LH concentration at the beginning of the photorefractoriness. In the middle of June it decreases strongly in females whereas it does so only slightly in males. In the last phase of postnuptial moulting (the finishing

stage of feather growth) the LH level of plasma grows, which again is more definite in female geese. In this period ganders are characterized by a slight but significant growth of the testosterone level /9/. In female geese, however, the level of sexual steroids does not increase at this time /22/. In feather follicles the quantity of androgen and oestrogen receptors grows in this period /21/.

The moderate and temporary reactivation of the gonadotrop-gonad system in ganders in the middle of the photorefractory period (August) is therefore related to feather growth and does not involve the beginning of spermatogenesis.

The LH levels of the two sexes coincide during most measurement. In ganders there is a slightly higher level of LH concentration at the beginning of the photorefractoriness and at the beginning of October, whereas in females the increase occurs in the middle of the egg-laying cycle. According to Dittami /8/ in the case of Anser indicus in the reproduction period the LH level of ganders is somewhat higher than that of the females. John et al. /15/ made similar observations in migratory Canada geese. None of the authors give an account of sexual differences at other times of the year.

Gonadotrophic hormone production in most bird species has a circadian fluctuation, which is characterized by a peak in the dark period and by lower values in the light period /2, 18, 23, 39/. In certain cases these changes show a regular diurnal rhythm /23, 27, 40, 43/. According to other data the regular pattern is divided by periodical "high" and "low" values and they cover the supposedly existing diurnal rhythm /3, 42/. The diurnal character of LH secretion can only be definitely proved in immature birds where the modifying effect of the sexual cycle does not cover the "basic rhythm" /2, 35, 40/. From our present results the LH level of plasma shows circadian fluctuations: LH concentrations in both males and females are shown to be higher at night than during the day. According to Cosinor analysis the LH fluctuation has a rhythmic character in female geese, but it does not conform to this rhythm in ganders.

Five minutes after the application of the superactive GnRH analogue ("Ovurelin") the LH concentration of plasma reached its highest value both in ganders and females. We found as early a reaction as did Wingfield et al. /41/ in Zonotrichia and Sharp & Lea /30/, Sterling & Sharp /34/ in domestic hens. LH returns to its original level 3 hours after injection -- slower than the LH level of the domestic hen after the application of GnRH of

chickens or pigs /34/. The increase of LH concentration in ganders is approximately twice as large as in females. The effect of GnRH on geese varies as a result of sexual differences to an extent similar to the variance found between hens and cocks /31/.

The study of the LH level of plasma in domestic geese has shown that the LH preparation applied from chickens together with the antibody which forms a heterologous system, can be successfully used. Our data show similar circadian and seasonal fluctuations in the LH level of plasma as described in studies about other bird species. Nevertheless, further experiments will have to decide whether the LH level of plasma can also be characterized by pulsatile, episodical and short-term changes.

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HISTOCHEMICAL CHARACTERISTICS OF THE EGG MEMBRANES OF PORTUNUS PELAGICUS (L.)

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Incubated eggs of berried female crab Portunus pelagicus are hanged to the pleopodal setae by an attaching membrane. Each egg is protected by inner chorion and middle chitinous membrane. The middle chitinous and the outer attaching membranes form together a distinct case surrounding each egg. This case resists acids and alkalies and appears impermeable to water, salts and dye substances. The histochemical results provide evidence as to the origin of the chorion and suggest its function. The chitinous membrane is tanned, calcified and contains chitin. The attaching membrane is of lipoid nature and appears to be rich in polyphenols and phenolase enzyme suggesting the presence of phenolic tanning. The histochemical results confirm the cuticular origin and nature of the egg case. The tegumental glands of the pleopods are involved in the formation of the outer attaching membrane and/or at least the phenolase enzyme necessary for phenolic tanning. Larval release is a result of molting if the egg case is considered as a part of the outer cuticular exoskeleton of Portunus.

Keywords: Crab — egg — membrane histochemistry — Portunus pelagicus

Introduction

Berried female crabs incubate their fertilized eggs for a time, during embryogenesis, on their pleopods until the time of hatching /1/. The origin and nature of the membranes surrounding the crustacean eggs received some attention /2–5/.

In our laboratory /5, 7/ some light was sked on the different membranes of both oocytes and eggs of the crabs Portunus and Carcinus using phase contrast and scanning electron microscopes.

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The formation of the chorion within the ovary of Homarus was identified ultrastructurally /8/. The lipoid nature of the inner chorion of Portunus and Homarus was demonstrated /5, 8, 9/. The chitinous membranes /2, 5/ of decapod eggs are oviductal in origin in the view of Yonge /10/ and vaginal based on concepts of El-Sherief et al. /5/ and El-Sherief /11/. The origin of the attaching membrane of Portunus egg is pleopodal and is for hanging /5/. The significance of the outer egg membranes is protection of the egg during the long period till hatching. So, the aim of the present work is to provide some necessary information about the nature and the chemical constituents of the egg membranes.

Materials and Methods

The edible crab, Portunus pelagicus (L.), is a common representative of the brachyurans in Alexandria coast. The ovigerous females were collected during spawning periods (May and September) /11/ at Abu-Kier gulf. Live females 6-8 cm in width were maintained in a holding tank containing filtered sea water which was changed every 24 h. To inhibit the bacterial growth, 0.25 ml of penicillin-streptomycin of 250 mg concentration was added to each liter of filtered sea water /12/. The photoperiod was 12 h, thereby providing light 50% of the time. The diameter of the egg including outer egg membranes was measured in an unfixed fresh condition under bright field illumination and phase contrast optics. For histological examination of the egg, parts of newly laid spawn (reddish orange) were fixed in 5% neutral buffered formaldehyde or Carnoy fixative then dehydrated and embedded in paraffin wax. Sections were made at 10 µm. The histochemical reactions were carried out on fresh unfixed cryostate sections of the spawn. The resistance of the egg membranes to acids and alkalies was tested using different concentrations of hydrochloric acid, sulphuric acid and potassium hydroxide. The chemical characteristics of the egg membranes were studied using different histochemical tests mentioned in the text (Table 1). Chitin was detected by the chitosan method modified by Peters /13/ and Rajeswari & Ravindranath /14/ for soft invertebrate skeletal structures. The timing of alkaline hydrolysis was done from 25 to 15 minutes as recommended by the latter authors /14/. Permeability experiments were carried out using 0.1% aqueous solution of basic fuchsin (BDH), toluidine blue and methylene blue. The staining of the yolk mass has been taken as an indication for the passage of the used dyes through the outer egg membranes. Staining with alizarin red was used for the detection of calcium deposited in the egg membranes /15/.

Results

Newly berried female crabs Portunus pelagicus (L.) appear with large reddish orange spawn on their pleopods. Incubated eggs are hanged to the setae of the pleopods (Fig. 1). They are spherical with about 275 µm

Table 1

Histochemical characteristics of the egg membranes and the tegumental glands of the pleopods of Portunus pelagicus (L.)

Staining method	References	Material demonstrated	Egg membranes						Tegumental gland
			chorion		chit. m.		attach. m.		
			inn.	out.	inn.	out.	inn.	out.	
1. Mercuric bromphenol blue (Mbb)	Mazia et al. /16/	proteins	—	BBB	BB	BBB	BBB	—	BBB
2. Aqueous bromphenol blue (Abb)	Mazia et al. /16/	basic proteins	—	BBB	BB	BBB	BBB	—	BBB
3. Deamination + Abb	Mazia et al. /16/	basic proteins	—	—	—	—	—	—	—
4. Toluidine blue (TB) (standared)	Pearse /17/	acidic groups (metachromatic)	—	VV	V	V	VV	—	VV
5. Xanthoproteic test	Lillie /18/	phenyl group of proteins	—	OO	OO	OO	OOO	O	OOO
6. Millon reaction (Bensley and Gersh modification)	Pearse /17/	protein containing tyrosine	—	PP	PP	PPP	PPP	P	PPP
7. Nitrosonaphthol method (Udenfried; Cooper and Stoward)	Pearse /17/	tyrosine	—	YY	YY	YYY	YYY	Y	YYY
8. DMAB-nitrite method (Adams)	Pearse /17/	tryptophan	—	—	b	b	b	—	b
9. Tryptophan reaction (Adams)	Pearse /17/	tryptophan	—	—	V	VV	V	—	VV
10. Oxidized tannin Azo method (OtA) (Dixon)	Pearse /17/	tannophilic proteins	—	PP	PP	PP	PP	P	PP
11. Ninhydrine-Schiff reaction (Yasuma and Itchikawa)	Pearse /17/	protein bound amino group	—	PP	P	PPP	PPP	—	PP
12. Sakaguchi reaction	Pearse /17/	arginine	—	OO	O	O	O	—	O
13. Ferric chloride test	Smyth /19/	diphenols	—	—	—	—	—	—	—
14. Ferric chloride test	Dennel /20/ Krishnan /21/	free ortho-dihydroxy phenols	—	—	—	—	—	—	—
15. Argentaffin reaction	Lison /22/	phenols; quinones; polyamines	—	—	++	++	++	+	++

Table 1 (contd.)

Staining method	References		Material demonstrated	Egg membranes						Tegumental gland
				chorion		chit. m.		attach. m.		
				inn.	out.	inn.	out.	inn.	out.	
16. Catechol technique	Smyth	/19/	phenolase	—	—	—	BB	BBB	B	BBB
17. Lead acetate test	Pearse	/17/	SH and S-S groups	—	±	—	RR	—	R	RR
18. Periodic acid-Schiff test (PAS) (McManus)	Pearse	/17/	hexose containing mucosubstances (MPS)	RR	RRR	RR	RRR	RR	R	RRR
19. Diastase + PAS	Hotchkiss	/23/	acid MPS	RR	—	R	R	RR	R	RR
20. Schiff's test	Pearse	/17/	free aldehydes	—	BB	—	—	BB	B	BB
21. Best's carmine (Bc)	Pearse	/17/	glycogen	—	PP	PP	PP	PP	P	PPP
22. PAS (4 h in periodic acid)	Hotchkiss	/23/	acidic mucopoly-saccharides (MPS)	DM	—	—	—	DM	DM	DM
23. PAS after acetylation	Pearse	/17/	acidic MPS	P	—	P	±	P	P	PP
24. PAS after acetylation and deacetylation	Pearse	/17/	acidic MPS	M	—	M	M	M	MM	MM
25. Alcian blue-PAS (Mowry)	Pearse	/17/	acidic and neut MPS	bb	RR	bb	b	bb	bb	bbb
26. Alcian blue method (Steedman)	Pearse	/17/	acidic MPS	bb	—	b	bb	—	bbb	bbb
27. Alcian blue 2.5% procedure	Pearse	/17/	acidic sulphated MPS	—	—	—	—	b	b	b
28. Alcian blue 1.0% procedure	Pearse	/17/	sulphated MPS	—	—	—	—	bb	—	bb
29. Alcian blue at 0.6 M $MgCl_2$ and 0.8 M $MgCl_2$	Scott et al.	/24/	acidic sulphated MPS	—	—	—	—	bbb	b	bbb
30. Chitosan test	Campbell Peters Rajeswari & Ravidranath	/25/ /13/ /14/	chitin	—	—	bbb	b	—	—	—
31. Sulphation method (Kramer and Windrum) + Alcian blue (pH 3.6)	Pearse	/17/	chitin sulphate	—	—	bb	b	—	—	—
32. Sudan black B (SBB)	Lillie	/18/	lipids	±	+	BB	B	—	BBB	BBB

Table 1 (contd.)

Staining method	References		Material demonstrated	Egg membranes						Tegumental gland
				chorion		chit. m.		attach. m.		
				inn.	out.	inn.	out.	inn.	out.	
33. Hot acetone +SBB	Lillie	/18/	lipids and bound lipids	—	—	BB	—	—	BBB	BBB
34. Nile blue method (Cain)	Pearse	/17/	neutral and acidic lipids	—	—	—	—	—	bb	bb
35. Hot pyridine extraction + SBB (Baker)	Pearse	/17/	glycolipids and phospholipids	+	+	—	—	—	—	—
36. Nile blue method (Menschik)	Pearse	/17/	phospholipids	b	b	—	—	—	—	—
37. Salt extraction (Holczinger and Balint)	Pearse	/17/	lipoproteins	—	—	bb	—	—	bbb	bbb
38. Periodic acid Schiff after pyridine ext.	Baker	/26/	glycolipids	—	—	—	—	—	—	—
39. Sudan black B (Boyliss Adams BSB)	Lillie	/18/	lipids	—	—	BB	B	—	BB	BB
40. Acetone-SBB (Berenbaum)	Pearse	/17/	bound lipids	—	—	BB	B	—	BB	BBB
41. Performic acid-Schiff (Lillie)	Pearse	/17/	unsaturated lipids and phospholipids	—	—	—	—	—	—	RR
42. Liebermann-Burchardt reaction	Pearse	/17/	cholesterol and its esters	—	V	—	—	—	—	—
43. Alizarin red	Stock	/15/	calcium ions	—	—	—	RR	R	RR	RR

B — black; b — blue; DM — dark magenta; M — magenta; O — orange; P — pink; R — red; V — violet; Y — yellow. The repetition of each symbol indicates intensity of this colouration

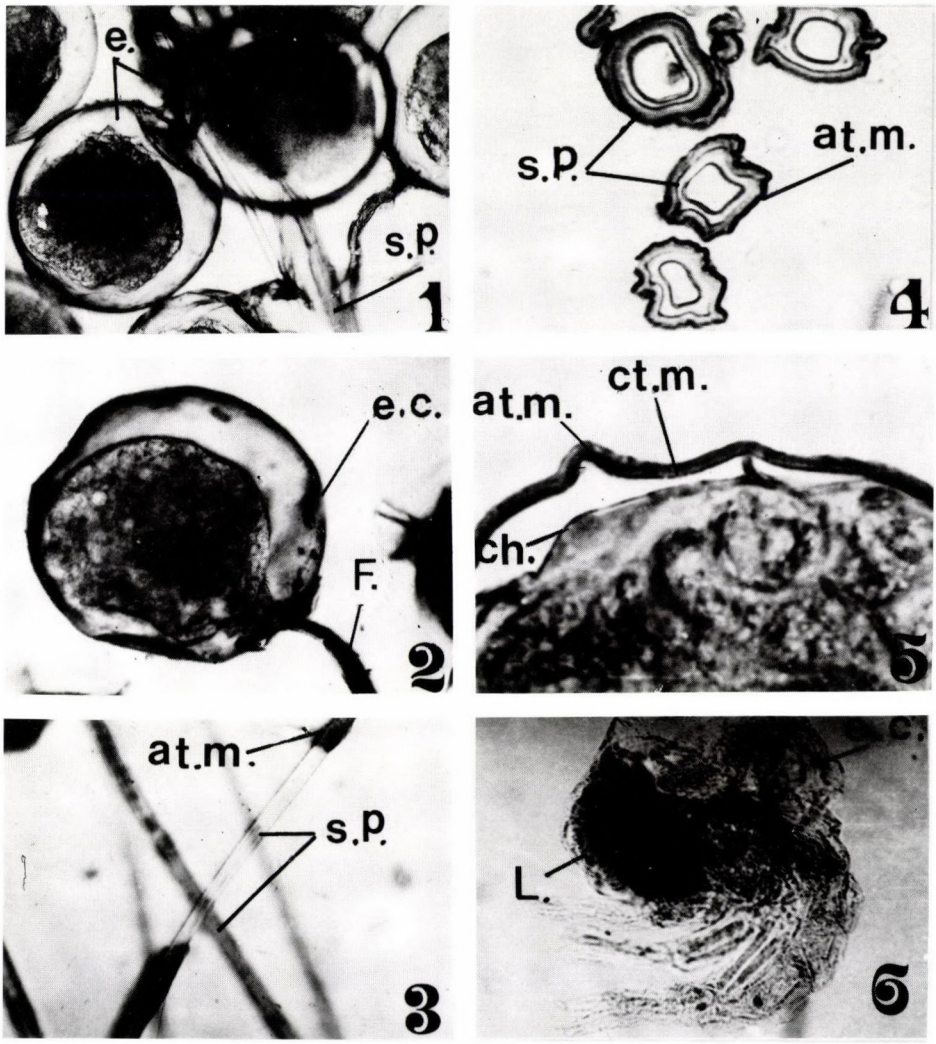

Fig. 1. Stained whole mount preparation of freshly incubated eggs hanged on the setae of the pleopods of Portunus. e.: egg; s.p.: seta of the pleopod. x100

Fig. 2. Whole mount preparation of unstained incubated egg of Portunus showing the outer case of the egg and its funiculus. e.c.: egg case; F.: funiculus. x130

Fig. 3. Magnified portion of the whole mount preparation of the spawn of berried female showing the outer attaching membrane on the pleopodal seta. at.m.: attaching membrane; s.p.: setae of the pleopods. x120

Fig. 4. Transverse section of the pleopod of Portunus showing its setae while covered with the outer attaching membrane. s.p.: setae of the pleopods; at.m.: attaching membrane. x800

diameter. The outer egg membranes and the perivitelline space are included in these measurements. Figure 2 shows a distinct case around the incubated egg. At the base of each egg, its case extends to form somewhat long funiculus which attaches it to the seta of the pleopod. Phase contrast and stereoscopic examinations showed that the outer egg case not only forms the funiculus, but it also extends to cover the seta itself (Figs 3 and 4). Histological preparations of the spawn showed that the egg is enveloped within three distinct membranes, the inner chorion and two different outer membranes (Fig. 5). These outer membranes are the middle chitinous membrane and the outer attaching membrane /5/ which form together the case around the incubated egg (Fig. 5).

The chorion of the egg of Portunus measures about 0.9 μm in thickness. The histochemical results proved that it consists of inner and outer layers. Protein tests indicated that the inner chorion is non-proteinous, while the outer one contains tyrosine and little basic proteins. In addition, tannophilic protein, reactive NH_2 group and arginine were also indicated in it (Table 1). The staining methods for carbohydrates proved the mucoid nature of the chorion, while the chitin tests indicated the absence of chitin in both layers of the chorion. The metachromatic reaction of the chorion and its diastase resistance suggested the presence of non-sulphated AMP. The reduced staining after methylation confirmed these results. From the above data, it can be reasonably suggested that the basophilia of the chorion is probably due to AMP.

The chitinous membrane of the egg case is the middle membrane around the egg. It measures about 3.6 μm in thickness. The resistance of this membrane to acids and alkalies and its strong reduction to ammoniacal silver hydroxide are well observed. According to the acceptance to the used stains and the histochemical results, this membrane is differentiated into two layers: the outer and inner chitinous layers (Fig. 5). Chitin tests confirm the presence of marked amount of chitin in the two layers of the chitinous

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Fig. 5. Magnified portion of section taken through freshly incubated egg of Portunus showing the inner chorion and the different layers of its outer case. ch.: chorion; ct.m.: chitinous membrane; at.m.: attaching membrane. x1000

Fig. 6. Stained preparation of greyish spawn of Portunus showing the first larval stage during larval release. Notice that the egg case is still attached to the larva. e.c.: egg case; L.: larva of Portunus. x160

membrane but it is more abundant in its inner layer. However, proteins are abundant in this membrane and more tyrosine in its outer layer. On the other hand, no free phenols were detected either histochemically or after prolonged extraction with the suitable solvents. However, phenolase enzyme and substantial amount of calcium were also detected in the chitinous membrane.

The outer attaching membrane appears impermeable as indicated by the non-diffusion of toluidine blue, basic fuchsin and aniline blue. The unaltered state of the inner yolk mass of the egg proves this result. It also appears impermeable to salts and water, and resists acids and alkalies. Lipids shown by Sudan black test in this membrane lose their ability to stain by Liebermann--Burchardt test, presumably as a result of polymerization of steroid lipids. The protein moiety, on the other hand, is characteristically rich in phenolic groups (Table 1). No free phenols could be detected, but the presence of phenolase can demonstrate the occurrence of tanning in the attacing membrane. This was further confirmed by the positive results of Millon's and argentaffin reactions indicating the presence of aromatic substances.

From the tabulated results (Table 1) it is interesting to point to the great similarity between the chemical characteristics of the attaching membrane and the detected secretions of the tegumental glands of the pleopods. The great abundance of calcium salts was also noticed in both.

Shortly before molting and when the spawn gets deep greyish, two events take place; the spawn hatches, and the larvae release (Fig. 6).

Discussion

The egg is a highly organized cell that is prepared for union with sperm and for withstanding long periods of either desiccation or other environmental conditions. Most egg cells are surrounded by one or more membranes. Chorion is such a secondary membrane formed by the follicle cells /7, 8, 27/. Tertiary membrane is superimposed upon the secondary membrane and acquired, sometimes after fertilization as the egg passes along the genital ducts /27/.

In the previous work of El-Sherief et al. /5/ the tertiary membranes of the egg of Portunus pelagicus were differentiated into the middle chitinous and the outer attaching membranes. The present work clarifies that

these two membranes form together a distinct case around the incubated hanged egg of Portunus.

Talbot /8/ showed by ultrastructural study two layers of the chorion of Homarus oocyte. The present histochemical results differentiated the chorion of Portunus egg into the inner lipoid layer and the outer proteinous one. Chorion of Portunus egg is free from chitin while it was detected in the corresponding membranes of the egg of Homarus vulgaris and Chirocephalus diaphanus /10, 29/. Since AMP was detected histochemically in the follicle cells of anomuran crab Clibanarius /30/, and the chorion of decapod oocytes is derived from the associated follicle cells /7, 8, 27/, the presence of AMP in the chorion of the egg of Portunus provides a supportive evidence for the contribution of the follicle cells in the formation of the chorion. Among the well known functions of AMP in vertebrates is that of allowing a ready diffusion of dissolved substances between cells and capillaries /31/. AMP, however, has been demonstrated on the surface of cells active in pinocytosis /32/. The results of previous works together with the present histochemical evidences may lead to the suggestion of the permeable nature of the chorionic membrane of the egg of Portunus. The resistance of the outer case to acids and alkalies and its strong reduction to ammoniacal silver hydroxide are considered as main criteria which are always associated to phenolic tanning in arthropod cuticule and other invertebrate structures /32/. Phenolic tanning is a process which leads to the formation of tanned proteins (sclerotein) through quinone structure by the aid of the polyphenol oxidase enzyme /19/. However, El-Sherief /11/ found that the epithelium of the vagina of Portunus forms continually a thick cuticular lining. From the above results we can suggest the cuticular origin and nature of the chitinous membrane of the egg of Portunus. The presence of calcium in this layer may support the above suggestion.

The outer attaching membrane of the egg of Portunus corresponds to the outer membrane of Homarus vulgaris egg /2/. El-Sherief et al. /5/ mentioned that the tegumental gland of the pleopods of Portunus is the principal site of formation of this membrane. The previous works on the tegumental glands record a wide range of functions performed by them. Yonge /10/ has given comprehensive account of earlier works and suggested that the tegumental glands are primarily concerned with the secretion and preservation of the epicuticle. The intimate association of these glands with the cuticle led Dennell /20/ to point out that "it is difficult to avoid the view that in crustaceans the activitiy of the tegumental glands is closely connected with

the structure of the cuticle". He suspected that if they are not concerned directly with the secretion of the cuticle, they may yet be related to the subsequent hardening by the elaboration of the oxidase involved in tanning. He found that in Carcinus maenas it secretes a polyphenol oxidase and is concerned with an aspect of cuticular development. Continuous secretory activity of the tegumental glands in the crab Callinectes sapidus was observed by Johnson /34/ throughout the molting cycle which led him to suggest that the repair of the epicuticle might be occurring continually. El-Sherief et al. /5/ added that this gland plays an obvious role during the time of spawning. It becomes highly active in this time and appears loaded with copious amount of secretion. The relationship to epicuticle production and tanning was strongly suggested /34/. In decapods, tanning increased in epicuticle where the epcuticle is subjected to wear /35/.

From the previously discussed results it can be concluded that the chemical stability of the outer membranes of the incubated egg of Portunus may be an enzymically catalyzed tanning process. In agreement with observation on other tanned structures /36/, the outer membrane of Portunus egg proved to consist of lipoproteins which are involved in the hardening process. This was also observed in the outer layers of the spermatophore of Penaeus /37/. On the other hand, the great abundance of calcium salts in the attaching membrane gives another evidence for the similarity of the egg case of Portunus and the crustacean cuticle /34/. For the function of calcium ion, Jura & George /38/ suggested its ability to chelate toxic ions and other substances diffusing into the egg shell.

The present study has emphasized the fundamental similarity of the crustacean cuticle to the outer egg case of Portunus. It, however, confirms the occurrence of phenolic tanning in both layers of the case and refers to the prime cause of hardening for phenolic tanning and calcification. Such study, also, may provide a useful idea regarding the nature of protein stabilization of the egg membranes and their relationship with the correlated tegumental glands. However, investigations on the chemical nature and the mode of stabilization of the egg case of other crustacean will be of interest.

If it is acceptable to suggest the cuticular nature of the egg case of Portunus, it is also a trial to attempt to postulate the correlation between the time of molting of mother Portunus and the time of hatching of the incubated eggs.

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POSTEMBRYONAL DEVELOPMENT OF THE TESTES IN COTTON LEAF WORM, SPODOPTERA LITTORALIS (BOISD.) (NOCTUIDAE, LEPIDOPTERA)

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During postembryonal development of males of Spodoptera littoralis the paired four-follicular larval testes undergo fusion and torsion, forming in the prepupal stage one gonad composed of eight testicular follicles. From the 6th larval till early pupal stage, the interior of the testicular follicles is divided into the following zones: 1) germarium with apical complex (an apical cell and two kinds of spermatogonia); 2) a zone, in which the single spermatogonia become surrounded by somatic cells, thus forming spermatogonial cysts; 3) a zone in which the spermatogonia inside the cysts undergo six incomplete mitotic divisions to form a syncytium of 64 spermatocytes (eupyrene spermatocytes with spherical nuclei or apyrene ones with polymorphic nuclei); 4) a zone, in which the spermatocytes transform into eupyrene or apyrene spermatids (256 per one cyst). In the mid-period of pupal stage two events occur: the apical cell in germarium degenerates and the eupyrene spermatogenesis ends. The apyrene spermatogenesis starts in the 6th larval instar and ends in the late pupa. In the late pupal and young imago testis, apyrene spermatozoa cysts form a compact layer under the gonadal wall, whereas the eupyrene cysts are loosely scattered in the central region of testicular follicles. The flagellum of the eupyrene spermatozoon is characterized by one mitochondrial derivative, by axonemal microtubules containing electron-dense material and by two kinds of appendage structures on the surface: lacinate appendages and satellite bodies. The flagellum of apyrene spermatozoon possesses two mitochondrial derivatives. It has neither extracellular appendages nor electron-dense material in microtubules. In seminal follicles the apyrene spermatozoa acquire a thick coating exhibiting periodic structure.

Keywords: Lepidoptera — testis — development — spermatogenesis

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Introduction

The present work describes the postembryonic development of testes in Spodoptera littoralis (Noctuidae) from the last instar larvae throughout the pupal stages to the young adults, i.e. comprises the period of complete metamorphosis of the moths. The research was made using light and electron microscopy and provides a basis for further ultrastructural studies with the purpose to give a detailed description of spermatogenesis in S. littoralis. The knowledge of the processes connected with the reproduction of this species, a common pest of agricultural crops, can be useful in devising a method of fighting this moth.

Materials and Methods

Animals and their maintenance

Sixth-instar larvae, prepupae, pupae and adults of the Egyptian cotton leaf worm Spodoptera littoralis were used in this study. They came from a laboratory population maintained over a number of generations under the following conditions: an artificial photoperiod LD 12:12, constant temperature 26 ^{o}C and relative humidity 65%. Larvae were fed on leaves of Taraxacum officinale. Newly emerged imagines were fed on 20% honey-water.

Light microscopy

The testes were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.4) and postfixed in 1% osmium tetroxide. After dehydration the testes were embedded in Epon 812. Semi-thin sections were stained with toluidine blue and Azur II. For studies of general morphology, the testes were fixed in Bouin's fluid, embedded in paraffin, sectioned at 6 µm and stained in Delafield's haematoxylin-eosin.

Electron microscopy

For fine structural observations the testes and seminal vesicles were fixed as for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Tesla BS 500 electron microscope.

Testes for scanning electron microscopy were prepared as previously described /12/.

Results

1) The 6th larval stage

The testes of S. littoralis lie underneath the dorsal integument of the fifth abdominal segment. In the larval stage they are paired, kidney-shaped and touch each other with their concave sides. Each testis is divided into four chambers, called testicular follicles.

The somatic envelope of the testis is composed of two layers: outer (capsular layer), enclosing the gonad, and inner (follicular layer), forming the walls of four testicular follicles. The adjoining walls of the testicular follicles form three inner septa, which separate the follicles. Numerous tracheae of various size penetrate into the envelope of the gonad and into the inner septa (they are visible in all stages of testis development (see Figs 3, 4d).

Within each testicular follicle there are zones related to the consecutive stages of germ cell development. In the germarium located right under the wall of the testis there is the apical complex (Fig. 1), made up of the centrally situated apical cell and a compact group of tear-shaped spermatogonia surroinding it. The large apical cell develops numerous processes penetrating between the gonial cells. Two types of the gonial cells can be distinguished within the apical complex: one of them has an intensely stained cytoplasm, the other (more distant from the apical cell) has light cytoplasm.

Beneath the apical complex there is a zone, where the single spermatogonia become surrounded by somatic cells, thus forming spermatogonial cysts (Fig. 1). Then the spermatogonia undergo mitotic divisions (Fig. 2e) with incomplete cytokinesis, which results in the formation of a syncytium in each cyst. As our observations show (see below), each spermatogonium in the cyst divides six times and forms a clone of 64 cells, which then transform into primary spermatocytes, connected by intercellular bridges (Fig. 2d). The spermatogonia are usually tightly packed in the cysts, while the primary spermatocytes are arranged along the wall of the cyst in a single cell layer (Fig. 1, inset). As a result, a space is left in the centre of the spermatocyte cysts, in which numerous protrusions representing growing flagella are visible (Fig. 2a).

Some spermatocyte cysts contain spermatocytes with polymorphic nuclei (Fig. 2c). These are apyerene cysts, while the remaining ones, containing typical spermatocytes with spherical nuclei, are eupyrene cysts (Fig. 2b).

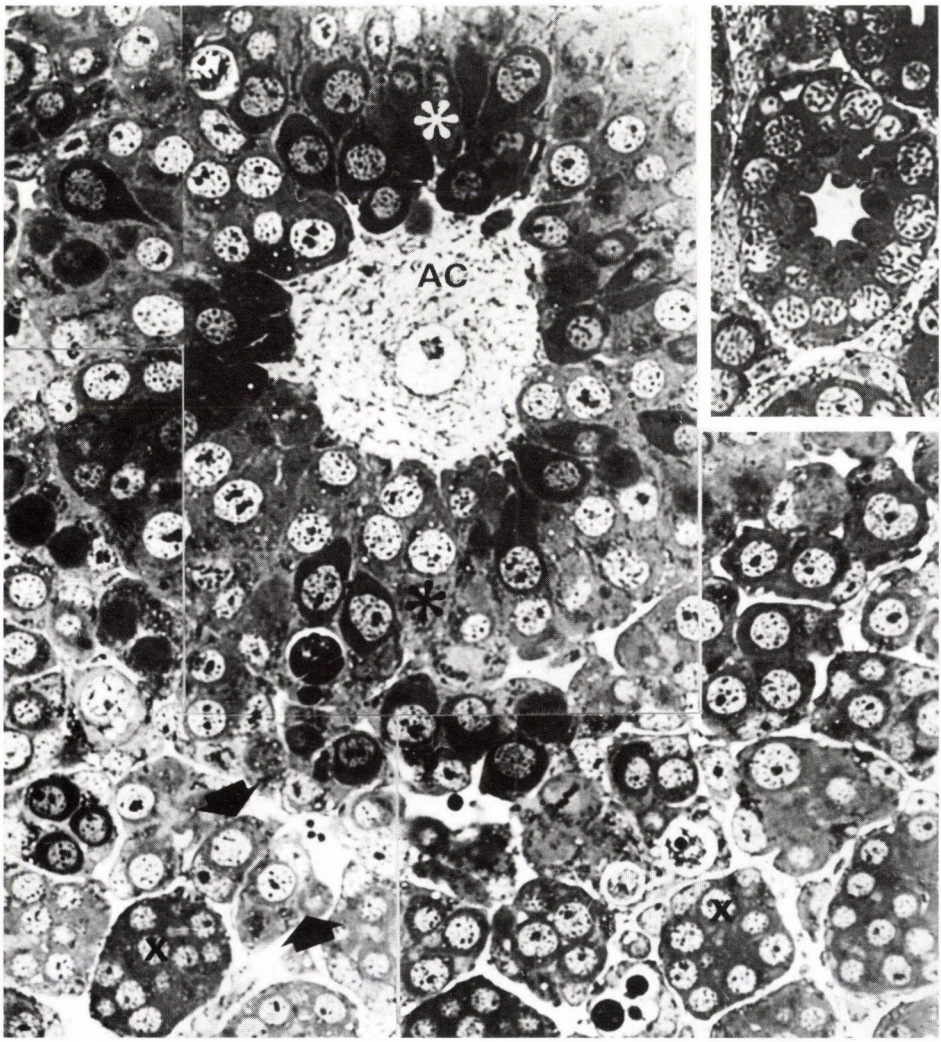

Fig. 1. The 6th instar larva. Apical complex: apical cell (AC) and zone of primary spermatogonia with light (black asterisk) and dark (white asterisk) cytoplasm. Arrows point to the region where the cysts are formed. (X) points to cysts with clone of spermatogonia after mitotic divisions. Inset shows later cyst with spermatocytes, arranged along the wall of the cyst (x960)

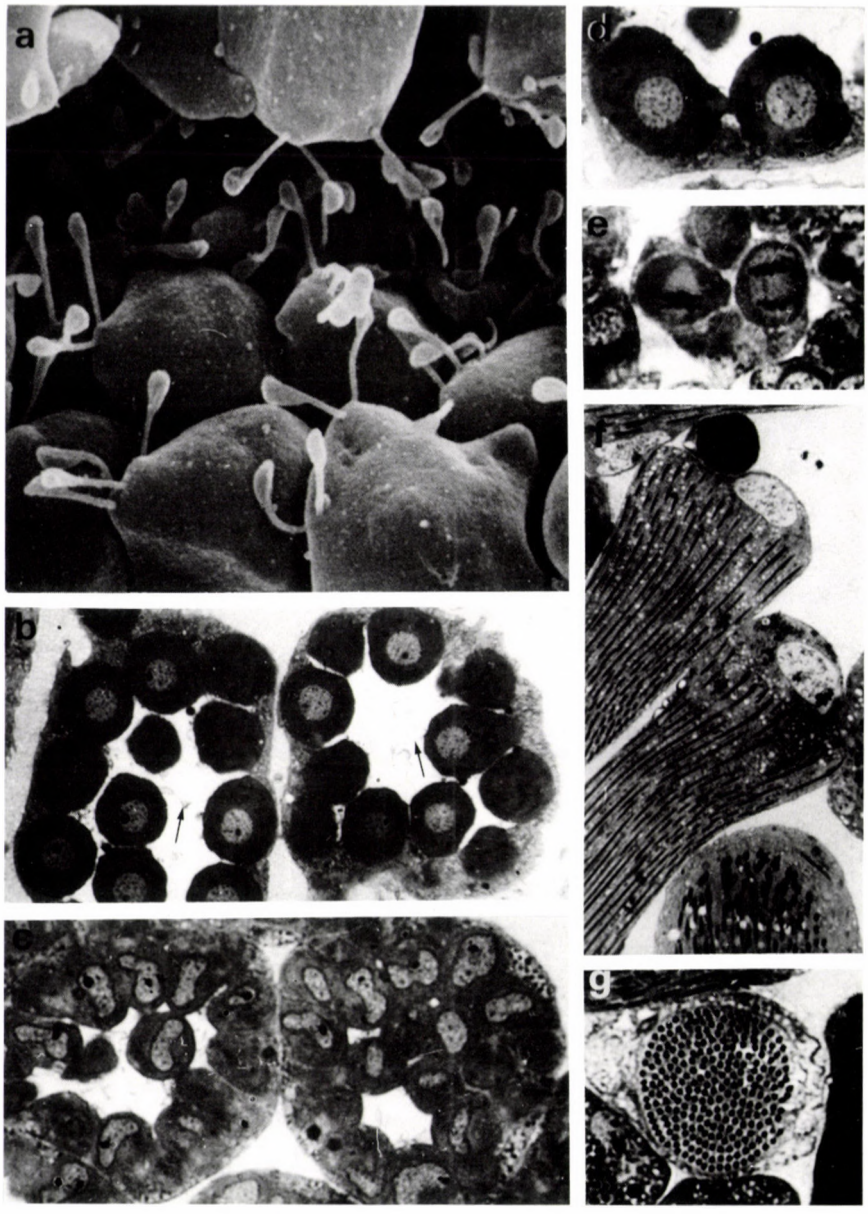

Fig. 2. a) Scanning electron micrograph of the interior of the eupyrene primary spermatocyte cyst. Numerous protrusions representing growing flagella are visible in the cyst lumen (x3000); b) Cysts containing eupyrene spermatocytes with arising flagella (arrows) (x960); c) Cysts containing apyrene spermatocytes. Note polymorphism of the spermatocyte nuclei (x1200); d) Spermatocytes joined together by the cell bridge (x1450); e) Spermatogonia during mitotic divisions (x1200); f) Two eupyrene spermatidal cysts on the longitudinal section. "Cup cyst cell" with large clear nucleus is seen at the pole of each cyst (x960); g) Cross-section of the eupyrene sperm cyst. 256 spermatozoa can be identified in this section (x1100)

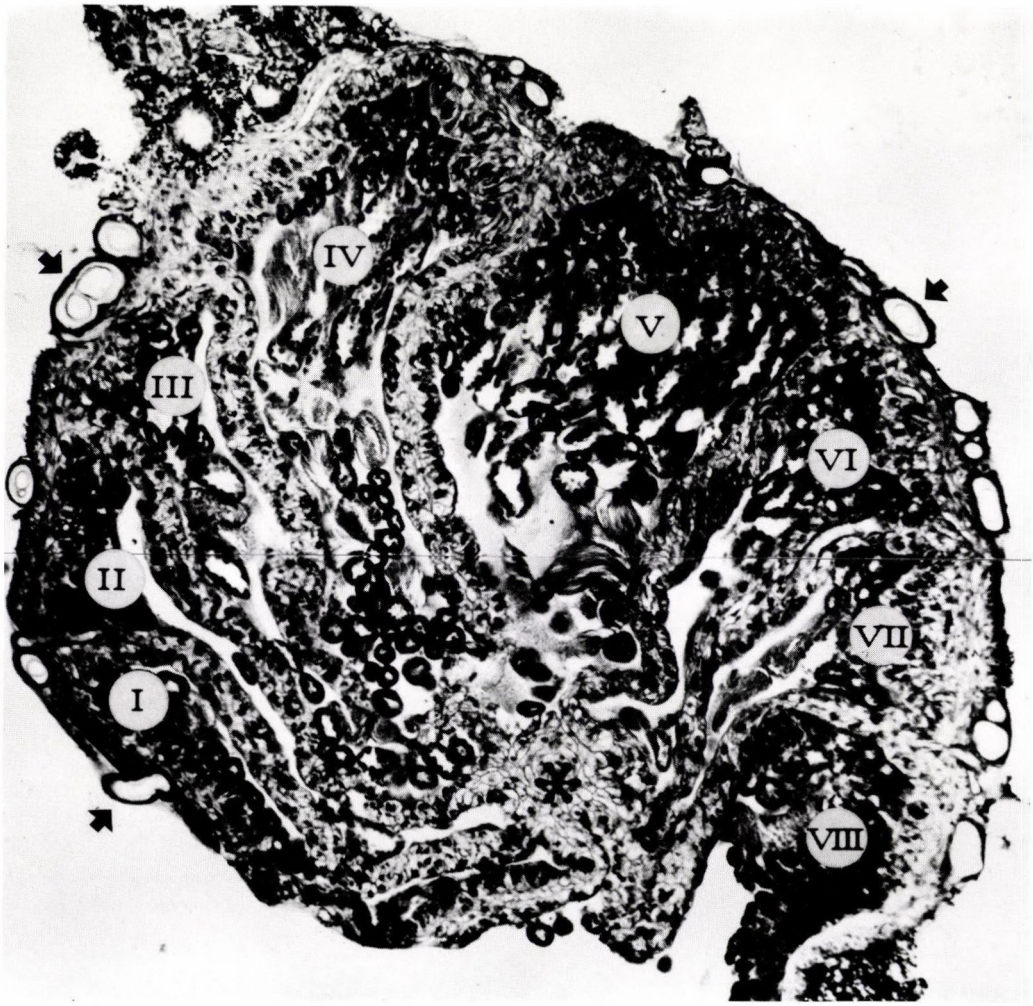

Fig. 3. Section of the prepupal testis, which consists of eight follicles (I—VIII) in result of fusion of two larval testes. Asterisk marks the place where eight interfollicular septa touch each other. This place is also a point around which the testicular follicles twist during torsion. Tracheae — arrows (x250)

In consequence of meiotic divisions the spermatocytes are transformed into spermatids. The spermatidal cysts in various stages of differentiation occupy the central and the distal part of the testicular follicle.

2) The prepupa

In the prepupa stage, which lasts about two days, the two larval testes merge forming a single testis. As a result of this process the prepupal gonad contains eight testicular follicles (Fig. 3), each of which has a separate vas efferens. On the surface of the testis there is a lighter stripe, which indicates the place of fusion of the two larval testes. In the following phase the testis undergoes the process of torsion: the testicular follicles twist around the place where the interfollicular septa touch each other (Fig. 3). It follows from the analysis of serial sections of prepupal and pupal testes that only the distal part of the gonad, i.e. that part from which the seminal ducts originate, is submitted to torsion. The interior of the testicular follicles undergoes no significant changes during the course of fusion and torsion; the follicles are still divided into zones related to the consecutive stages of the germ cell development.

3) The 8-day-old pupa

The stage of the pupa lasts two weeks. No apical cell has been found on serial sections of the testicular follicles in the 8-day-old pupa (the cell is still present in the 4-day-old pupa, but it is markedly smaller than in the larval testes). A group of spermatogonia is visible, which we consider to be the remnant of the disintegrating apical complex. Near the apical part of the testicular follicles numerous apyrene and a few eupyrene spermatocyte cysts can be observed. The lumen of the testicular follicles is filled mostly with spermatidal and spermatozoic cysts (apyrene and eupyrene). The apyrene cysts (thinner and more elongated than the eupyrene ones) have a more peripheral location. The eupyrene cysts, situated more centrally, exhibit distinct polarization, resulting from the existence of the cyst cell with a large and light nucleus, which is found at the pole of each cyst (the "cup cyst cell") (Fig. 2f). The heads of spermatids and spermatozoa are always turned in the direction of this cell.

Cross sections of the eupyrene cysts have made it possible to count the number of germ cells in one cyst (Fig. 2g). There are 256 of them in each cyst. The number proves that each definitive spermatogonium undergoes six mitotic divisions, which produce 64 primary spermatocytes. The spermatocytes are then subjected to meiotic divisions, so that a mature cyst contains 256 spermatozoa.

The spermatozoic cysts, found in this stage, appear already in the initial stages of pupal life (they are not observed in a 1-day-old pupa, but a testis of 4-day-old pupa contains them).

4) The 1-day-old imago

Only spermatidal and spermatozoic cysts are present in the lumen of testicular follicles (eupyrene spermatocyte cysts are to be observed only up the 8-day-old pupal stage; apyerene spermatocyte cysts stop to be generated between 8- and 12-day-old pupal stage). The imago gonad exhibits peripheral location of the apyrene cysts, like in the pupal testis. They form a characteristic compact layer right under the wall of the gonad (Fig. 4a). Their arrangement is more regular than that of eupyrene cysts -- they usually lie parallel to the wall of the gonad (Fig. 5a). The apyrene cells are closely packed in the cysts (Fig. 4b), more closely than the eupyrene ones (Fig. 4c).

Comparing the flagella of eupyrene and apyrene spermatozoa, one can notice considerable differences. The eupyrene spermatozoa (Fig. 5c) possess one kidney-shaped mitochondrial derivative and contain electron-dense material in nine additional axonemal microtubules. On the surface of the cell membrane of the flagellum there are two kinds of additional appendage structures. The first (the lacinate appendages) consists of radially arranged laminar structures, which in transverse sections appear to be composed of alternating electron opaque and electron lucent areas. The second (satellite body) is a moderately electron-dense structure, attached to the flagellum at the point, where the mitochondrial derivative touches the axoneme. The apyrene spermatozoa (Fig. 5b), which are characterized by two mitochondrial derivatives in the flagellum, have no lacinate or satellite appendages or any other extracellular coating material on the surface. The peripheral microtubules of flagellar axoneme never display the dense centre, which characterizes these microtubules in eupyrene sperm.

As further observations have shown, sperm cysts disintegrate in the distal part of the testicular follicles. Cyst walls disappear and the liberated spermatozoa leave the testis and pass into the ducts of the reproductive tract, migrating towards the seminal vesicle. In the semical vesicle the apyrene spermatozoa acquire a thick coating in the form of an electron-dense sheath exhibiting periodical structure (Fig. 5d).

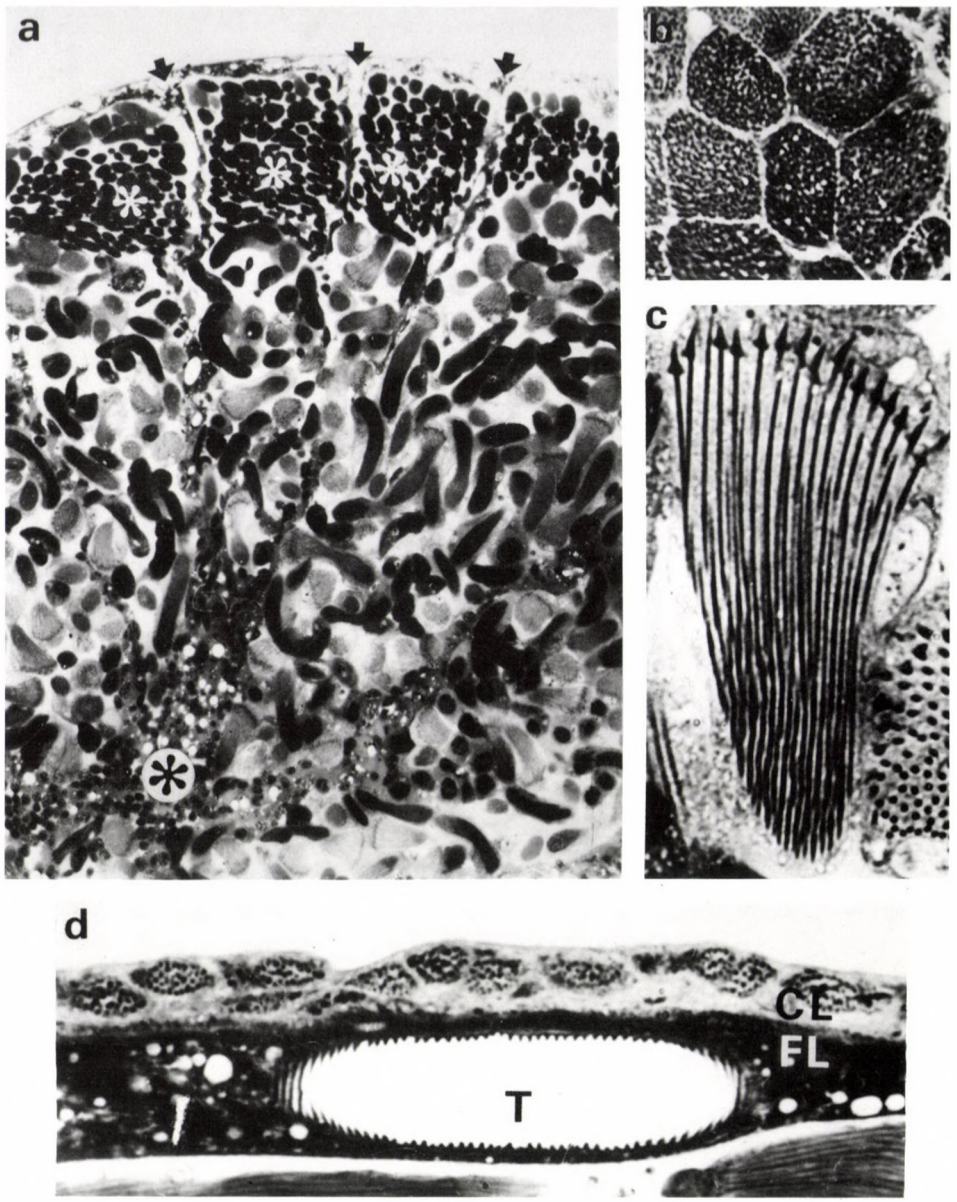

Fig. 4. a) Paraffin section of the imago testis. Interfollicular septa (arrows) are radially arranged and merge together (large asterisk). Apyrene sperm cysts (small asterisks) form compact layer right under the testis wall. Eupyrene sperm cysts are loosely scattered in the central region of testis (x510); b) Transverse section through the group of the apyrene cysts with closely packed spermatozoa (x1200); c) Longitudinal section through the eupyrene sperm cyst. Note the arrow-shaped sperm heads (x1300); d) The testicular wall of the 1-day-old imago. CL — capsular layer, FL — follicular layer, T — trachea (x1200)

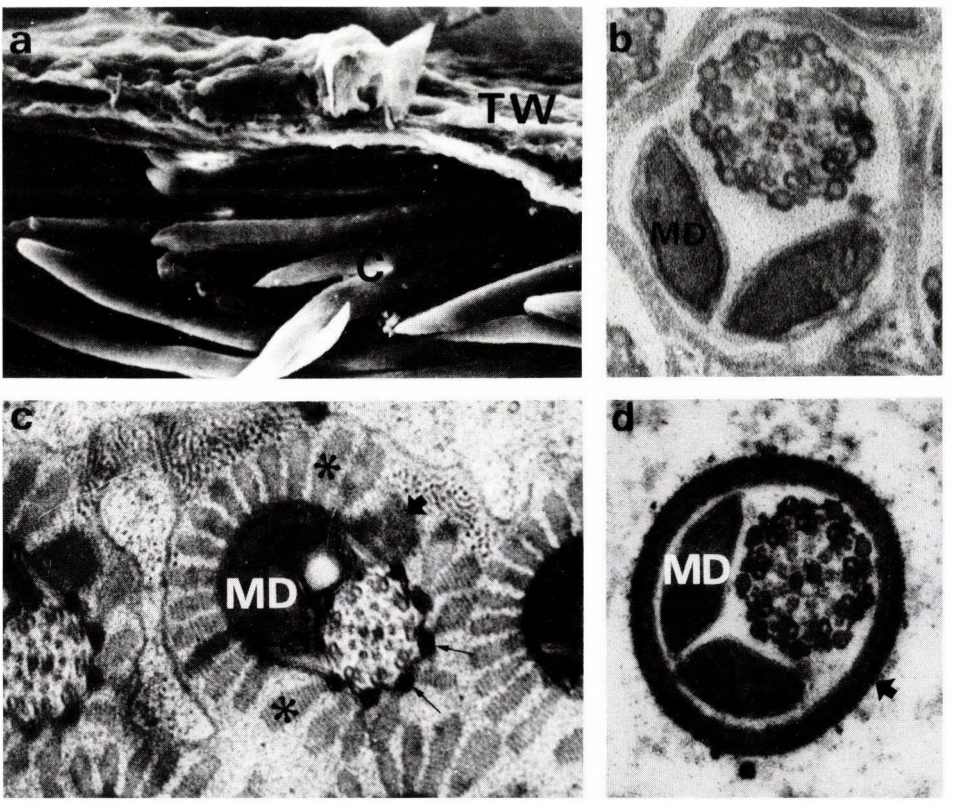

Fig. 5. a) SEM micrograph. Parallel arrangement of apyrene sperm cysts (C) beneath the testicular wall (TW) (x750); b) Transverse section of flagellum of apyrene spermatozoon. It may be distinguished from the eupyrene one by the presence of two mitochondrial derivatives (MD) and the absence of both dense material in microtubules and additional appendages on the surface. (Compare with c) (x100.000); c) Transverse section of the flagellum of eupyrene spermatozoon. Kidney-shaped mitochondrial derivative (MD), lacinate appendages (asterisks), satellite body (thick arrow) and accessory microtubules filled with electron-dense material (thin arrows) are visible (x75.000); d) Transverse section of the flagellum of apyrene spermatozoon from seminal vesicle. Note thick electron-dense sheath with periodical structure (arrow) surrounding the flagellum (x90.000)

Discussion

1. General morphology of testes

In the course of metamorphosis, the paired larval testes of S. littoralis undergo a complete fusion forming a single testis, which is submitted to the process of torsion. The mechanism and significance of testis fusion

and torsion observed in Lepidoptera have not yet been fully elucidated, but experiments have shown the inductive role of ecdysone, which is secreted by prothoracal glands /21, 22/.

The results of the present work show that only the distal part of testis in S. littoralis, from which the seminal ducts emerge, is submitted to torsion (in this part the inner septa, which separate testicular follicles, are curved). Therefore we suppose that the testis torsion is caused by the action of pressuring forces, in consequence of the elongation and translocation of seminal ducts (the walls of seminal ducts are connected with the inner septa of testis, therefore the translocation of seminal ducts can cause partial twisting of the inner septa).

In testes of S. littoralis the fusion and torsion take place in the prepupal stage, as in a number of other lepidopteran species /3, 4, 5, 14, 17, 18, 21/.

2. Structure of the testicular follicle

The interior of the testicular follicles of S. littoralis exhibits a division into the zones corresponding to different stages of germ cell development. In the larval, prepupal and early pupal stages the apical part of each testicular follicle (germarium) is occupied by the apical complex, which is made up of the centrally situated apical cell and gonial cells surrounding it.

According to Roosen-Runge /27/ the apical cell degenerates in Lepidoptera in the prepupal stage. The apical cell in germarium of S. littoralis, however, can be observed till a young pupa. But one can observe that in the pupal testis of S. littoralis the apical cell is markedly smaller than in the larval testis, which is certainly a symptom of its gradual degeneration occurring in the middle pupal stage.

Articles published so far differ considerably in identifying the stages of the gonial cells surrounding the apical cell. For example Hannah-Alava /13/ defines three types of spermatogonia in the testes of insects: the apical cell is immediately surrounded by 1) primordial (predefinitive) spermatogonia; these cells produce, 2) primary (indefinitive) spermatogonia, which, in turn, give rise to 3) secondary (definitive) spermatogonia. As our observations show, the apical complex of S. littoralis comprises at least two types of gonial cells, differing in cytoplasm stainability. In our opinion the cells containing light cytoplasm, which are more distant from

the apical cell, may represent mature (definitive?) spermatogonia (the cells will be a subject of our further ultrastructural research).

Below the apical complex there is a zone in which spermatogonial cysts are formed. After the accepted model there are two possible modes of the formation of spermatogonial cysts. In one, even a single definitive spermatogonium becomes surrounded by somatic cells /27/; in the other, spermatogonia are enveloped as a group after one or more cell divisions /1/. We observed that in S. littoralis the first mode of the formation of spermatogonial cysts occurs.

According to other authors /23, 27, 31/ each definitive spermatogonium in testis of Lepidoptera undergoes 6 mitotic divisions within the lumen of spermatogonial cysts, which results in the formation of 64 primary spermatocytes per cyst. Our observations confirm this number in S. littoralis. From the number of spermatozoa in a cyst (256) it can be concluded that six successive spermatogonial divisions take place in each cyst before meiosis and, as a result, 64 primary spermatocytes are formed.

3. Eupyrene and apyrene spermatogenesis

From numerous studies (for example: /5, 6, 7, 16, 30/) it follows that two types of spermatogenesis can be distinguished in Lepidoptera: the eupyrene spermatogenesis, which produces nucleated spermatozoa, and the apyrene spermatogenesis, producing anucleated spermatozoa. It is known that the eupyrene spermatozoa are capable of fertilization, but the functions of apyrene spermatozoa, whose nuclei disintegrate into micronuclei and are then excluded from the cell /10, 19, 31/ have not yet been fully elucidated (see /30/). We suppose that the polymorphic shape of nuclei of apyrene spermatocytes observed in S. littoralis can be interpreted as a symptom of the disintegrating processes mentioned above.

It is supposed that both kinds of spermatozoa derive from the same kind of bipotential primary spermatocytes, and that the mechanism regulating the switchover of spermatocyte commitment from eupyrene to apyrene differentiation is connected with pupation /6, 7, 20, 28/. Studies on S. littoralis have so far defined the timetable only of eupyrene spermatogenesis. According to Gelbič and Metwally /11/ and Salama et al. /29/ eupyrene spermatogenesis starts in the 4th larval instar of S. littoralis and ends in an adult testis. We have observed, however, that eupyrene spermatogenesis ends earlier, i.e. in the pupal stage. An 8-day-old pupa contains remnants of the

disintegrating apical complex and only a few eupyrene spermatocytes. In late pupal and adult testes we have observed only spermatidal and spermatozoa cysts. Our observations have shown also that mature eupyrene spermatozoa appear only in the pupa of S. littoralis, whereas according to Salama et al. /29/ they are present in the last 6th larval instar and according to Gelbič and Metwally /11/ they appear in the prepupa. The discrepancies mentioned above may have been caused by the different living conditions of specimens used in the researches (diet, photoperiod, etc.).

We observed that the switchover from the eupyrene to the apyrene kind of spermatogenesis in S. littoralis takes place in the last 6th larval instar. The end of apyrene spermatogenesis occurs in the pupal stage, which is in contrast with results obtained from other lepidopteran species, in which the apyrene spermatogenesis still continues in the imago /19, 20, 28/. As it can be noticed, apyrene spermatogenesis in S. littoralis ends later than the eupyrene spermatogenesis.

One of our interesting observations in late pupal and adult testis was that cysts with apyrene spermatids and spermatozoa formed a compact layer closely under the wall of the gonad, along its entire circumference. Such a location of apyrene cysts has not been described in Lepidoptera so far.

The structure of spermatozoon flagella in S. littoralis is on the whole similar to those of other lepidopteran species /8, 9, 15, 24, 25, 26/. We noticed, however, that the satellite bodies are structurally uniform and exhibit no substructural elements, so the term "reticular appendages", introduced by André /2/, seems inappropriate. After Riemann and Thorson /25, 26/ we prefer to use the term "satellite body", as it says nothing of the structure of the appendage. It follows from our observations that eupyrene spermatozoa of S. littoralis contain one kidney-shaped mitochondrial derivative. Most probably the situation here corresponds to that described in other species of Lepidoptera, where two unequal derivatives fuse almost completely to form a single body /15/.

The structure of eupyrene and apyrene sperm axoneme of S. littoralis is also similar to that of other Lepidoptera with only one difference -- eupyrene sperm axoneme does not contain electron-dense material in the central pair of microtubules.

Acknowledgements

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NO EFFECT OF VANADATE ON THE CENTROMERE SEPARATION SEQUENCE

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Alteration of the centromere separation sequence may lead to aneuploidy and may be an indicator of chromosome instability. The aim of this study was to examine whether this phenomenon could be influenced by a factor known to affect cell division. Human lymphocyte cultures were exposed to Na-vanadate in various concentrations and durations. As compared to controls, the inhibitory effect of vanadate manifested itself in a decrease of mitotic index values but the centromere separation sequences remained unchanged, i.e. chromosomes 2, 17-18 were the first, 1, 16 and the acrocentrics the last to separate in both the control and vanadate-treated cultures. The findings support the suggestion that the centromere separation sequence is hardly influenced by environmental factors but rather is a species specific, genetically determined phenomenon.

Keywords: Centromere separation sequence — cell division — vanadate

Introduction

The existence of a "normal" sequence of centromere separation in human mitoses has repeatedly been described /10, 14/. According to some observations this may play a role in non-disjunction causing aneuploidy: too early or too late separation may lead to trisomy or monosomy of the given chromosome /5, 9, 15/. This raised the question whether this phenomenon could be influenced by mutagenic factors. To our knowledge only a few studies have dealt with this problem so far. Singh and Miltenburger /13/ and Miltenburger

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et al. /12/ examined the effect of cyclophosphamide and isoniazid on the centromere separation sequence in Chinese hamster spermatogonia and bone marrow cells, respectively, and found no alteration after exposure to these drugs. At the same time, Bajnóczky et al. /1/ demonstrated significant changes of the centromere separation sequence in lymphocytes of in vivo prednisolone-treated infants. No increase of chromatid or chromosome gaps, breaks, rearrangement figures, dicentrics or sister chromatid exchanges were observed in these babies. This is of special importance because Bühler et al. /3/ claimed that similarly to structural aberrations and sister chromatid exchanges also premature centromere divisions are indicators of chromosome instability. A similar conclusion was drawn by Goswami et al. /7/ when examining the victims of the gas-explosion in Bhopal.

In this study we made an attempt to analyze the centromere separation sequence in human lymphocytes exposed to vanadium, a well-known cytotoxic agent /6/.

Materials and Methods

Lymphocyte cultures from a healthy 23-year-old male subject with normal karyotype were cultivated for 72 hours in McCoy 1A medium. Mitotic arrest was made by adding 0.125 μg/ml colchicin for the last 2 hours, KCl was used for hypotonic shock, and the fixed cells were stained with Giemsa.

To examine the effect of vanadate, 5% $NaVO_3$ was given to the cultures in various amounts and for various times in the following combinations: 0.51, 1.02 and 2.56 μmol/ml, respectively, each for 2.5 hours, 2.56 μmol/ml for 3 and 4 hours, 5.12 μmol/ml for 2.5 and 4 hours, and 2.56 μmol/ml for 6 hours. Since vanadate concentrations above 2.56 μmol/ml and expositions of more than 4 hours proved to be so toxic that the preparations contained no evaluable mitoses, only the lowest and highest exposures allowing normal mitoses were further investigated. Simultaneous cultures of the same blood sample not treated with vanadate served as controls.

Mitotic index was determined by counting at least 2000 cells from five different fields and expressed as number of mitoses at any stage per 1000 cells.

The sequence of centromere separation was characterized by the centromere separation index (CSI) introduced by Vig /14/. By this method a score of 0 was given to the centromeres that had not divided at all, those that had just begun separation scored 1, and those showing clear separation were scored 2. The scores obtained were pooled and divided by the elements of the group of chromosomes. These totals were then divided by the largest number in the series; thus relative values (CSI) against a given value of 1 for the earliest separating pair were gained. The higher the CSI for a chromosome, the earlier was its position in the separation sequence. In order to see the separation more clearly, the chromosomes were not banded and this meant that only the main groups and the most characteristic individual chromosomes could be identified.

Results

The findings in the three groups, i.e. low and high vanadate exposures and control, are summarized in Table 1. As shown by the figures, the mitotic indices were significantly lower in the vanadate-treated cultures than in the untreated controls. Apart from two gaps, no breaks and other structural aberrations were found in any of the slides examined.

Table 1

Mitotic index and centromere separation index (CSI) values in control lymphocyte cultures and in those of slight and intensive vanadate exposure

	Control	Vanadate-treated cultures	
		0.51 µmol/ml 2.5 h	2.56 µmol/ml 4 h
Mitotic index (1/1000)	49.5	26.0[a]	24.5[a]
CSI			
Number of mitoses	95	91	93
Chromosome or group			
1	0.11	0.15	0.08
2	1.00	0.99	1.00
3	0.47	0.51	0.47
4-5	0.61	0.60	0.58
6-X-12	0.50	0.56	0.52
13-15	0.00	0.01	0.01
16	0.02	0.08	0.03
17-18	0.97	1.00	0.93
19-20	0.45	0.50	0.44
21-22-Y	0.00	0.01	0.02

[a] $p < 0.001$ against control. The differences in CSI values of the three groups are statistically not significant.

The relative CSI values of both the slight and heavy exposures are almost identical with those of the control group. Since individual chromosomes could not be identified, only the mean values of chromosome groups are given, which demonstrate very similar tendencies of separation. As in the controls, also in the cells treated with vanadate chromosomes 2, 17-18 showed a very early division, whereas chromosomes 1, 16 and the acrocentrics were the last to separate. This sequence corresponds to the findings of several earlier studies from different laboratories.

Discussion

Vanadate is regarded as an inhibitor of cell development and division, especially of chromosomal movement /4, 8/. The decreasing mitotic index values referred to such an inhibition also in the present study.

At the same time, vanadate did not alter the sequence of centromere separation. These findings have at least two implications:

1. Different vanadium compounds may have different effects on cell metabolism and division /6/. The present data show that also Na-vanadate is toxic as far as slowing down the division of cultured lymphocytes and lowering their mitotic rate.

2. It has been shown in previous studies that varying factors of cell culture and preparation, such as temperature, medium, culture time, colchicin, hypotonic shock, and calcium, do not alter the sequence of centromere separation /2, 9/. The fact that even toxic levels of vanadate were ineffective in this respect provides further evidence for the suggestion that the centromere separation sequence is hardly influenced by environmental factors but rather is a species specific, genetically determined phenomenon.

Since our recent observation suggested a possible new aspect of out-of-phase centromere separation causing aneuploidy /11/ analysis of possible exogenous and endogenous factors influencing the separation sequence seems to be constantly needed.

Acknowledgements

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THE AGE DEPENDENCE OF GUSTATORY HABITS — A FAMILY STUDY

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The authors continued their earlier studies in the field of human food favouritism. They collected family samples from two Hungarian towns. For the comparison of food choices of adults and children a quantity called Adults' Food Preference Score (AFPS) was constructed which seemed to be suitable to discriminate the gustatory habits. A family analysis has shown that the food preferences of mothers and children are much more connected than those of fathers and children.

Keywords: Population genetics — family study — food preferences — Adults' food preference score

Introduction

The first population genetic investigations in Hungary for phenylthiocarbamide (P.T.C.) taste sensitivity evaluated by mathematical-statistical methods were carried out by Forrai and Bánkövi /1/; the first Hungarian twin studies for P.T.C. taste character and the connections among P.T.C. tasting and food choice were also published by the same authors /3, 4/.

As regards the latter investigations, it could be demonstrated by the adaptation of a Food Like/Dislike Chart (FLDC) constructed by the above authors that gustatory habits of MZ and DZ twins significantly differ from each other. The details of the concept of the "Taste Concordance Point" (TCP) was introduced /3/.

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Akadémiai Kiadó, Budapest

The adult twin pairs mentioned above were also studied for taste sensitivity to P.T.C. /4/.

In order to compare the food choices of the P.T.C. tasters and non-tasters another quantity called "Tastership Test Point" (TTP) was earlier introduced /2/. The adult twin pairs mentioned above (namely members of the MZ pairs) were also studied in this respect /4/.

As a final result of these studies it could be stated that 1) gustatory habits are presumably influenced by genetic factors; 2) P.T.C. taste character and food preferences are not completely unrelated characteristics.

It is well known also among inexpert people that children and adults prefer different foods. A good example for the food preferences of the different age groups has been shown by Judith J. Wurtman /6/. The work quoted is dealing with eating habits in a qualitative way.

Materials and Methods; Results and Discussion

First of all, one must emphasize that food preferences and eating itself are not the same. The slogan "food only nourishes when it is eaten" is true in the same way: food preferences are different from the foods which are really eaten.

The aim of the present investigation was to measure the differences among food preferences of children and adults in a quantitative way. For this purpose, populations from two Hungarian towns, Dunakeszi and Szombathely have been chosen. The choice of these towns was merely influenced by technical reasons; it can be regarded as a "random selection" from population genetic aspects.

The data originated from family surveys: in Dunakeszi 52 families (101 adults, 75 children), in Szombathely 51 families (102 adults, 92 children) were tested. Families including at least one child (aged from 6 to 19 years) were selected.

All the test persons were offered a questionnaire, a "Food Like/Dislike Chart" (see Table 1) and asked to give answers independently from one another to each of the 30 foods of their likes and dislikes: "I like it", "I dislike it", "I cannot decide". Thus no foods were actually applied to taste. The FLDC was constructed on the basis of former experiences /2, 3, 4/.

Table 1

Food Like/Dislike Chart for 30 different foods and drinks

Anchovy paste	Honey
Asparagus soup	Horseradish
Bacon	Kale
Beans (vegetable dish)	"Körözött" (spiced sheep-cheese with butter)
Beer	Mayonnaise
Beetroot salad	Mushrooms
Bitter chocolate	Poppy
Brawn	Pumpkin (vegetable dish)
Brown bread	Sardine
Carrots (vegetable dish)	Small salty cakes
Cheeses	Smoked tongue
Coffee with milk	Spinach
Ewe-cheese (curded)	Sweets
Fish	Vinegar
French beans (vegetable dish)	
Grape-fruit	

A contingency test was applied relating to the connection between "adultness" and food preferences for each element of the reduced FLDC. The scheme of the contingency table is shown in Table 2; N(k) means the number of definite answers, i.e., the number of all test persons minus those who declared their uncertainty by "I cannot decide" given for the k-th food or drink (k = 1, 2,...,30).

Table 2

Scheme of the contingency table for the k-th food/drink

Answer	Adult	Child	Total
"I like it"	N_{11} (k)	N_{12} (k)	N_1 (k)
"I dislike it"	N_{21} (k)	N_{22} (k)	N_2 (k)
Total	N_1 (k)	N_2 (k)	N (k)

To discriminate adults and children on the basis of their food likes/dislikes a quantity called Adults' Food Preference Score (AFPS) was applied. The AFPS was determined as a function of the frequencies of the outcomes within both adults and children. Namely, for the k-th element of the FLDC (k = 1,2,...,30) the AFPS (k) was defined as follows:

$$AFPS(k) = \begin{cases} P_1(k), & \text{for the answer: "I like it",} \\ 0, & \text{for the answer: "I cannot decide",} \\ P_2(k), & \text{for the answer: "I dislike it",} \end{cases}$$

where

$$P_j(k) = Q_j(k) - C(k),$$

$$Q_j(k) = c \log \frac{N_{j1}(k)}{N_{j2}(k)} \qquad (c > 0),$$

$$C(k) = \frac{1}{N(K)} (Q_1(k)N_1(k) + Q_2(k)N_2(k),$$

$$(j = 1,2;\ k = 1,2,\ldots,30).$$

The role of the constant C(k) consists of making the average AFPS(k) (with respect to the test persons considered) equal to zero for any food (k = 1,2, ...,30), i.e. the equalities

$$P_1(k)N_1(k) + P_2(k)N_2(k) = 0 \qquad (k = 1,2,\ldots,30)$$

obviously hold. The value of c and the base of the logarithm influence only the range of the point scale and can be fixed arbitrarily; in our computations c = 1000 and the logarithm of base 10 were used, and the AFPS(k) values were rounded to integers. A positive sign of $P_1(k)$ indicates that the k-th food was liked better by the adults than by the children.

The AFPS(k) values were calculated for each food and drink of the FLDC. On the basis of the results the FLDC was reduced: the "best discriminating" 24 foods and drinks were retained and renumbered according to the descending order of χ^2-values, produced by the contingency test.

The AFPS(k) values for this reduced FLDC are contained in Table 3. The AFPS of a test person was defined by the sum of AFPS(k) values corresponding to the answers given by the test person in respect of each element of the reduced FLDC, i.e.

$$AFPS = \sum_{k=1}^{24} AFPS(k).$$

Consequently, the value of the AFPS may be regarded as an estimator of the "adult" or "child" character of a person, on the basis of the reduced FLDC.

Table 3

Adults' Food Preference Score for foods and drinks of the reduced FLDC

k	Name of food/drink	P_1 (k)	P_2 (k)	χ^2 (k)	$100 \times \{Prob(\chi_1^2 > \chi^2 (k))\}$
1	Beer	657	-731	105.71	0.00
2	Sweets	-435	709	61.64	0.00
3	Brawn	247	-455	40.75	0.00
4	Mushrooms	122	-673	32.48	0.00
5	Horseradish	121	-605	29.36	0.00
6	Smoked tongue	209	-284	17.40	0.01
7	Beetroot salad	110	-314	13.55	0.05
8	Grape-fruit	-154	243	12.08	0.09
9	Honey	-120	320	11.15	0.13
10	Pumpkin (vegetable dish)	118	-245	10.98	0.14
11	Carrots (vegetable dish)	-304	77	9.11	0.30
12	Beans (vegetable dish)	56	-350	8.06	0.49
13	Bacon	79	-258	7.98	0.51
14	Vinegar	88	-222	7.49	0.64
15	French beans (vegetable dish)	64	-281	7.26	0.72
16	"Körözött"	64	-281	7.08	0.79
17	Sardine	101	-176	6.55	1.03
18	Mayonnaise	80	-161	4.79	2.71
19	Ewe-cheese (curded)	103	-111	3.79	4.88
20	Coffee with milk	-70	162	3.76	4.96
21	Poppy	-57	195	3.55	5.64
22	Asparagus soup	173	-59	2.07	14.64
23	Kale	45	-121	2.03	15.08
24	Cheeses	19	-255	1.97	15.72

The above described method was used in several steps. At first, the two populations were investigated separately. On the basis of a preliminary analysis -- as similar tendencies were found in both towns -- the samples were joined; this step seemed to be important because of the moderate size of the samples.

The joined sample consisting of 104 children (aged 6-14 years), 63 adolescents (15-19) and 203 adults (above 24 years) was analyzed in two versions. In Version 1 the children and the adolescents were joined. As in this group the correlation between age and AFPS proved to be significant ($R = 0.185$, $p < 0.017$), the AFPS values were recalculated by neglecting the data for adolescents (Version 2). In Table 3 and Table 4 the results of

Table 4

Frequency distributions of test persons according to AFPS

	Children	Adolescents	Adults
below -5400	—	—	—
-5400 — -5000	1	—	—
-5000 — -4600	1	—	—
-4600 — -4200	2	1	—
-4200 — -3800	6	—	—
-3800 — -3400	6	1	—
-3400 — -3000	8	2	1
-3000 — -2600	7	4	1
-2600 — -2200	15	6	—
-2200 — -1800	9	9	5
-1800 — -1400	11	5	6
-1400 — -1000	10	5	5
-1000 — -600	7	6	10
-600 — -200	8	4	18
-200 — 200	6	8	14
200 — 600	3	4	15
600 — 1000	3	2	18
1000 — 1400	1	4	31
1400 — 1800	—	2	23
1800 — 2200	—	—	13
2200 — 2600	—	—	15
2600 — 3000	—	—	13
3000 — 3400	—	—	7
3400 — 3800	—	—	5
3800 — 4200	—	—	3
above 4200	—	—	—
Total	104	63	203
Mean	-1902	-1073	976
Median	-1998	-1105	1143
Std. deviation	1394	1379	1442

Version 2 (104 children versus 203 adults) are contained. In Table 4 also the frequency distribution of AFPS for the adolescents is shwon (although the adolescents did not take part in the formation of AFPS values). It must be noted, however, that the AFPS results were fairly similar for Version 1 and Version 2.

It can be seen from Table 4 that the AFPS values have a fairly strong discriminating character between children and adults, namely low AFPS values referred mostly to children while high ones mostly to adults. The frequency distribution of adolescents is placed between those of children and adults, being much nearer to the former.

P.T.C. taste sensitivity and food preferences were also compared in both adults and children. Connections found in the present sample were weaker than those in the earlier works of the authors. In spite of this fact the P.T.C. taste character might have been a source of inhomogeneity in the formation of the AFPS values. Thus, the mean AFPS values of tasters and non-tasters were calculated (both in adults and children). In both cases differences of about 200 units were found which can be neglected as compared to the difference of the mean AFPS values of adults and children (being 2878 units, see Table 4).

The AFPS values were applied to testing the connections of gustatory habits within families, namely a comparison of the AFPS values of parents with those of their children (including adolescents) was made. The correlation between mothers and children proved to differ significantly from zero ($R = 0.22$, $p < 0.0016$), while for the case of fathers and children an uncertain result was found ($R = 0.096$, $p > 0.22$).

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BOOK REVIEWS

HUMAN ETHOLOGY

I. Eibl-Eibesfeldt

Aldine de Gruyter, New York (1989), 848 pages, US $ 69.95

In the last decades one could learn several opinions and definitions about the human behaviour, including such a drafting: Human ethology is a speculation about the place of Homo sapiens in nature. Human ethology is a dynamically developing branch of science which has six Nobel Prize winners. The author dedicated his book to one of them, to his "beloved friend and teacher, Konrad Lorenz".

In his book the author, one of the most outstanding human ethologists of our age, gives a nearly complete overview on the subject, based on his four-year field studies. He could use his earlier zoological observational methods and could build up his comparative developmental social psychology. He was able to test some hypotheses of evolutionary biology empirically, using human organisms. He carried out his field studies in the last two decades in the Australo-Pacific region, in Southern Africa and in South America (where he produced ca. 200 km of 16 mm films).

This imposing book, which has been published in the series "Foundations of Human Behavior", contains 10 chapters, within 49 sections. Each chapter begins with a shorter or longer treatise about the item and the author gives appropriate examples, based mostly on his own researches. There are a lot of citations, originated from the author's scientific predecessors, and a large number of excellent photos, real masterpieces of work. Each section finishes with a summary.

The reviewer (who is a human biologist) appreciates the section of sex-typical behaviour and gender roles which is a good overview of the evidence from both human and non-human primate studies. Similarly, the immense quantity of photos presenting a large variety of Homo sapiens living in the above-mentioned regions of the world, is a pleasure for a European physical anthropologist. It is the author's merit that he calls our attention to such connections which have never been described.

On the other hand, it is necessary to remark that this book is based on the author's earlier monographs, and several important and successful authors of the last decade and their works, results are not mentioned. The works from the German language literature are abundantly cited, however, others are represented modestly (e.g. E. O. Wilson only with 4 works). The book has a very large Bibliography of books and papers (68 pages!) and films, as well as a detailed Author Index and Subject Index.

It is hard to decide: is this book a research monograph or a textbook. In the reviewer's opinion, Professor Eibl-Eibesfeldt gave a lucky alloy of these two, it is a detailed synthesis of the human ethology. The elegant getup of the book praises Aldine de Gruyter Publishers.

O. G. Eiben
(Budapest)

Akadémiai Kiadó, Budapest

ALLGEMEINE BIOLOGIE

Eike Libbert

7th edition. (UTB) Gustav Fischer Verlag, Jena (1991), 533 pages
208 figures, 15 tables. DM 34.80

Written as a comprehensive textbook for undergraduates (students) of biology and medicine, the book contains 11 chapters in a well-ordered arrangement with up-to-date information on general biology, physiology, biochemistry, microbiology, genetics, cytology, ethology and ecology. The latter deals especially with a chapter of man's interaction with the environment. Every chapter of this book, written by specialists of the topic, is well understandable, the contents are well explained and illustrated by 208 good quality and instructive figures and 15 tables. References are organized according to the chapters of the book, giving a comprehensive list of the most important additional textbooks of the special disciplines. A detailed index helps the reader for better orientation and search of special biological items. A synopsis of the plant and animal kingdom is added at the end of the book. Especially beginners will appreciate this short introduction to general biology.

J. Nemcsók
(Szeged)

DRUGS OF ABUSE, IMMUNITY and IMMUNODEFICIENCY

(Advances in Experimental Medicine and Biology, Vol. 288)

Ed. H. Friedman, S. Specter and T. W. Klein. Plenum Press (1991),
xii + 317 pages

This volume is based on the program of the International Conference on Drugs of Abuse, Immunity and Immunodeficiency, held in Clearwater Beach, Fla. December 13–15, 1989. The volume begins with 2 reviews, one on the subject of immunopharmacology and immunotoxicology, the other on the neuroimmunopharmacologic effects of drugs of abuse, giving a good background of these subjects for the reader. The volume is then divided into groups of chapters dealing with marijuana, cocaine, opiates, alcohol and various drugs. These are then generally subdivided into human and animal studies.

The section on marijuana begins with chapters describing immune deficiency in marijuana smokers, and a chapter reporting the association of marijuana smoking and cancer. The section continues with studies involving the effects of THC (delta-9-tetrahydrocannabinol) on cultured human leukocytes and contains reports on the modulation by the drug of natural killer function, neutrophil function, and secretion of cytokines by human leukocytes. The human studies are followed by animal models in marijuana research.

The next section of the volume deals with the area of immunomodulation by cocaine, reporting on the disturbances of human peripheral T cell phenotypes in cocaine and heroin addicts, and demonstrating that cocaine can directly modulate the function of human and mouse lymphocytes in cultures.

The section on opiates begins with a review on immune alteration associated with heroin abuse and methadone maintenance, followed by chapters on the influence of opiates on human leukocytes in vitro. The rest of the

section involves animal studies on the influence of opiates and their analogs on lymphocyte proliferation and the interaction of endorphins with lymphocytes.

The effect of alcohol on the immune response of experimental animals is discussed in the next chapters, starting again with a review of the subject followed by studies on the effect of prenatal alcohol exposure on murine lymphocyte populations. The other two chapters in this section deal with the influence of alcohol on leukocyte host defences in the lung and liver. The final section deals with putative immunomodulators ranging from isobutyl nitrite to the herpes virus.

The studies reported here clearly show that drugs of abuse modulate the function of the immune system. This volume should be of interest to immunologists, pharmacologists, toxicologists and public health workers who rehabilitate drug abusers.

Anna Bíró
(Göd)

ZOOLOGIE (ZOOLOGY)

H.-A. Freye, L. Kämpfe and G.-A. Biewald

9th edition. Gustav Fischer Verlag, Jena (1991), 605 pages
169 figures, 38 tables, DM 36.80

The book as a member of the series of university pocket books (UTB 1657) represents a newly edited, enlarged version of the original book, "Zoology", first published about 30 years ago, in 1963. It is an important handbook for those who are involved in teaching or studying zoology, and who know German. This 9th edition deals indeed, in a comprised form, with almost all the important fields and aspects of zoology, and includes also new and up-to-date chapters, dealing with the relationship of animals to their environment, and to humans.

The book consists of 10 chapters, all well structured and with a number of informative figures of good quality. Chapter 1 deals with the position of zoology within natural sciences; Chapter 2 is devoted to cytology and histology; Chapter 3 contains genetics; Chapter 4 deals with reproduction and development; Chapter 5 describes the evolution of the animal kingdom; Chapter 6 is a brief summary of basic zoological taxonomy; Chapter 7 is devoted to comparative physiology; Chapter 8 deals with the biology of behaviour; Chapter 9 deals with the position and relationship of animals in and to their environment, and finally Chapter 10 deals with the relationship of animals to humans.

K. Elekes
(Tihany)

MICROBIAL AUTOREGULATORS

A. S. Khokhlov

Harwood Academic Publishers, Chur—Reading—Paris—Philadelphia—Tokyo—Melbourne (1991), 450 pages

It was not easy to be a scientist in the so-called "socialist" countries. The original Russian version of this book was published in 1988, there was already a two-three-year gap between writing the monograph and the Russian publication. At last in 1991, it was translated (by E. M. Suscenko) and published in English. (It is of great importance that the English edition is not only a translation because to each chapter an addendum is attached with references to publications until 1989.) It is still a thought provoking book, an original approach. This is a very special and pioneering work. I wish to emphasize: it is really a new book, not a combination of chapters from sources by other authors. The author Prof. A. S. Khokhlov is a well known investigator of the chemistry of natural products, mainly antibiotics. In the late sixties he discovered factor A, the "autoregulating factor" in Streptomyces griseus, the first chemically known low molecular weight compound which induced sporulation (and streptomycin production) in some Streptomyces asporogenus, streptomycin non-producing mutants. The main merit of this book is that the author, starting with his own discovery, collected facts from a sea of publications sorting out their connections to the phenomenon of autoregulation of life processes by small molecules in microorganisms. The result is most stimulating. It turns out that there are many different biological phenomena, like spore production in prokaryotes, sexual differentiation in yeasts, antibiotic production in prokaryotes and eukaryotes, morphogenesis, etc., that are all regulated by autoregulatory molecules which are chemically as diverse as are the biological phenomena they produce.

Studying the biological role of factor A and other compounds which affect cytomorphological development, the author realized that there was an analogy between the "autobioregulators" of higher organisms and the metabolites which appeared during cultivation as mediators of interaction of different cells or parts of the same colony.

What is an autoregulator? It does not comprise compounds of similar structure, understandably, because it is the biological phenomenon that is common: interaction of cells which was the basis of selecting the chemical compounds by Prof. Khokhlov. Interactions in microbial systems are of different nature that is reflected in the 16 chapters of the monograph. Sex regulators of Streptomyces faecalis (dealing with mating type regulators), autoregulators from Vibrio fischeri, Cosynthetic Factor I, Factor C, Factor A and Analogous Lactone Type Autobioregulators from Actinomycetes, Factor B, Pamamycin and Other Autoregulators of Streptomyces alboniger, Anteridiol and Dehydro-oogonioles, Sirenines, Trisporic Acid and Related Regulators of Mucorales, Sex Pheromones (Hormones) of Yeasts (Saccharomycetes), Lipopeptide Sexual Hormones (Pheromones), Sclerosporines and Other Sporulation Regulators from Sclerotinia fructicola, Autoregulators of Cellular Slime Moulds (Acrasiomycetes), Effect of Antibiotics upon the Organisms which Produce the Same, Microbial Autoregulators Not Yet Investigated in Detail.

The difficulty in covering the vast area of biological phenomena connected to the one common object — autoregulators — is not felt. Indeed, the book is easy to read, clearly and concisely written, and the English translation is correct.

Not only are facts described but the contradictory results are also critically discussed. The author's vantage point is that he is a chemist and mainly deals with the purity of the natural products and the structure of the new compounds and less with the genetics and metabolic connections of the problems described in the book.

The generality of the biological role of an autoregulator is often questioned throughout the book because a marker, e.g. spore formation, may ensue in some mutants even in the absence of the autoregulator that normally is necessary for spore production in the wild type strain.

I think that autoregulators are signal molecules which trigger evolutionary fixed metabolic pathways. In the absence of specific signals non-specific factors may trigger (perhaps less efficiently) the same pathway. Such results do not contradict the significance of autoregulators in natural regulatory processes.

I highly recommend this monograph to microbiologists, geneticists, biochemists, developmental biologists, etc. worth reading because it evokes further thinking and research.

G. Szabó
(Debrecen)

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IMRE TÖRŐ

(1990—1993)

A leading personality of the Hungarian biological sciences, Imre Törő, passed on 27th September 1993, one day before his 93rd birthday. His life was closely bound to the twentieth century with its two world wars, ups-and-downs of the Hungarian history. He was born in 1900 in Debrecen, a prominent and large city in the Eastern part of Hungary, but his family originated from Nagyvárad, a city with rich cultural traditions in Transylvania. He spent his highschool years in the Debrecen Calvinist College, a highly appreciated school in the country. Partly as a result of the human tragedies during World War I, he decided to become a doctor. He started his medical studies at the Medical Faculty of the Péter Pázmány University in Budapest and continued them at the Debrecen University after the Medical Faculty had been established. Following his graduation in 1926, he became member of the Department of Anatomy and Biology at this university and decided to devote his life to basic research and teaching. His professor at that time was

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Tivadar Huzella, an internationally known and recognized scientist and pioneer of experimental biology of his time. The thinking of the young Törő was greatly influenced by the modern views of his master and he early became interested in the in vitro behavior of cells as well as in developmental processes. His study trips at the Department of Biology in Berlin-Dahlem (1929—1930), at the Department of Anatomy of the Columbia University in New York, as well as in the Marine Biological Laboratory in Woods Hole (1936—1938) under the leadership of famous scientists like O. Mangold, A. Fischer, Ch. Goss, G. Harrison and R. Chambers taught him how pure cell cultures can be isolated and later confronted with each other to study the interaction of tissues and the organisatory factors during ontogenesis. His interest in embryonic development and developmental mechanics prompted him to publish a comprehensive textbook on embryology.

He became chairman of the Department of Anatomy at the Debrecen University in 1947 and member of the Hungarian Academy of Sciences in 1946. In 1950 he was invited to be chairman of the Department of Histology and Embryology of the Budapest University of Medicine (later Semmelweis University) where he remained until his retirement in 1971. The department under his leadership played a prominent role in introducing and spreading modern methodical approaches like tissue culture, microcinematography of cells in culture, histo- and cytochemistry and electron microscopy. His main field of interest was the histophysiology of the reticulo-endothelial system which early led him to the investigation of the thymus. Indeed, he was among the first ones to discover the important role of the thymus in immune defense mechanisms. Long before the discovery of the T and B lymphocytes, he assumed that the lymphocytes of the thymus ("thymocytes") differ from ordinary lmyphocytes and furnished histochemical evidence for this. Observing the interactions of the thymic cells in culture, he early discovered the nursing role of the thymus epithelial cells for lymphocytes.

Imre Törő played a leading role in development of biological sciences in Hungary. The introduction of biology in the teaching of medical students was one of his many achievements. Ten thousands of medical students used his textbooks on histology and embryology in their studies. He greatly contributed to the foundation of the Biological Section of the Hungarian Academy of Sciences in the fifties and to the revitalization and development of the Hungarian Biological Society of which he was president during the sixties. He played a prominent role in the foundation of the Society of Hungarian Anatomists, Histologists and Embryologists in 1966 of which he was

elected president (1966—1971). The sections Histochemistry as well as Embryology and Teratology of this society were also initiated by him. He participated in establishing the Institute of Experimental Medicine of the Hungarian Academy of Sciences and became the director of its Department of Morphology for many years. His outstanding organisatory abilities were acknowledged by electing him the Rector of the Semmelweis University (1961—1964). His name was closely connected with the foundation and editorial work of the journals Acta Biologica Hungarica and Acta Morphologica Hungarica (president of the Editorial Board). His activities were honored by many national and international awards, honorary memberships in national academies and universities abroad.

The history of biological sciences in Hungary could not be written without the name of Imre Törő. Hardly any branch of biology could be found in Hungary where he had not left traces of his active and outstanding personality. Recollecting at his 80th birthday he wrote (Acta Biol. Hung. 31, p. 12, 1980): "I had more than 50 years at my disposal to accomplish my endeavours. I think that, as far as allowed by historical events, I have made good use of this rather long time and have been of service to my country, my people and fellowmen." We thank him for the immense work he did for the cause of Hungarian biology and will never forget him.

Pál Röhlich

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A PHARMACOLOGICAL DISCRIMINATION OF TWO BEHAVIORAL FORMS OF THE PARADISE FISH (MACROPODUS OPERCULARIS)

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Intracranial injections of 5 μg/fish (equivalent to 2 nmol/g) of the dopamine agonist Apomorphine into the paradise fish brain considerably decrease the occurrence of escape behavior with a parallel increase of time spent in swimming. All other elements of the behavioral repertoire are unaffected. The simultaneous administration of 0.5 μg/fish (equivalent to 0.15 nmol/g) of the dopamine antagonist Pimozide abolishes this effect. In a second experiment a dose dependent decrease in escape behavior was obtained with a parallel increase of swimming. On the bases of these pharmacological data it is concluded that the escape and the swimming reactions are truly independent units but their regulation might be closely related.

Keywords: Ethology — behavior — behavioral units — orientation — paradise fish — Apomorphine

Introduction

In previous experiments /4/ it was found that various interactions of the paradise fish with its environment could be characterized by a system of behavioral units defined on the ground of body orientation and movements. It was also found that the distribution of these units is strongly influenced by the local environment /5/. In an unfamiliar restricted place the predominant form of activity was escaping (ESC), i.e. the paradise fish tried to avoid that place by attempting to swim through the glass walls. After some hours this reaction was habituated and the fish showed normal

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swimming (SWI) instead /6/. That swimming was substituted by approach (APR) and orientation (ORI) in the presence of a harmless fish species /2/ or by fin erecting display (DIP) nearby an attacking pike /1/. The proportion of most behavioral forms could have been altered by operant conditioning although some constraints on learning were also observed, e.g. it was more easy to increase the occurrence of that avoidance element being spontaneously elicited by a given type of environment than those ones most characteristic in other type of environment /3/.

When swimming (SWI) and escape (ESC) behaviors were considered both behavioral units were expressed in normal or fast swimming, however swimming was not characterized by oriented behaviors while escape may be considered as an attempt to swim "through" the transparent wall of the aquarium. Transparency of the wall is a necessary condition for performing ESC because covered walls inhibited it /6/. The presence of a conspecific outside the wall draws the paradise fish in proximity of that wall in an attempt to escape. In the presence of a predator ESC was performed by reaching the opposite wall. In spite of these differences in orientation the distinction between the two behavioral units could be questioned. ESC can be considered as a form of SWI interrupted by the transparent wall. So the independent nature of these units needs further proof.

Various drugs cause changes in the movement patterns of animals /10/. Apomorphine, for example, initiates stereotyped movements in mammals /11/, decreases aggressive behavior and increases swimming activity in fish /8/. Therefore it is a likely candidate to differentiate between ESC and SWI if their regulations are really different. The aim of the present experiments was to examine the effect of Apomorphine on the behavior units of paradise fish.

Material and Methods

Experimentally naive males of an outbred population of paradise fish (Macropodus opercularis L.) served as subjects. The fish were bred in our laboratory, weighted 7.1 $\pm$ 0.6 g and were 180-210 days old at the time of the experiments.

The 80 l glass aquaria in which they were housed in groups of thirty were well filtered and the temperature was held constant (28 ^{o}C). Each unit contained waterplants (Hygrophila polisperma) planted in clay pots. The animals were fed daily on specially prepared fish food. Two days before the experiments the fish were housed individually in 6 l filtered aquaria containing a piece of waterplant.

In the first experiment the following five experimental groups, each containing 14 individuals, were used:

Control (CONT), without any injections;

Apomorphine (APO), injected with Apomorphine (Sigma Inc.); at a dose of 5 µg/fish (equivalent to 2 nmol/g fish) administered intracranially (i.c.);

Pimozide (PIM), injected i.c. with Pimozide at a dose of 0.5 µg/fish (equivalent to 0.15 nmol/g fish);

Apomorphine + Pimozide (A+P), injected i.c. with 5 µg Apomorphine and 0.5 ug Pimozide/fish; and

Saline (SAL), injected with 5 µl of 0.65% sodium chloride intracranially.

In a second experiment 60 paradise fish divided into six equal groups were used. The dose dependent effect of Apomorphine was studied by injecting different doses of 0, 0.2, 0.5, 1.0, 2.0 and 4.0 µg/g fish body weight, respectively. The drug was administered intraperitoneally to achieve a prolonged constant reaction level.

Before any injections a Finquel (Ayerst Laboratory Inc.) bath (50 mg/l) was used for 1 minute for anesthesia in both experiments, until all movements (including gill-rakers) stopped. Ethological observations were made 30 minutes after injections.

For observations the fish were individually housed in a 20x20x20 cm transparent aquarium one by one for five minutes. In both experiments the behavioral units were recorded behind a green plastic screen on a small computer (Atari 400) which was programmed to accept keyboard inputs as codes for behavioral units (for details see /9/). Twenty-five earlier defined uncorrelated /4/ units were measured to characterize the behavior of paradise fish. The time percentages were calculated and data with group means higher than 1 t% were analyzed by one-way ANOVA and post-hoc Duncan multiple range tests in cases of significant differences. These units were:

Swimming (SWI) is common swimming without any specific spatial orientation; performed by normal or fast movements with all fins;

Escaping (ESC) is represented by swimming to and for along the sides the aquarium, the fish being perpendicularly oriented in relation to the glass side, the mouth frequently touching or biting the glass;

Moving (MOV) is a slow change in body position;

Air-gulping (A-G) is a characteristic anabantoid reaction, the fish swims to the surface and gulps air (being a single act it was measured in its frequency/min);

Standing in three different positions: Standing under the surface (S-S); Hanging in midwater (HIM); and Resting on the ground (RES);

Freezing (FRZ) reaction: all fins are motionless, only the eyes and the gill rakers are moving.

All other behavioral forms were sporadic hence were omitted from the analysis.

Results and Discussion

In the first experiment the paradise fish were placed in a novel restricted aquarium and they showed a lot of escape reaction. The performance of ESC (Fig. 1a) was highly inhibited by Apomorphine, a dopaminergic agonist. One-way ANOVA showed that this decrease was significant

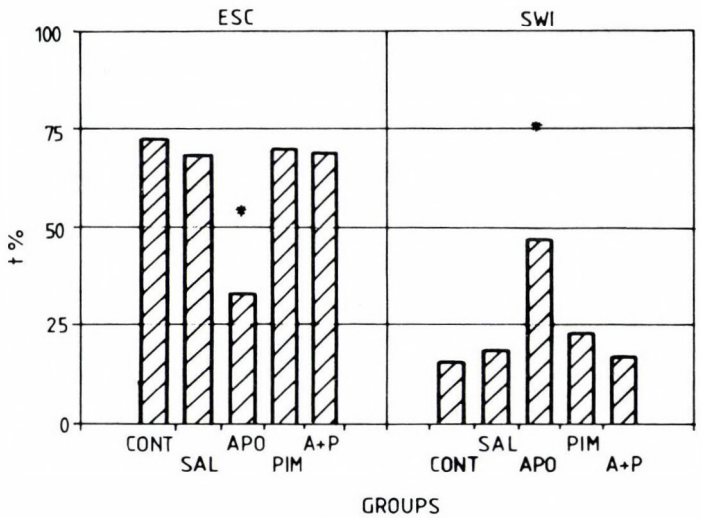

Fig. 1. Effects of Apomorphine (5 µg/fish) and Pimozide (0.5 µg/fish) injections on the performance of behavioral units of the paradise fish. Drugs were administered 30 min. before the 5 min. observational test in an unfamiliar small aquarium. Bars represent time in percentage spent in escape (ESC) and swimming (SWI). Group means ± SE are shown (N = 14); * indicates significant difference by Duncan range test at $p < 0.05$ level. (CONT = control; SAL = saline; APO = Apomorphine; PIM = Pimozide; A+P = Apomorphine + Pimozide group)

($F(4,65) = 16.97$, $p < 0.001$, omitting APO group $F(3,51) = 0.26$, n.s.; Duncan ranges of significance are 33.3 and 68.7-72.7). In contrast with ESC, the performance of SWI (Fig. 1b) significantly increased in the Apomorphine-treated group ($F(4,65) = 11.39$, $p < 0.01$, without APO group: $F(3,51) = 1.09$, n.s., Duncan ranges of significance are 15.4-23.0 and 47.0). The simultaneous addition of Pimozide, a classical dopaminergic antagonist, completely prevented the effects of Apomorphine. The other units seemed to be unaltered by the Apomorphine treatment, no significant effects were found (Table 1).

In the second experiment the dose curve of Apomorphine was measured and performance of ESC and SWI are discussed (Fig. 2) because time percentages of these two forms always exceeded 90% and the other elements were sporadic. The performance of ESC decreased gradually with increasing dosages of Apomorphine and at the same time the less oriented SWI became more and more frequent.

Table 1

The effect of various treatments on the behavior of paradise fish

	G R O U P S					
BEHAV. UNITS	Control (CONT)	Saline (SAL)	Apomorphine (APO)	Pimozide (PIM)	Apom.+Pimozide (A+P)	ANOVA
						F(4,65) =
MOV	3.3	2.8	3.4	2.2	3.6	1.07
(SE)	1.0	0.7	0.6	1.0	1.1	n.s.
A-G^	4.9	6.2	5.1	3.9	4.1	2.04
(SE)	2.5	3.0	2.3	3.1	2.3	n.s.
S-S	2.0	2.5	3.0	2.6	2.5	1.18
(SE)	0.6	0.7	0.7	0.5	0.6	n.s.
HIM	2.9	2.6	3.5	2.2	2.6	1.23
(SE)	0.6	0.4	0.7	0.8	0.4	n.s.
RES	1.1	0.7	1.5	0.4	1.2	2.11
(SE)	0.8	0.7	0.5	0.5	0.8	n.s.
FRZ	1.5	2.4	2.7	1.4	2.5	2.36
(SE)	0.5	0.7	0.8	0.8	0.5	n.s.

Group means and SE are shown. n.s. = non-significant differences

These experiments clearly showed that ESC and SWI react quite oppositely to the Apomorphine treatment, thus confirming our previous findings concerning the ethological nature of these behavioral units. Experiments with predators /1/, other frightening stimuli /5/, a harmless testfish /2/ and also with a conspecific /6/ showed that the ESC movements always had a definite orientation, either by approaching positive or by retreating from the source of negative stimuli when they might be interpreted as prolonged attempts to swim back to the home aquarium /5/. In case of SWI such an orientation was not found, at least not in small aquaria where environmental stimuli usually elicited other behavioral forms such as orientation or social display /6/. Swimming is the most usual reaction in a home tank where the habituated animals do not show fear induced reactions. The Apomorphine treatment resulted in a depressed level of fear thus eliciting fearless forms of behavior such as SWI /12/. However, the ESC and SWI units should be closely related because they changed in an antagonistic way after the Apomorphine treatment, whilst the frequency of all other units did not change. These observations fit in well with those obtained with other species. Munro /8/ found decreased aggressiveness in morphine treated fish,

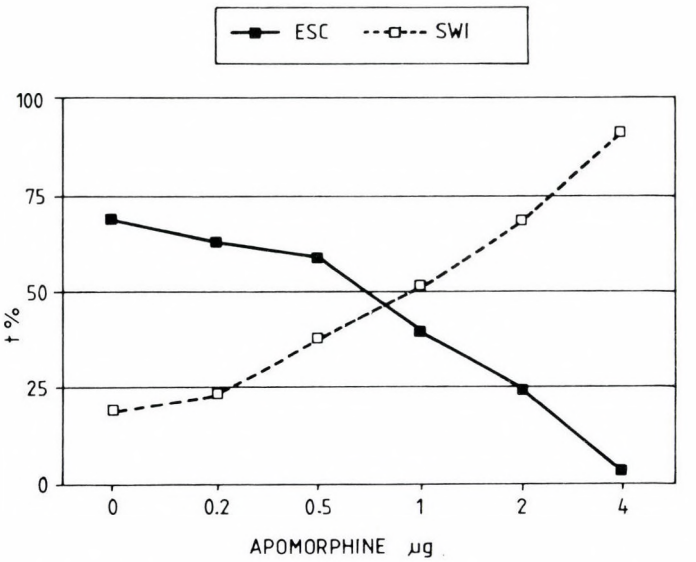

Fig. 2. Effect of the various dosage of Apomorphine on the performance of the two most common behavioral units, escape (ESC) and swimming (SWI), of the paradise fish. Apomorphine was administered intraperitoneally to achive a more stable reaction level. Values represent mean relative time percentage spent in ESC and SWI. Group means ± SE are shown (N = 10)

while Freed and Yamamoto /7/ showed that dopamine metabolism was strongly linked to the speed and to the direction of movements in the rat. Apomorphine which is a classical dopaminergic agonist seems to decrease the rate of occurrence of oriented behaviors and to enhance stereotyped movements both in mammals /10, 11/ and in fish as indicated by our experiments.

Acknowledgement

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THE PHOTOREFRACTORINESS IN DOMESTIC GOOSE: EFFECT OF GONADS AND THYROID ON THE DEVELOPMENT OF POSTBREEDING PROLACTINEMIA

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Blood samples were taken from adult male and female geese, and from gonadectomized (GX), thyroidectomized (TX) and gonadectomized+thyroidectomized (GX+TX) ones during the reproduction peak period (March 16), at the beginning of photorefractoriness (June 14), in the second half of the photorefractory period (August 14), and at the beginning of the postrefractory period (October 2). Surgeries were carried out at the age of 10-13 weeks. The birds were kept under natural light conditions. From the blood plasma prolactin (PRL), luteinizing hormone (LH), testosterone (T), progesterone (P_4), 17 β-oestradiol (E_2) as well as thyroxine (T_4) and triiodo-tironine (T_3) were determined by RIA methods.

At the beginning of photorefractoriness in intact geese the LH and E_2 levels significantly decrease, relative to sexually active period, whereas the P_4 remains at a high level. In ganders the LH shows only a slight decrease but the T level is significantly lower than in spring. The PRL, T_4 and T_3 levels increase after reproduction and give a peak value in both sexes. Both in GX ganders and geese decreased T, P_4 and T_3 levels are observable and the PRL is decreased in females, and the LH is increased in both sexes. Gonadectomy does not influence the E_2 in females, the PRL in males and the T_4 in both sexes. In TX birds the PRL is higher in March and lower in June than the control level, but these changes are significant only in females. In TX females the P_4 level is lower during reproduction and at the beginning of photorefractoriness. The T and E_2 levels do not change during the photorefractoriness.

In the second half of photorefractoriness a low PRL and P_4 level, an increasing LH, T and medium high T_4 and T_3 levels characterized the intact male and female geese. In GX animals an increase of LH levels is occurred in both sexes. The T_4 is higher in castrated-, and lower in ovarectomized geese. In TX birds the P_4 is higher than in controls, but the difference is significant only in males. The levels of PRL, LH, T and E_2 remain unchanged in TX animals.

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At the beginning of the postrefractory phase the T (in males) and the P_4 and the T_4 level increase in both sexes. The PRL and LH show a low value. In GX animals the high LH level refer to the increased sensitivity of hypothalamo-gonadotropic system, because of the absence of negative feed-back of sexual steroids.

Out results refer to the fact that the high PRL level, which is typical at the beginning of photorefractoriness, is thyroid dependent in geese, and the P_4 may also play a role in its formation. The lack of testicles does not influence this state. The later phase of photorefractoriness can be described by a hormonal condition, different from the former one: the PRL decreases in both sexes and a high thyroid hormone level and a transitional slight reactivation of gonadotropic-gonadal system occur then.

Keywords: Photorefractoriness — domestic goose — gonads — thyroid — prolactinaemia

Introduction

The annual reproductive cycle of birds living in temperate and cold zones terminates in summer due to photorefractoriness /11, 12, 23/. Photorefractoriness is characterized by a rapid decline in FSH, LH and gonadal steroid levels as well as by a rapid gonadal regression /18, 25, 31, 36, 37/.

The physiological mechanism(s) underlying the initiation of photorefractoriness is unknown. It may involve alteration in neural, neuro-endocrine and endocrine pathways that mediate long-day length information. Hypothalamic involvement has been suggested by the findings in the Starling where hypothalamic GnRH is nondetectable in photorefractory animals compared to that in photosensitive birds /5/. Changes in hypothalamic sensitivity to gonadal steroids have been implicated for the induction of the photorefractory state in Red Grouse /30, 34, 35/, Peking Drakes /21/, Mallards /17/ and in various species of song-birds /4, 24, 40, 41, 42/. Evidence has been presented that prolactin plays a role in the induction of photorefractoriness; based on experiments on the Starling /3, 4, 5, 15, 18/ and the Partridge /31/ in which a close temporal correlation between seasonal plasma prolactin peak and the induction of photorefractoriness was observed. There is also some evidence that circulating levels of thyroid hormones (possibly T_4) seem to elevate when birds are subjected to day lengths that cause refractoriness. After thyroidectomy in Starling /15, 39, 44/ and Japanese Quail /13/ there was no formation of photorefractoriness. One of the possible points of effect of thyroid hormones is presumably the increase of prolactin secretion: in thyroidectomized Starling the long-day photoperiod does not increase the prolactin level of the plasma /15/.

Studies from our laboratory on domestic geese raised under natural lighting conditions show photorefractoriness to be initiated in June at which time copulatory activity of ganders stops, plasma testosterone level falls to a low value, the egg laying of females ends, and postnuptial moulting becomes intensive /6, 26/.

In the present study, we determined plasma levels of LH, prolactin (PRL), testosterone (T), progesterone (P_4), 17β-oestradiol (E_2), thyroxine (T_4) and triiodothyronine (T_3) in intact male and female geese, gonadectomized, thyroidectomized and gonadectomized + thyroidectomized male and female geese during peak reproductive activity, at the beginning and the middle of the photorefractory period, and at the beginning of autumnal sexual reactivation. On the basis of changes in hormone levels, conclusions were made regarding the role of prolactin, the gonad, and the thyroid in photorefractoriness.

Material and Methods

The experimental geese (Hungarian white species) were hatched at the beginning of May 1988. In July and August (at the age of 10-13 weeks) some of them were orchidectomized and ovariectomized (in equithesin + lidocain local anesthesia, in males bilateral, in females unilateral laparatomy with thermocauterization at the end of the intervention). A week after gonadectomy some male and female animals were surgically thyroidectomized (equithesin + local lidocain anesthesia, interclavicular intervention; after ligating the main blood vessels, the thyroid gland was excised, and thermocauterization was applicated at the end of the intervention). The efficacy of operations was examined by autopsy and histological control after experiment.

The animals spent autumn and winter in an open-run. The natural photoperiod can be characterized by the date of the shortest day (Dec. 22: 8,5L-15,5D) and of the longest one (June 21: 16L-8D) on our experimental area. The animals were fed with water and granulated goose food (17% raw protein content) ad libitum. The egg-laying of control geese began at 17 February and it finished at 10 June. The average egg-production was 31 eggs/goose. In autumn a sporadic egg-laying occurs in the control group between 25 September to 8 December. The average egg production was only 1.9 eggs/goose.

A phase-shift of egg-laying was observed in thyroid-ectomized geese. The egg-laying began in this group at 30 January and finished at 28 May. The intensity of egg-production was lesser than in controls: only 9.2 eggs/goose.

A continuous brooding-egg-sitting behaviour did not develop neither in control, nor in thyroidectomized groups.

Seasonal taking of blood started in January 1989 and were carried out every 10 days on average between 9-11 a.m. till the end of February 1990. Blood was drawn into heparinized polyethylen tubes. After centrifugation,

the plasma was immediately stored at the temperature of -20 ^{o}C till usage. For prolactin determination we liophilized the plasma aliquotes.

We measured the hormone levels of the plasma with RIA methods (LH: /32/, modified for goose, according to /19/, PRL: /9/, P_4: /1/, E_2: /22/, T: /20/, T_4 and T_3 with human RIA KIT produced by the Isotope Institute of the Hungarian Academy of Sciences). In the case of T_4 and T_3 KIT we ensured the validity of the method with a continuous control of NSB values which were unique or under average as well as with the application of T_4 and T_3 released goose plasma.

With all RIA methods we achieved an intra- and interassay accuracy which was below 10%, expressed in coefficiency of variation.

We assessed our results by ANOVA on the base of MINITAB statistical computer soft wear.

Results

1. Prolactin (PRL)

1.1. Ganders

At the beginning of photorefractoriness (June 14) in control birds (n = 10) it is of the same value as at the peak of the spring cycle (March 16). In the second half of photorefractoriness (August 14) PRL concentration decreases intensively and the decrease of the level continues at the beginning of the autumnal postrefractory period as well (October 2).

Castration results in a lower PRL level than in the sexually active intact animals (n = 18) (March 16). At the beginning of photorefractoriness the PRL increases, relative to the spring castrated animals, then similarly to intact control ganders, it decreases, but at the beginning of postrefractory period, it does not show any further decrease.

During the spring sexual cycle in thyroidectomized ganders (n = 8) the PRL concentration is similar to that in control birds. At the beginning of photorefractoriness a decline can be observed which continues in the second half of photorefractory period as well. At the beginning of postrefractory phase the PRL level tends to rise.

In castrated + thyroidectomized ganders (n = 9) the PRL level is the highest during the spring sexual cycle, then it significantly decreases at the beginning of photorefractoriness. Its level is unchanged in August and at the beginning of postrefractory period (Fig. 1).

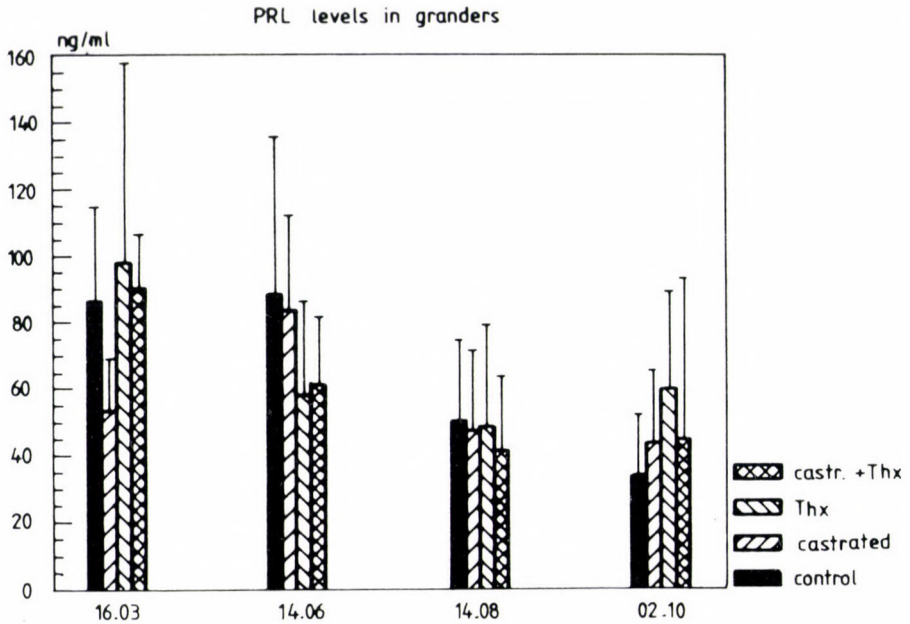

Fig. 1. Prolactin levels in ganders. Calendar date of taking blood samples from intact control and operated animals (means and standard deviations). Levels of significance are indicated on Tables 1 and 2

1.2. Geese

During the spring cycle the PRL level of control animals (n = 9) is the same as of ganders. At the beginning of photorefractoriness (it coincides with the formation of "brooding tendency") PRL concentration increases significantly, then in the later period of photorefractoriness there is an intensive decrease. At the beginning of postrefractory period the PRL level is unchanged.

In ovariectomized animals (n = 10) there are changes similar to the ones in castrated animals: the level is lower than in control birds, which is typical of the sexually active period, increases intensively at the beginning of photorefractoriness, then it decreases in August and rests on this low level at the beginning of postrefractory period.

The PRL level of thyroidectomized geese (n = 8) is very high in spring. During the photorefractory period the hormone concentrations significantly, than slowly declines, then it tends to rise at the beginning of postrefractory phase.

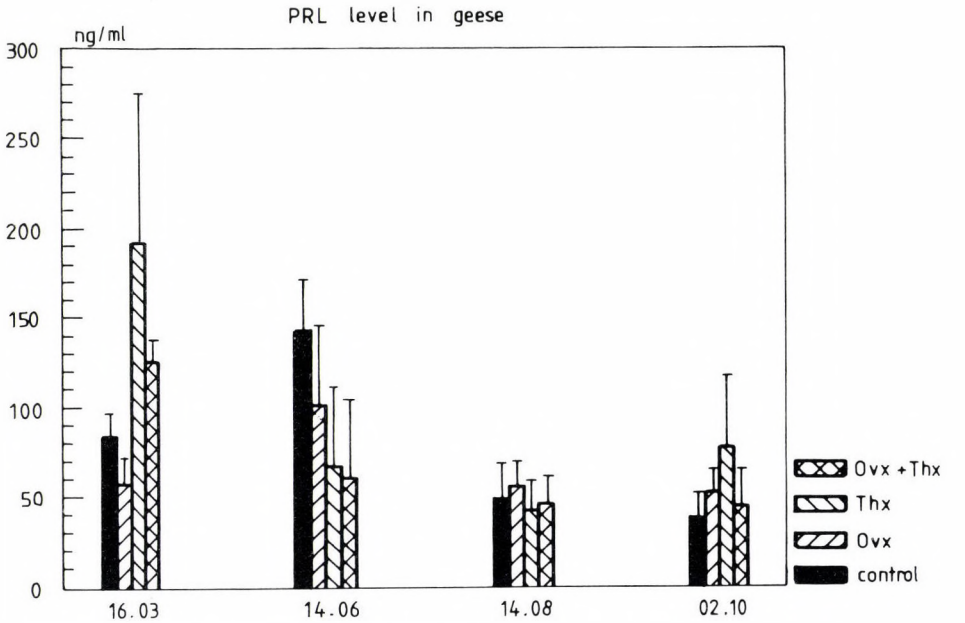

Fig. 2. Prolactin levels in geese. Calendar date of taking blood samples from intact control and operated animals (means and standard deviations). Levels of significance are indicated on Tables 1 and 2

The high PRL plasma concentration of ovariectomized + thyroidectomized animals (n = 8) in March decreases significantly at the beginning of photorefractoriness, then it remains at low level (Fig. 2).

2. Luteinizing hormone (LH)

2.1. Ganders

In control birds (n = 10) the LH level shows only a slight, but not significant fluctuation during the examined period because of the high individual variation. It is relatively higher during the peak period of the spring reproduction cycle and in the second half of photorefractoriness.

In castrated animals (n = 18) the LH level is significantly higher during the period of sexual activity, then it gradually increases during photorefractoriness. At the beginning of postrefractory phase the LH concentration significantly decreases, but in comparison with intact, control animals it remains significantly high.

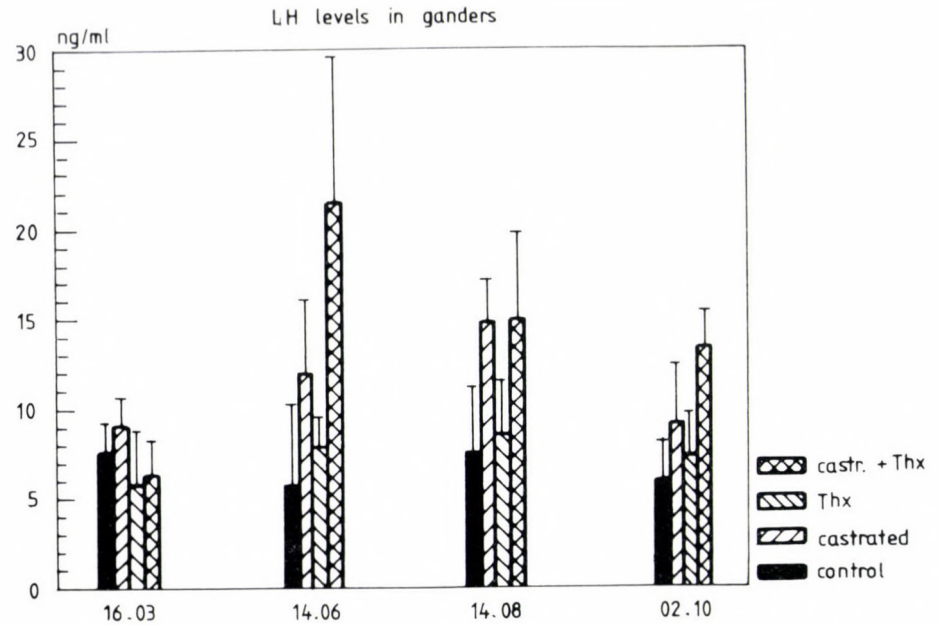

Fig. 3. Luteinizing hormone levels in ganders. Calendar date of taking blood samples from intact control and operated animals (means and standard deviations). Levels of significance are indicated on Tables 1 and 2

The LH level of thyroidectomized ganders (n = 8) is not differ from the intact control animals during the examined period.

The LH plasma concentration of castrated + thyroidectomized animals (n = 9) does not differ from the one in control ganders during the sexually active spring period. At the beginning of photorefractory phase it increases fairly intensively, then its later period it decreases (but even then it is much higher than the control level). At the beginning of postrefractory period the LH level does not change (Fig. 3).

2.2. Geese

During the spring egg-laying period the LH level of control animals (n = 9) is higher than that of ganders. At the beginning of photorefractoriness the LH decreases intensively, but in its second half it increases considerably. At the beginning of postrefractory period a further strong decline can be observed.

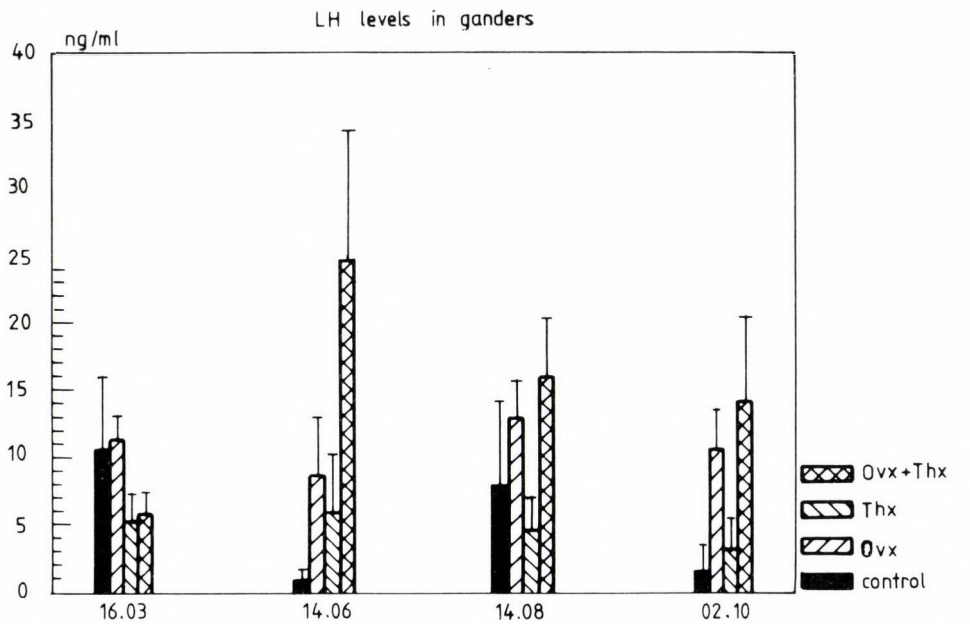

Fig. 4. Luteinizing hormone levels in geese. Calendar date of taking blood samples from intact control and operated animals (means and standard deviations). Levels of significance are indicated on Table 1 and 2

During the spring reproduction cycle in ovariectomized animals (n = 10) the LH level does not differ from control geese. Its concentration does not change during the examined period. At the beginning of photorefractoriness the LH level is higher than in control birds. At the beginning of the post-refractory period it will be also considerably higher than the control value.

During the spring cycle the LH plasma concentration of thyroidectomized geese (n = 8) is considerably lower than in intact control animals. At the beginning of photorefractoriness it remains at the same level, then in the second half of the period and at the beginning of the postrefractory phase it decreases slowly.

The LH level of ovariectomized + thyroidectomized geese (n = 8) is lower during the sexual cycle than in controls. At the beginning of the photorefractory phase the LH concentration increases very intensively, then in the second half of the phase it decreases significantly. At the beginning of the postrefractory period the LH level remains at a still high value (Fig. 4).

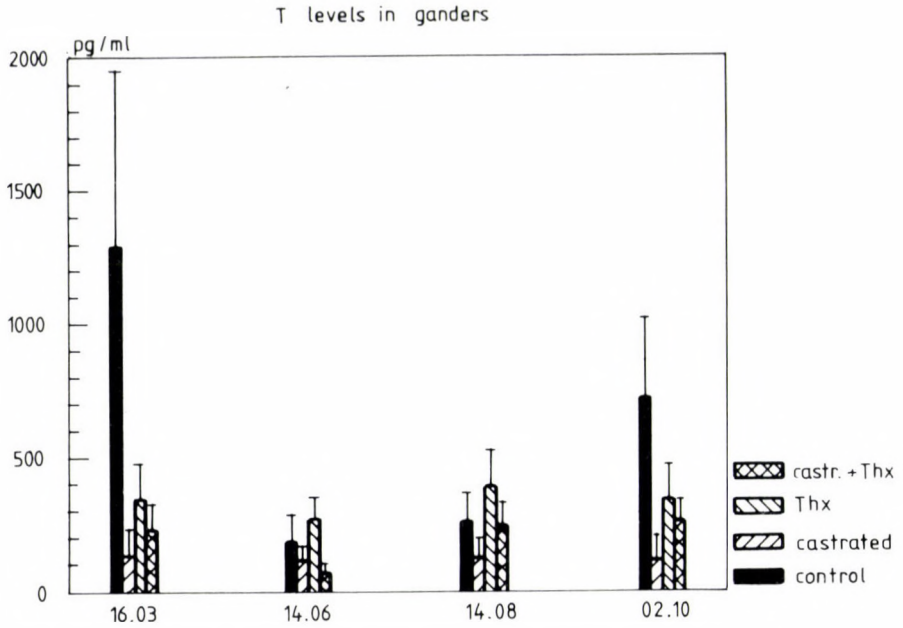

Fig. 5. Testosterone levels in ganders. Calendar date of taking blood samples from intact control and operated animals (means and standard deviations). Levels of significance are indicated on Tables 1 and 2

3. Testosterone (T)

During sexual activity the T plasma level is high in control ganders (n = 10), then at the beginning of photorefractoriness it sharply declines. The androgen concentration remains at this low level in the second half of photorefractoriness as well, than at the beginning of postrefractory phase it starts to rise sharply again.

During the whole period tested a very low, non-fluctuating T level is characteristic in castrated animals (n = 18).

During breeding time, both during photorefractoriness and postrefractory phase it is low and slightly fluctuates in thyroidectomized ganders (n = 8). The androgen concentration is still higher in thyroidectomized ganders than in castrated animals.

In castrated + thyroidectomized animals (n = 9), similarly to castrated ganders very low T plasma concentrations are present. It sharply decreases at the beginning of photorefractoriness and a rise can be observed in the

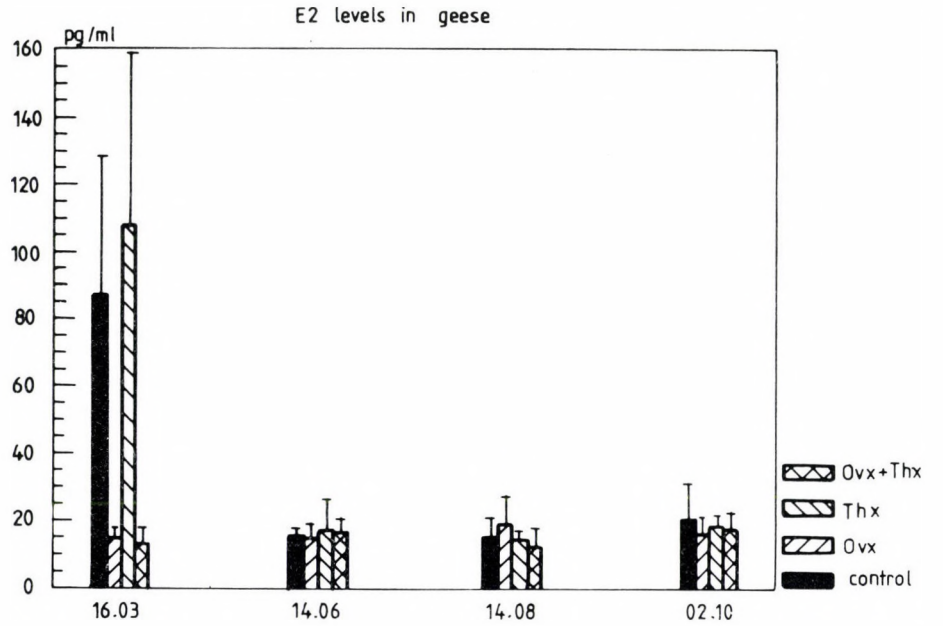

Fig. 6. 17 β-oestradiol levels in geese. Calendar date of taking blood samples from intact control and operated animals (means and standard deviations). Levels of significance are indicated on Tables 1 and 2

second half of photorefractoriness. Similarly, the same relatively high T level is observable at the beginning of postrefractory period (Fig. 5).

4. 17 β-oestradiol (E_2)

In control geese (n = 9) the highest E_2 level can be found during the spring cycle. It sharply declines at the beginning of photorefractoriness, then it remains at this very low level, however a slight rise can be observed at the beginning of postrefractory period.

In ovariectomized animals (n = 10) the E_2 plasma concentration shows a very low plateau during the examined period.

In the sexually active period of thyroidectomized geese (n = 8) there is a high E_2 level which is comparable to controls. At the beginning of photorefractoriness it sharply declines, then the E_2 level remains at this low value during photorefractoriness, and at the beginning of postrefractory period.

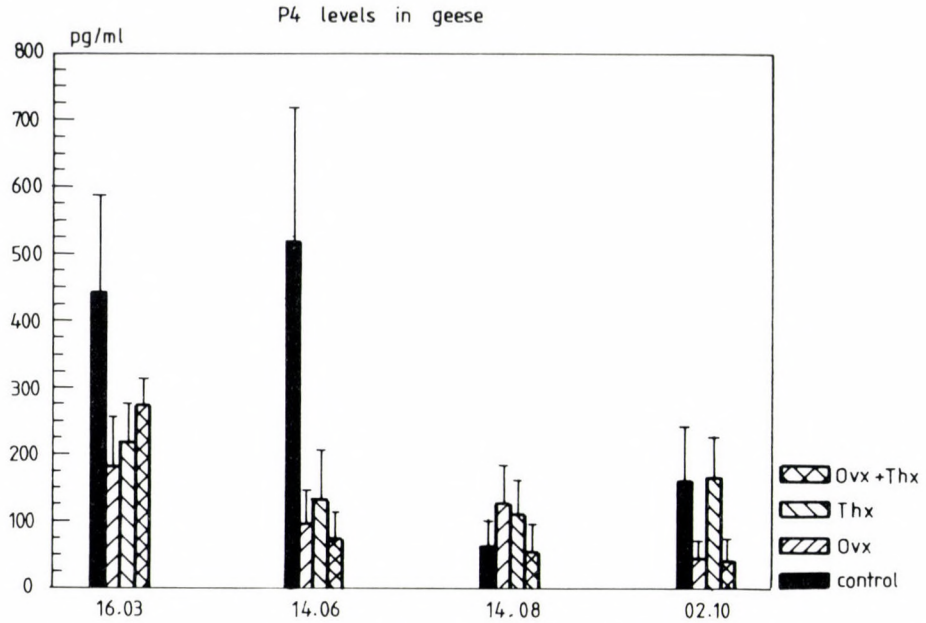

Fig. 7. Progesterone levels in geese. Calendar date of taking blood samples from intact control and operated animals (means and standard deviations). Levels of significance are indicated on Tables 1 and 2

The E_2 plasma concentration of ovariectomized + thyroidectomized geese (n = 8) is at the same very low level as of the ovariectomized animals during the whole tested period (Fig. 6).

5. Progesterone (P_4)

5.1. Geese

At the beginning of photorefractoriness the high P_4 level of the spring cycle in control geese (n = 9) does not change. In the second half of photorefractory period the P_4 concentration sharply declines, then it starts to rise at the beginning of the postrefractory phase.

In ovariectomized geese (n = 10) the P_4 level falls into 40% of the control value in the spring sexual period, and it keeps falling at the beginning of photorefractoriness.

In the second half of photorefractoriness the P_4 level does not change, then at the beginning of the postrefractory phase there is a significant decline.

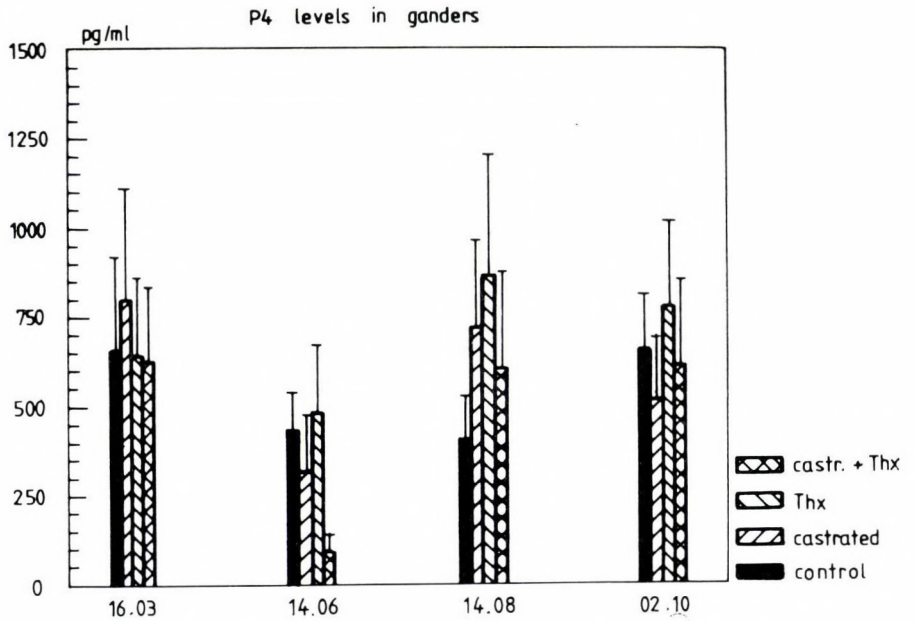

Fig. 8. Progesterone levels in ganders. Calendar date of taking blood samples from intact control and operated animals (means and standard deviations). Levels of significance are indicated on Tables 1 and 2

At the spring cycle in thyroidectomized geese (n = 8) the P_4 levels are in the 40-50% of the control ones. During the photorefractoriness the P_4 concentration continuously decreases, and at the beginning of postrefractory period it tends to increase.

During the spring breeding time in ovariectomized + thyroidectomized geese (n = 8) the P_4 level is 50% of the control one, and it decreases intensively at the beginning of photorefractoriness and remains at this low level later on (Fig. 7).

5.2. Ganders

During breeding time in control ganders (n = 10) a higher P_4 level can be measured than in egg-laying geese. At the beginning of photorefractoriness there is a significant decrease here, and this lower level characterizes the later period as well. At the beginning of postrefractory phase tehere is an intensive rise of P_4.

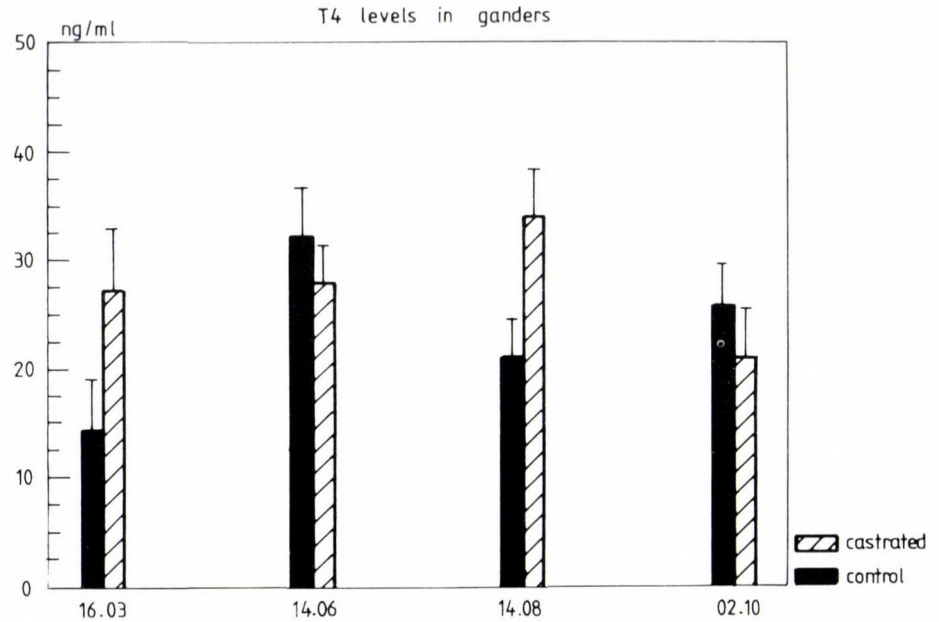

Fig. 9. Thyroxine levels in ganders. Calendar date of taking blood samples from intact control and operated animals (means and standard deviations). Levels of significance are indicated on Tables 1 and 2

During the spring cycle in castrated ganders (n = 18) the P_4 concentration does not differ from the control animals. The hormone level decreases sharply at the beginning of photorefractoriness, but during the later phase it increases significantly and this tendency continues at the beginning of postrefractory period, too.

The P_4 plasma level of thyroidectomized (n = 8) and castrated + thyroidectomized geese (n = 9) is similar to the castrated ones: it is not different from the control value in spring, then sharply declines at the beginning of photorefractoriness, and it increases in its later phase. In autumn its concentration does not change (Fig. 8).

6. Thyroid hormones (T_4, T_3)

6.1. Ganders

During reproduction peak periode the level of T_4 is relatively low in controls (n = 10). At the beginning of photorefractoriness its level

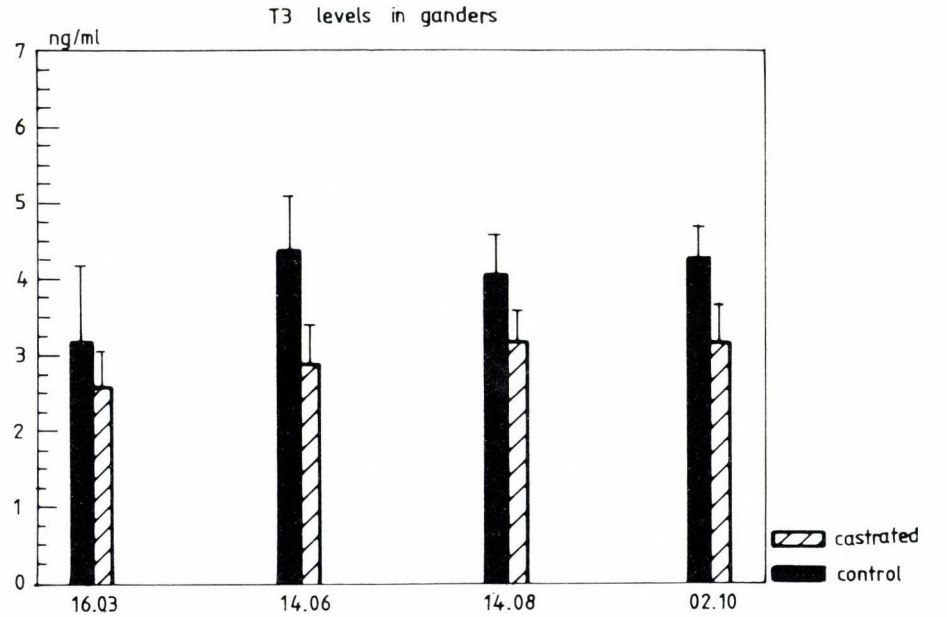

Fig. 10. Triiodo-tironine levels in ganders. Calendar date of taking blood samples from intact control and operated animals (means and standard deviations). Levels of significance are indicated on Tables 1 and 2

increases. In the second half of photorefractoriness the T4 level is lower, then, at the beginning of the postrefractory phase an increase can be observed. The T_3 (n = 10) is formed similarly to the T_4 level in control ganders: it is characterized by a smaller peak value at the beginning of photorefractoriness, then by a high level of summer and the beginning of autumn.

In castrated animals (n = 18) during reproductive period a higher T_4 level can be found than in controls. An identical concentration can be observed at the beginning of photorefractoriness, and there is an increase in the second half of photorefractoriness. At the beginning of the postrefractory period the T_4 concentration falls. The T_3 (n = 18) is lower than the control level and it is lower than control during the refractory and postrefractory period (Figs 9, 10).

In thyroidectomized (n = 8) and thyroidectomized + castrated animals (n = 9) very low T_4 and T_3 levels can be found which do not show any fluctuation.

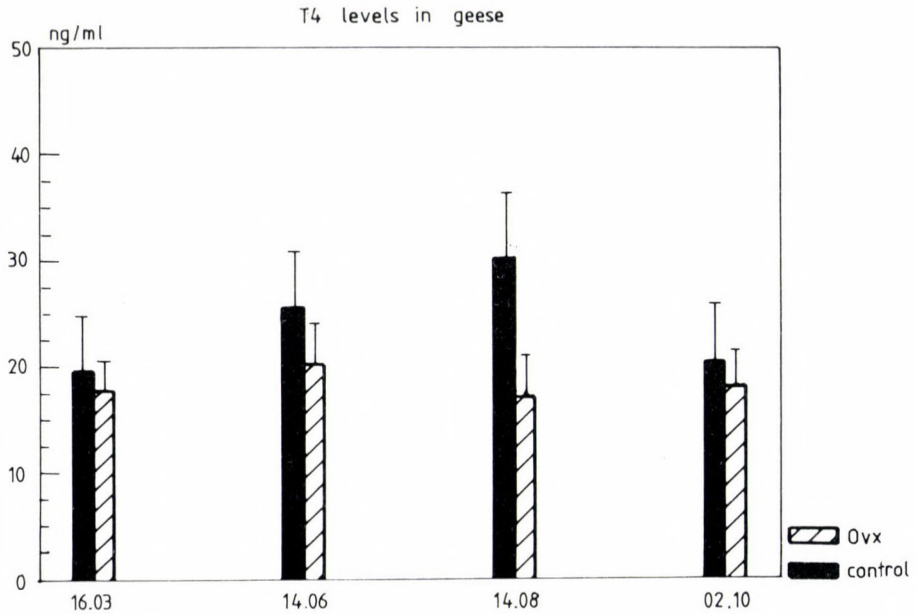

Fig. 11. Thyroxine levels in geese. Calendar date of taking blood samples from intact control and operated animals (means and standard deviations). Levels of significance are indicated on Tables 1 and 2

6.2. Geese

In control birds (n = 9) the T_4 level tends to increase during photorefractoriness and we can notice a significant peak value in its second half. At the beginning of the postrefractory phase the T_4 concentration significantly decreases.

The T_3 level (n = 9) shows a maximum at the beginning of the photorefractory period, it decreases in its second half, and it remains at this value at the beginning of the postrefractory phase as well.

The T_4 level of ovariectomized animals (n = 10) is the same as the control value in the reproductive cycle at the beginning of the refractory period and also during the postrefractory phase. It is significantly lower than in controls in the second half of photorefractoriness.

In the reproduction period the T_3 level (n = 10) is somewhat higher than the control one, and it does not fluctuate during the examined period. Its value is significantly lower than in control animals at the beginning of photorefractoriness (Figs 11, 12).

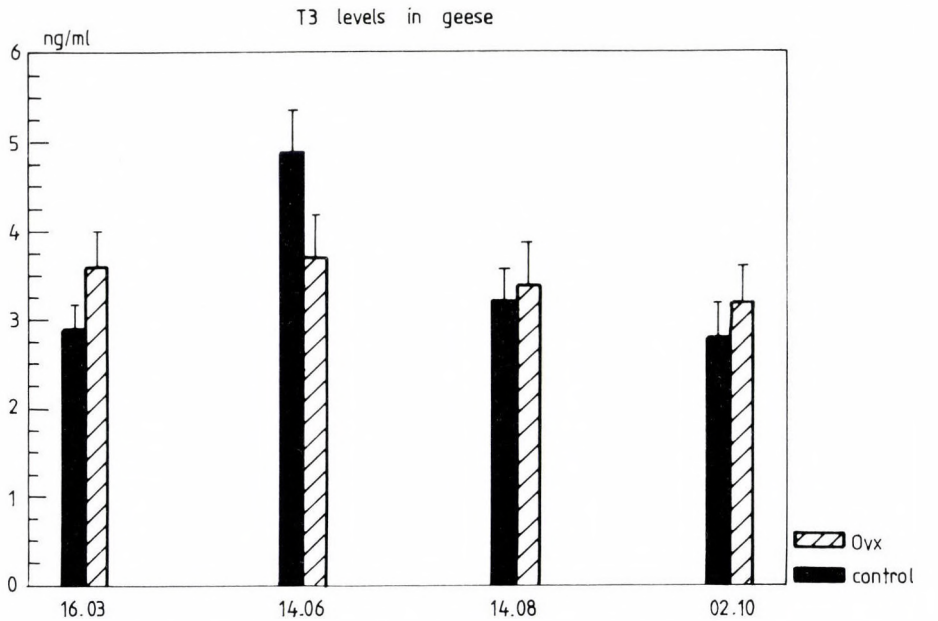

Fig. 12. Triiodo-tironine levels in geese. Calendar date of taking blood samples from intact control and operated animals (means and standard deviations). Levels of significance are indicated on Tables 1 and 2

In thyroidectomized (n = 8) and thyroidectomized + ovariectomized geese (n = 8) we have found a low T_4 and T_3 concentrations which is close to the limit of measurability (Tables 1, 2).

Table 1

Results of ANOVA in experimental group I. Different interventions in same period

	16 March		14 June		14 August		2 October	
	male	female	male	female	male	female	male	female
	PRL							
Contr.-GX	$P < 0.001$	$P < 0.01$	n.s.	$P < 0.05$	n.s.	n.s.	n.s.	n.s.
Contr.-TX	n.s.	$p < 0.05$	n.s.	$p < 0.01$	n.s.	n.s.	n.s.	n.s.
Contr.-GX+TX	n.s.	$p < 0.01$	n.s.	$p < 0.01$	n.s.	n.s.	n.s.	n.s.
GX-TX	$p < 0.01$	n.s.	p 0.05	n.s.	n.s.	n.s.	n.s.	n.s.
GX-GX+TX	$p < 0.001$	$p < 0.05$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
TX-GX+TX	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	LH							
Contr.-GX	$p < 0.05$	n.s.	$p < 0.01$	$p < 0.001$	$p < 0.001$	n.s.	$p < 0.05$	$p < 0.001$
Contr.-TX	n.s.	$p < 0.05$	n.s.	$p < 0.01$	n.s.	n.s.	n.s.	n.s.
Contr.-GX+TX	n.s.	$p < 0.05$	$p < 0.01$	$p < 0.001$	$p < 0.05$	n.s.	$p < 0.001$	$p < 0.001$
GX-TX	$p < 0.001$	$p < 0.001$	$p < 0.05$	n.s.	$p < 0.001$	$p < 0.001$	$p < 0.05$	$p < 0.001$
GX-GX+TX	$p < 0.01$	$p < 0.05$	$p < 0.05$	$p < 0.01$	n.s.	n.s.	n.s.	n.s.
TX-GX+TX	n.s.	n.s.	$p < 0.05$	$p < 0.001$	n.s.	$p < 0.001$	$p < 0.01$	$p < 0.01$
	T (male), E2 (female)							
Contr.-GX	$p < 0.001$	$p < 0.001$	$p < 0.01$	n.s.	$p < 0.001$	n.s.	$p < 0.001$	n.s.
Contr.-TX	$p < 0.01$	n.s.	n.s.	n.s.	n.s.	n.s.	$p < 0.05$	n.s.
Contr.-GX+TX	$p < 0.01$	$p < 0.001$	$p < 0.01$	n.s.	n.s.	n.s.	$p < 0.01$	n.s.
GX-TX	$p < 0.001$	$p < 0.05$	$p < 0.001$	n.s.	$p < 0.001$	n.s.	$p < 0.001$	n.s.
GX-GX+TX	$p < 0.001$	n.s.	n.s.	n.s.	$p < 0.001$	n.s.	$p < 0.001$	n.s.
TX-GX+TX	n.s.	$p < 0.05$	$p < 0.001$	n.s.	n.s.	n.s.	n.s.	n.s.
	P4							
Contr.-GX	n.s.	$p < 0.01$	$p < 0.05$	$p < 0.001$	$p < 0.01$	n.s.	n.s.	$p < 0.01$
Contr.-TX	n.s.	$p < 0.01$	n.s.	$p < 0.001$	$p < 0.01$	n.s.	n.s.	n.s.
Contr.-GX+TX	n.s.	$p < 0.05$	$p < 0.001$	$p < 0.001$	n.s.	n.s.	n.s.	$p < 0.01$
GX-TX	n.s.	n.s.	$p < 0.05$	n.s.	n.s.	n.s.	$p < 0.05$	$p < 0.001$
GX-GX+TX	n.s.	$p < 0.05$	$p < 0.001$	n.s.	n.s.	$p < 0.05$	n.s.	n.s.
TX-GX+TX	n.s.	n.s.	$p < 0.01$	n.s.	n.s.	$p < 0.05$	n.s.	$p < 0.001$
	T4							
Contr.-GX Contr.-TX Contr.-GX+TX GX-TX GX-GX+TX TX-GX+TX	$p < 0.001$	n.s.	n.s.	n.s.	$p < 0.001$	$p < 0.001$	$p < 0.001$	n.s.
	T3							
Contr.-GX Contr.-TX Contr.-GX+TX GX-TX GX-GX+TX TX-GX+TX	n.s.	$p < 0.01$	$p < 0.05$	$p < 0.001$	$p < 0.05$	n.s.	$p < 0.01$	n.s.

Table 2

Results of ANOVA in experimental-group II. Same interventions in different periods

	Control		GX		TX		GX+TX	
	male	female	male	female	male	female	male	female
	PRL							
16.03—14.06.	n.s.	$p<0.01$	$p<0.001$	$p<0.001$	n.s.	$p<0.01$	$p<0.01$	n.s.
16.03—14.08.	$p<0.01$	$p<0.001$	n.s.	n.s.	n.s.	$p<0.01$	$p<0.05$	$p<0.01$
16.03—02.10.	$p<0.001$	$p<0.001$	n.s.	n.s.	n.s.	n.s.	$p<0.05$	$p<0.01$
14.06—14.08.	$p<0.05$	$p<0.01$	$p<0.001$	$p<0.001$	n.s.	n.s.	n.s.	n.s.
14.06—02.10.	$p<0.01$	$p<0.001$	$p<0.001$	$p<0.001$	n.s.	n.s.	n.s.	n.s.
14.08—02.10.	$p<0.05$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	LH							
16.03—14.06.	n.s.	$p<0.001$	$p<0.05$	n.s.	n.s.	n.s.	n.s.	$p<0.01$
16.03—14.08.	n.s.	n.s.	$p<0.001$	n.s.	n.s.	n.s.	n.s.	$p<0.01$
16.03—02.10.	$p<0.05$	$p<0.001$	n.s.	n.s.	n.s.	n.s.	$p<0.01$	n.s.
14.06—14.08.	n.s.	$p<0.01$	$p<0.05$	n.s.	n.s.	n.s.	n.s.	$p<0.05$
14.06—02.10.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
14.08—02.10.	n.s.	$p<0.05$	$p<0.001$	n.s.	n.s.	n.s.	n.s.	n.s.
	T (male), E2 (female)							
16.03—14.06.	$p<0.001$	$p<0.001$	n.s.	n.s.	n.s.	$p<0.01$	$p<0.001$	n.s.
16.03—14.08.	$p<0.001$	$p<0.001$	n.s.	n.s.	n.s.	$p<0.05$	n.s.	n.s.
16.03—02.10.	n.s.	$p<0.001$	n.s.	n.s.	n.s.	$p<0.05$	n.s.	n.s.
14.06—14.08.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	$p<0.001$	n.s.
14.06—02.10.	$p<0.001$	n.s.	n.s.	n.s.	n.s.	n.s.	$p<0.001$	n.s.
14.08—02.10.	$p<0.001$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	P4							
16.03—14.06.	$p<0.05$	n.s.	$p<0.001$	$p<0.05$	n.s.	n.s.	$p<0.001$	$p<0.001$
16.03—14.08.	$p<0.05$	$p<0.001$	n.s.	n.s.	n.s.	$p<0.01$	n.s.	$p<0.001$
16.03—02.10.	n.s.	$p<0.001$	$p<0.01$	$p<0.01$	n.s.	n.s.	n.s.	$p<0.001$
14.06—14.08.	$p<0.001$	$p<0.001$	$p<0.001$	n.s.	$p<0.05$	n.s.	$p<0.001$	n.s.
14.06—02.10.	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.05$	n.s.	n.s.	$p<0.001$	n.s.
14.08—02.10.	$p<0.01$	$p<0.05$	$p<0.01$	$p<0.05$	n.s.	n.s.	n.s.	n.s.
	T4							
16.03—14.06.	$p<0.001$	n.s.	n.s.	n.s.				
16.03—14.08.	$p<0.01$	$p<0.05$	n.s.	n.s.				
16.03—02.10.	$p<0.001$	n.s.	n.s.	n.s.				
14.06—14.08.	$p<0.001$	n.s.	$p<0.01$	n.s.				
14.06—02.10.	$p<0.01$	n.s.	$p<0.01$	n.s.				
14.08—02.10.	$p<0.05$	$p<0.05$	$p<0.001$	n.s.				
	T3							
16.03—14.06.	n.s.	$p<0.001$	n.s.	n.s.				
16.03—14.08.	n.s.	n.s.	$p<0.01$	n.s.				
16.03—02.10.	$p<0.05$	n.s.	$p<0.05$	n.s.				
14.06—14.08.	n.s.	$p<0.001$	n.s.	n.s.				
14.06—02.10.	n.s.	$p<0.001$	n.s.	n.s.				
14.08—02.10.	n.s.	n.s.	n.s.	n.s.				

Discussion

The spring reproductive cycle of our experimental geese started in the middle of February, and copulation activities of ganders as well as egg-laying activity reached maximum in the second half of March.

During this time the intact ganders had moderately-elevated LH and high T levels and elevated LH, E_2 and P_4 levels were observed in females. However, slightly high levels of PRL, T_3 and T_4 were found in both sexes, too.

According to our previous data /6, 7, 26/ the long day photorefractoriness of domestic geese kept under natural light conditions starts at the beginning of June: egg-laying and copulation activity gradually stops, and postnuptial moulting, which started a bit earlier, becomes more intensive.

At the beginning of the photorefractory period the plasma levels of PRL increased in females and remained at high level in males, whereas the concentration of LH, T and E_2 decreased in both sexes. P_4 levels did not change in females but was slightly decreased in ganders.

At the beginning of photorefractoriness the high PRL level measured in geese and ganders confirms the data of literature, according to which in the bird species examined the appearance of photorefractoriness coincides with a significant increase of PRL both in male and female birds /4, 15, 18, 31, 43/.

According to our data at the beginning of photorefractoriness in female geese the increase of the PRL level is significantly higher than in ganders. Our experimental geese were not in brooding, egg-sitting condition in this period, but certain pattern of the behaviour and the relatively higher PRL levels indicate the transitory presence of this physiological event.

Similar increases in PRL levels were observed at the beginning of the photorefractory period in intact and gonadectomized birds. These findings implictate PRL but not gonadal steroids in the development of photorefractoriness (for review see Farner et al. /11/). However, we must reckon with permissive and synergistic effects of sexual steroids in PRL regulation. According to El Halawani et al. /10/, in the turkey the photoinduced increases in serum PRL level is partially due to ovarian steroids, primarily estrogens and to a lesser extent to the synergistic effect of P_4 /29/. However, in geese several indications implicate a greater role for P_4 than for E_2 in PRL regulation: (1) E_2 decreased while P_4 and PRL remained at high

level and increased at the beginning of the photorefractory period. (2) At the beginning of photorefractoriness the PRL levels of ovariectomized geese, but not of castrated ganders was reduced significantly compared to intact birds; implying that the remaining high P_4 levels associated with photorefractoriness in intact females may be also responsible for the increased PRL level at this time.

The role of the thyroid gland in the development of photorefractoriness has been established in Starlings /15, 39, 30/. The results of the present study show that the seasonal maximum of T_4 and T_3 levels occur at the beginning of the photorefractory period (middle of June). These findings confirm the results of studies on Peking Drakes /20/, Zonotrichia /33/, Rooks /25/, Pengiunes /16/, and Willow Ptarmigans /37/. Taken together these results indicate that the postbreeding thyroid activity occurs with a phase sift in relation to gonadal function and it is associated with the start of the postnuptial moult. It is worth mentioning that the levels of thyroid hormones do not change during photorefractoriness in Partridges /31/.

There appears to be a positive relationship between PRL and thyroid hormone levels and the induction of photorefractoriness. On the one hand we noticed that the seasonal maximum of T_4 and T_3 in geese was in the middle of June, at the same time as that of PRL. On the other hand, in thyroidectomized birds the June PRL peak — typical in intact and gonadectomized geese at the beginning of the photorefractory period — was missing.

But during the reproductive peak period in hypothyroid ganders and geese relatively high PRL levels show up. In females it is significantly higher than the control value. So the lack of thyroid hormones causing the "absence" of the PRL plasma increase — typical for the postbreeding period in both sexes -- through a "phase sift" produces a peak of PRL secretion in spring.

During the increasing and long-day photoperiod in spring the lack of thyroid hormones may directly affect the PRL secretion and probably not by influencing the gonadal activity. The PRL plasma level of gonadectomized + + thyroidectomized male and female geese is formed in the same way as that of the thyroidectomized animals. In this case there appears to be a negative relationship between PRL and thyroid hormones.

Regarding LH, thyroidectomy causes slighty fluctuating hormone levels, which are very similar to control values. However, the LH level reflects the circannual change of photoperiod in thyroidectomized + gonadectomized birds, i.e., it increases between March and 14 June and declines sharply during the

shortening of the daylength. This observation underlines the significance of the modulatory gonado-thyroid interaction in the seasonal regulation of LH secretion, also, thyroid can only modulate the LH production through directly influencing the gonadal function.

In our present experiment we have found the same high P_4 level in female geese at the beginning of photorefractoriness, which is characteristic at the peak of spring sexual activity.

Plasma P_4 level was significantly lower throughout the reproductive cycle in ovariectomized geese as compared to their respective intact controls. This level was further decreased during the initial phase of the photorefractory period. Accordingly it appears that the elevated P_4 levels at the onset of the photorefractoriness in intact geese are mainly of gonadal origin, and suggests that the regressed ovary is capable of secreting P_4 during this period /14/.

However, in ganders the high P_4 level of the reproductive period slightly decreases at the beginning of photorefractoriness. In male animals gonadectomy does not affect significantly the P_4 level. These findings suggest that the origin of circulating P_4 is different in male and female birds, and that the adrenals may be the main source of P_4 in ganders. The adrenals have been suggested to be the main source of plasma P_4 in domestic hens /38/, Starlings and Black-Headed Gulls of both sexes /26, 27, 28/. We suppose that the relatively high plasma P_4 level in both ganders and geese is related to the postnuptial moulting at the beginning of photorefractoriness.

The results of our present study point out that the hormonal status observed between the middle of June and the end of September (at the termination of photorefractoriness) is not homogeneous. Earlier studies on geese indicated a short, transitory increase in T level in August which coincided with an increase in T_4 and a decrease in T_3 /6, 7, 26/. In this study an increase in both LH (mainly in female geese) and T levels, and a very low PRL levels in this period has been found which does not mean a gonadal reactivation. The beginning of the later phase of photorefractoriness is indicated by the ending of postnuptial moult and characterized by an increase of body weight (late summer fattening). That means that the photorefractoriness of geese consists at least two phases, and both are related with the postnuptial moulting. At the termination of photorefractoriness (early October), there was a decrease in LH and PRL level and an increase in T level as well as in E_2 and P_4. In the postrefractory period the T_4 level

increases in ganders and decreases in females. In this period a significant percentage of ganders showed spontaneous sexual reactivation, however, ovarian recrudescence was rarely observed in females.

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IMMUNOCYTOCHEMICAL DEMONSTRATION OF PROGESTERONE AND ESTROGEN RECEPTORS IN FEATHERS AND SKIN OF ADULT HENS

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Clinical evidence indicates that ovarian steroids are involved in the control of moulting in the chicken. This immunocytochemical study investigates if feather papillae and growing feathers are target tissues for ovarian steroids. Progesterone (PR) and estrogen (ER) receptors were demonstrated using monoclonal antibodies in feathers and surrounding skin of laying hens. Both receptor types were present in the nuclei of dermal papillae and in the nuclei of the epidermal germinative layer cells of growing and full-grown feathers. In growing feathers most nuclei of the intermediate layer (ramogenic column, rachis, axial plate) were immunostained, but during the final stages of differentiation into barbules, only estrogen receptors remained prominent. Skin adjacent to feathers showed ER and PR receptors in nuclei of cells from epidermis, muscles and arteries.

During egg-laying pause, plasma progesterone levels decrease ten-fold and it is supposed that this results in a much greater endocrine efficiency of the remaining estrogen levels which are only reduced by 50% when egg-laying stops. The moult-inhibiting effect of progesterone in laying hens could be due to its well-established downregulation on estrogen receptors and therefore, on the endocrine effect of ER at cellular level in feather papillae. Such may account for the presence of both receptor types on the same feather cells, as observed in the present study.

Keywords: Immunology — steroid receptor — moulting

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Introduction

In the annual cycle of most bird species, moulting fits in a period of sexual inactivity, mostly just after the breeding season /14/. High levels of sex steroids during breeding mainly inhibit moult initiation /14, 23/ or are attributed a role in the differentiation of special characters of the plumage (sexual dimorphism; /23/). In henny-feathering traits of chickens, enhanced conversion of testosterone to estrogen in the skin is responsible for the female appearance of the plumage in the male /3/. In laying fowls, artificial induction of a 4-6 weeks pause in egg-laying, mainly by reduction of food & light, is a current management practice to improve laying in a subsequent second cycle. The egg-laying stop is accompanied by a variable extent of moulting /4/, and the resulting plumage renewal has long-term implication on the energetic efficiency of the birds /6/. There is much evidence that thyroid hormones are involved in the process of moult initiation and feather growth /23/. The sudden dramatic decrease of plasma progesterone and the increase of plasma thyroxin during the egg-laying pause are likely to be the causal factors of moult initiation /5, 7, 21/. However, it could not yet be established whether ovarian steroids have a direct effect on mitosis in feather papillae or whether the moult induction is indirectly due to the reduction of the negative feed-back of steroids on thyroid hormone levels or function.

This immunocytochemical study investigates if feather papillae and growing feathers are target tissue for ovarian steroids.

Material and Methods

Feather papillae and surrounding skin of brown laying hens (Warren SSL hybrids) were dissected and pre-fixed (1 h at 0 ^{o}C), before keratinized feather shafts could be dissected and removed microsurgically. Fixation continued for 2 h afterwards. At least one dimension of the final tissue blocks was less than 5 mm. In a first series of tissue samples, 5 fixatives were tested: a) Bouin Hollande Sublimate (BHS: 90% Bouin + 10% saturated $HgCl_2$); b) Glutaraldehyde (0.5% in PBS, PH 7.4); c) absolute ethanol + 1% acetic acid; d) Faglu (4% paraformaldehyde + 0.2% glutaraldehyde in 0.1 M PBS, PH 7.4); e) Baker's fluid (10% paraformaldehyde + 1% $CaCl_2$ in H_2O, PH 6.7). In a second series of tissues only the fixatives a, b and d were used, because ethanol and Baker's fluid had resulted in sections of poor histological quality.

The first series of tissues was dehydrated by a quick procedure: ethanol 70% (1 h), followed by a series of dioxane (4 x 1.5 h). Because this

resulted in very weak immunostaining in the control tissue, a slower procedure of dehydration was used in the second series: graded ethanol 50%, 70%, 90% (2 h each), ethanol 100% (overnight), 1/2 ethanol 100% + 1/2 xylene (10 h), xylene (overnight), 1/2 xylene + 1/2 paraffin (10 h), paraffin (overnight). Paraffin sections (7 µm) of the same tissue type, but differently fixed, were mounted side by side on the same slides. In a third series, five growth stages of primaries were taken from white broiler breeder hens, to overcome the problem of interference between the brown feather pigments and the similarly brown DAB/peroxidase immunostaining. This last series of tissues was fixed in BHS and dehydrated in graded alcohol (slow procedure).

After dewaxing and rehydration, BHS-fixed sections were further treated with lugol (2x2 min) and natriumthiosulfate (5%, 2 min) prior to being rinsed in tris-saline (pH 7.6). Incubation with primary antibodies (dilution 1/500 - 1/2000) lasted for 20 h. The peroxidase conjugated rabbit-anti-mouse secondary antibody (Dakopatts, Glostrup Denmark) was applied at 1/200 dilution for 1 h. The second series of tissues was furthermore pre-incubated during 20 min with pre-immune rabbit antiserum (1/20). Except for a preincubation with trypsin (0.1% in 0.1% $CaCl_2$, pH 7.8 for 20 min at 37 °C; only applied to the second series), and its blocking afterwards in PBS at 4 °C, all steps were carried out at room temperature. Visualization was carried out in a Tris buffer containing diaminobenzidine hydrochloride (Sigma) (25 mg/200 ml) and 0.02% H_2O_2 (5-15 min).

Mouse monoclonal antibodies against the progesterone receptor in chick oviduct were obtained from Dr. D.O. Toft, Mayo Clinic, Minnesota: the preparation and biochemical properties of the antibody are reported by Sullivan et at. /20/. The antibody used (PR 13) is an IgG_{2b} which recognizes both A and B forms of the progesterone receptor, but which does not recognize estrogen or corticosteroid receptors. Mouse monoclonal antibodies against the estrogen receptor in calf uterus were obtained from Prof. B. Moncharmont, Università di Napoli. The preparation, specificity and other biochemical properties of the antibody JS 34/32 are reported by Moncharmont et al. /11, 12/: in biochemical assays the antibody cross-reacts with chicken estrogen receptor.

Specificity of staining was checked in negative controls in which (1) a pre-immune rabbit-serum replaced the actual primary antibody, or (2) the primary antibody, the secondary antibody or the development in DAB/H_2O_2 were skipped, respectively. Chick oviduct and cow uterus served as positive control tissues. Anatomical terminology of skin and feather structures follows the atlas of Lucas and Stettenheim /9/.

Results

Specific immunostaining of steroid receptors appeared to be located exclusively in the nucleus. In general, BHS fixation resulted in the best immunostaining, particularly with progesterone receptors. The impact of fixatives on the results was much less with estrogen receptors. Though cross-reactivity of PR13 to bovine uterus was intense (Fig. 2), chicken oviduct showed only a weak specific immunostaining of progesterone receptors (Fig. 1). Estrogen receptors on the other hand became only immunostained following trypsin pre-treatment, even in the control tissue (bovine uterus)

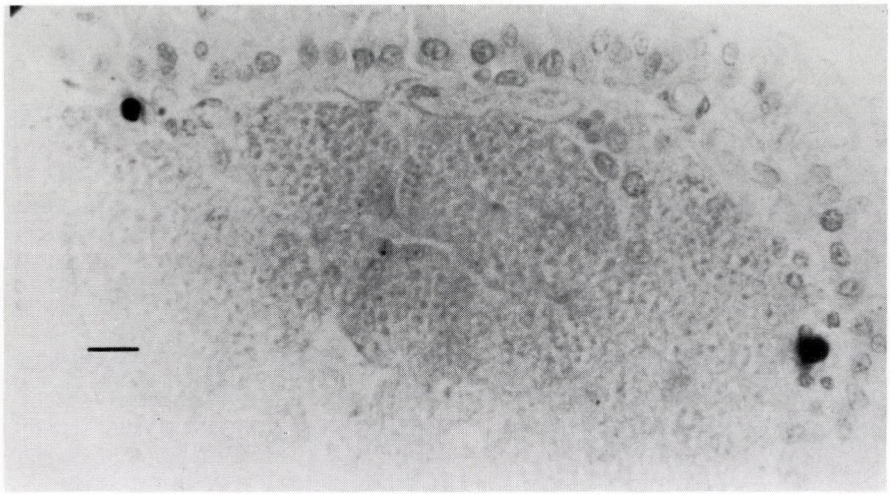

Fig. 1. Immunocytochemical staining of nuclear progesterone receptors in the oviduct epithelium of an adult laying hen (BHS fixation; bar = 10 μ)

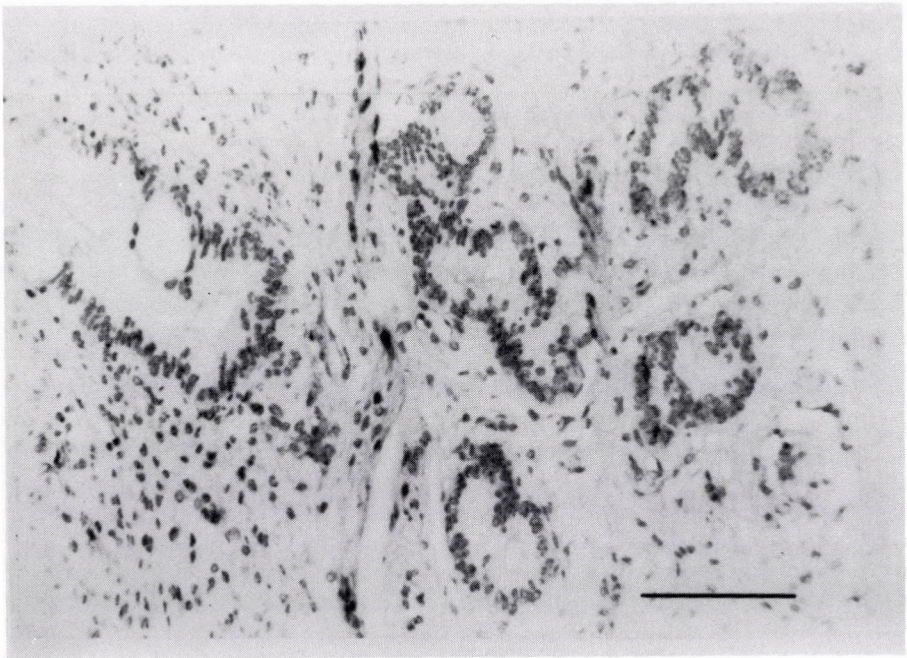

Fig. 2. Heterologous immunocytochemical staining of nuclear progesterone receptors in bovine uterus glands, following trypsin pre-treatment (BHS fixation; bar = 0.1 mm)

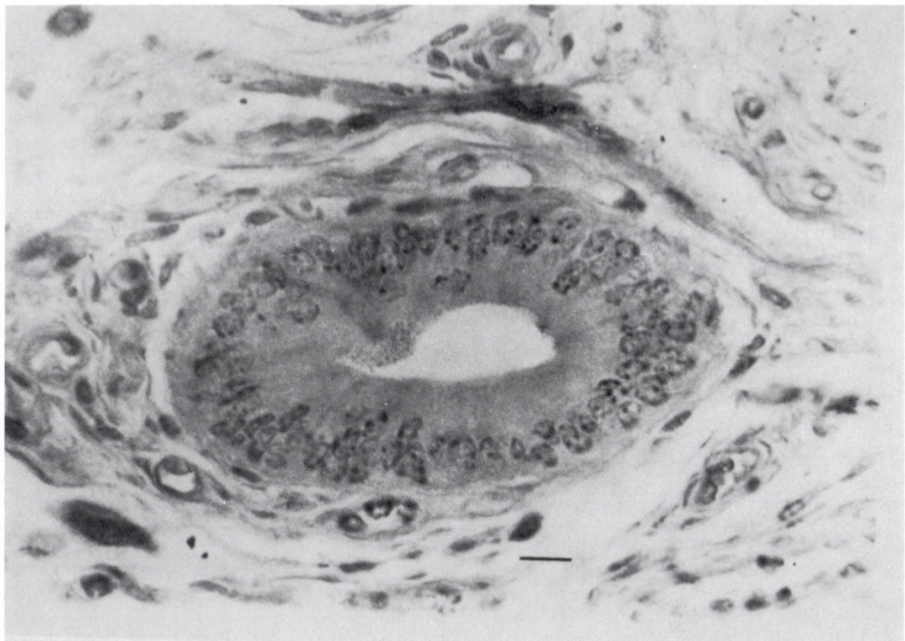

Fig. 3. Immunocytochemical staining of nuclear estrogen receptors in a bovine uterus gland, following trypsin pre-treatment (BHS fixation; bar = 10 μ)

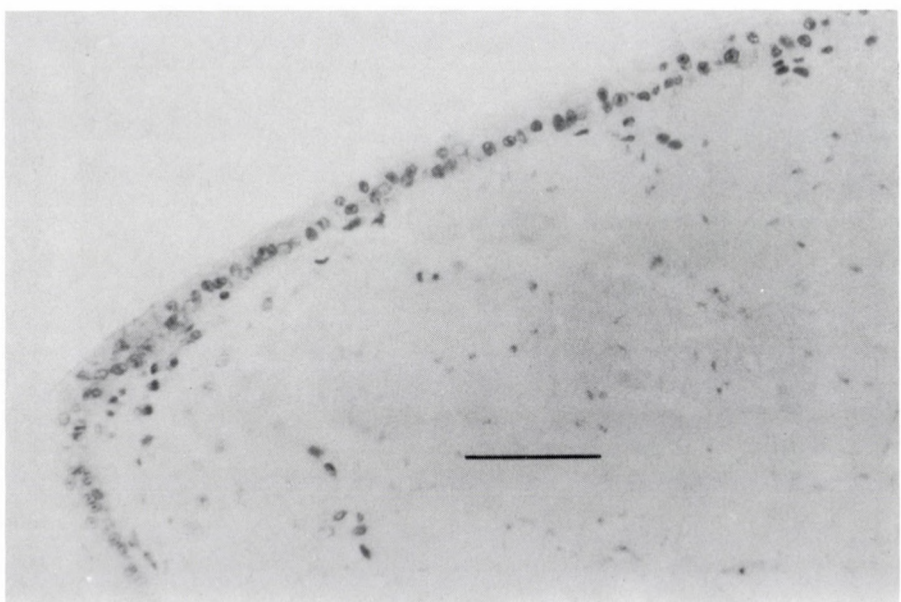

Fig. 4. Heterologous immunocytochemical staining of nuclear estrogen receptors in the oviduct of an adult laying hen, following trypsin pre-treatment (BHS fixation; bar = 50 μ)

(Fig. 3). Nevertheless, cross-reactivity with chicken tissues after trypsinisation was good (Fig. 4), and resulted in even clearer staining of estrogen than progesterone receptors.

Full-grown remiges (pre- and post-moult) had progesterone receptors in the nuclei of the dermal papillae and in the epidermal germinative layer (Fig. 5). Growing remiges and contour feathers had progesterone receptors in the nuclei of the epidermis of the follicles and in the proximal part of the epidermal collar during the earlier stages of differentiation into barbuli (Fig. 6). In the dermal pulp, covering the central, non-differentiated part of the growing feather, progesterone receptors remained more obvious throughout development (Figs 6 and 7). During the more advanced stages of differentiation of the intermediate layer into feather barbs and barbuli, the immunostaining became more difficult to interpret, because melanocytes begin to develop in the proximal part and pigments migrate through the collar towards the barbs (Fig. 8). It is especially hard to evaluate whether melanocytes themselves have progesterone receptors. In the tissue series from white broiler hens, which have only few dark pigments, there was evidence for the presence of progesterone receptors also in the ramogenic column, in the area of the rachis, and in the axial plate during the differentiation of the intermediate layer into barbules (not shown). During the final stages of differentiation, progesterone receptors can hardly be demonstrated in the intermediate layer (compare e.g. Figs 8 and 9).

Furthermore, several structures in the skin adjacent to a growing feather had progesterone receptors: especially the epidermis and muscles were positive (Fig. 10), but more localized, connective tissue deeper in the dermis was also stained. Arteries close to follicles of remiges and the axial artery in the pulp of growing feathers were also immunostained (Fig. 11).

Very similar to the pattern of progesterone receptors, estrogen receptors were present in the dermal papilla and the epidermal germinative layer of remiges in not moulting hens. Specific immunostaining of estrogen receptors was also found in the dermal pulp, in the proximal and central part of the epidermal collar, and in the epidermis of the follicle (not shown). However, in contrast to progesterone receptors, estrogen receptors remained obvious during the further differentiation of the epidermal collar: nuclei of barb cells and of cells in the ramogenic column, the axial plate, and the area of the developing rachis were clearly labeled (Figs 12 and 13). Although it is again hard to evaluate the state of immunostaining of the

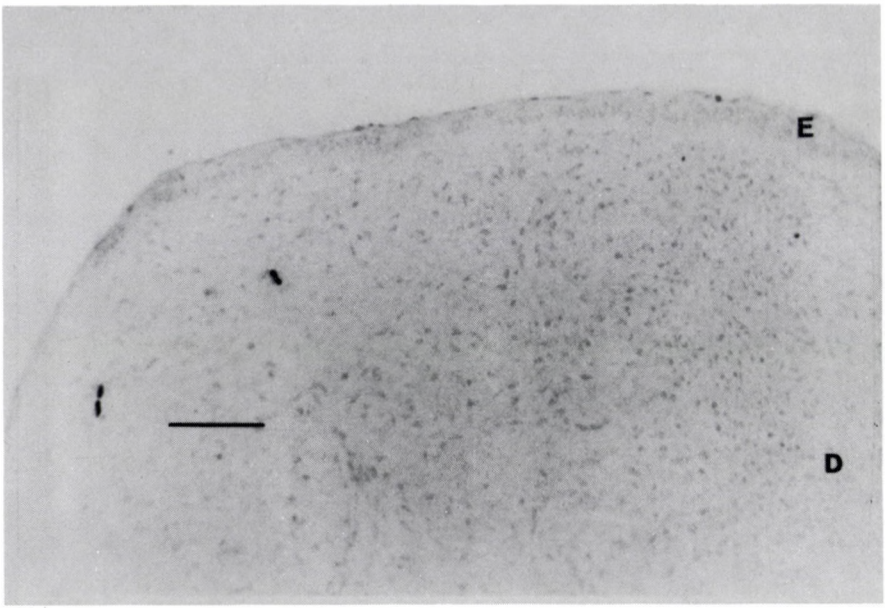

Fig. 5. Immunocytochemical staining of progesterone receptors in the nuclei of the dermal papilla (D) and the epidermal germinative layer (E) of the follicle of a full-grown primary of the adult hen (BHS fixation; bar = 50 μ)

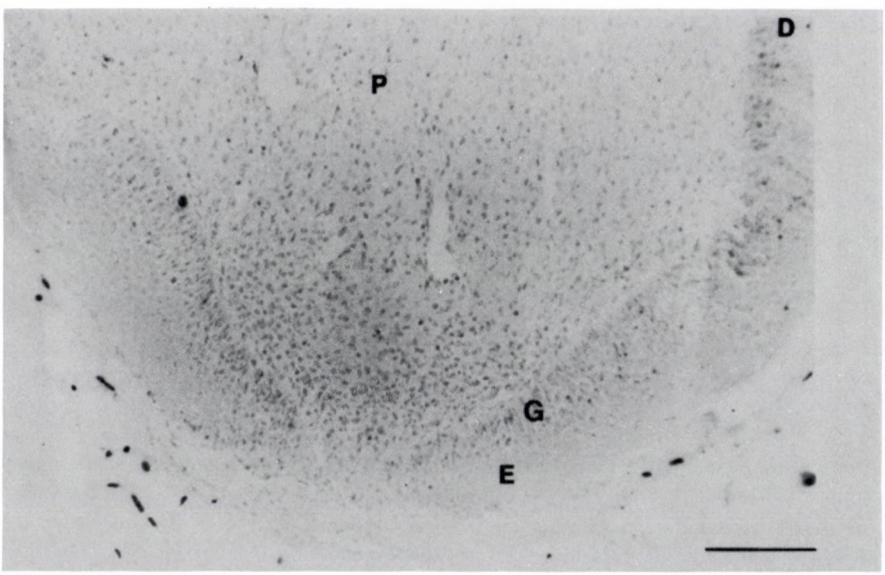

Fig. 6. Immunocytochemical staining of nuclear progesterone receptors in the basal part of a growing feather from the crural tract (longitudinal section) of the adult hen (BSH fixation). P = dermal pulp; E = epidermal collar with G = germinative layer; D = zone of differentiation in barbuli (bar = 0.1 mm)

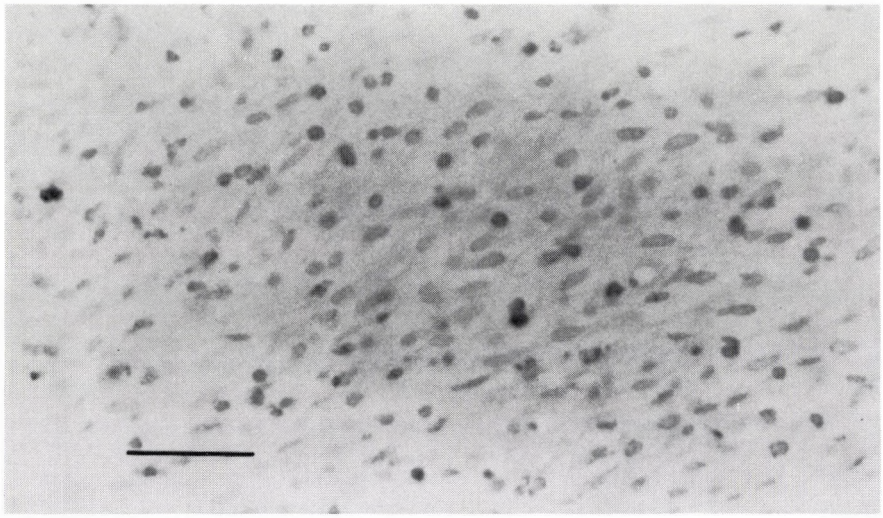

Fig. 7. Immunocytochemical staining of nuclear progesterone receptors in the dermal pulp of a growing filoplume adjacent to a primary (P9) in the adult hen (Glutaraldehyde fixation; bar = 50 μ)

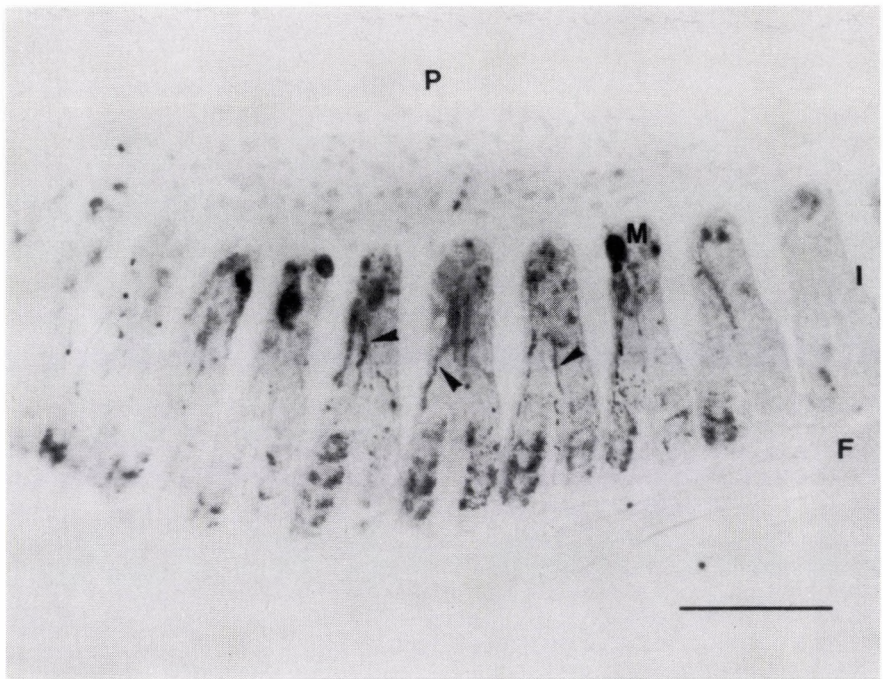

Fig. 8. Immunocytochemical staining of nuclear progesterone receptors in a growing feather from the crural tract in the adult hen (Faglu fixation). P = dermal pulp; I = intermediate layer, differentiating into barbs and barbules; M = melanocytes; arrows-migrating pigments; F = feather sheath (bar = 50 μ)

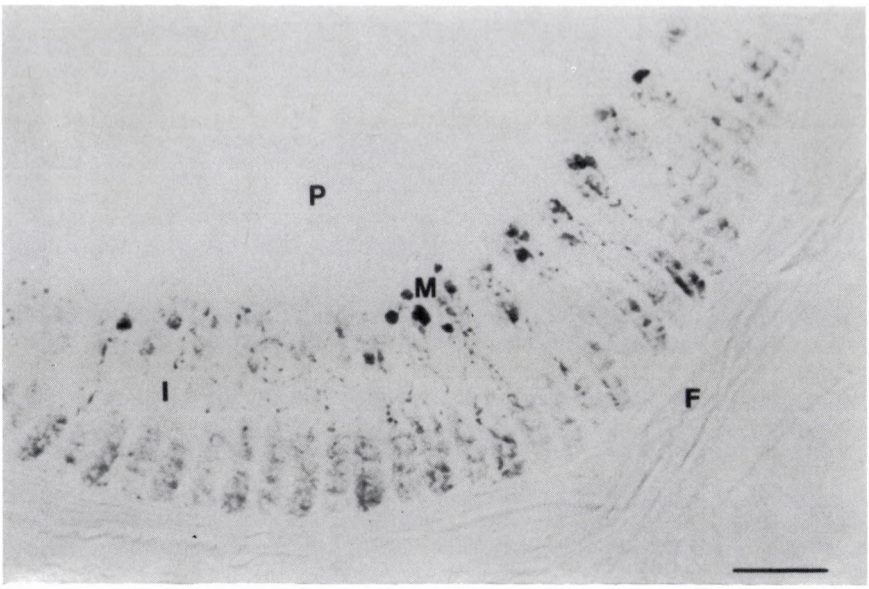

Fig. 9. Melanocytes and pigments in the intermediate layer of a growing feather from the crural tract in the adult hen. No primary or secondary antibody applied, neither developed in DAB/peroxide (Faglu fixation). P = dermal pulp; I = intermediate layer differentiating into barb and barbules; M = melanocytes; F = feather sheath (bar = 50 μ)

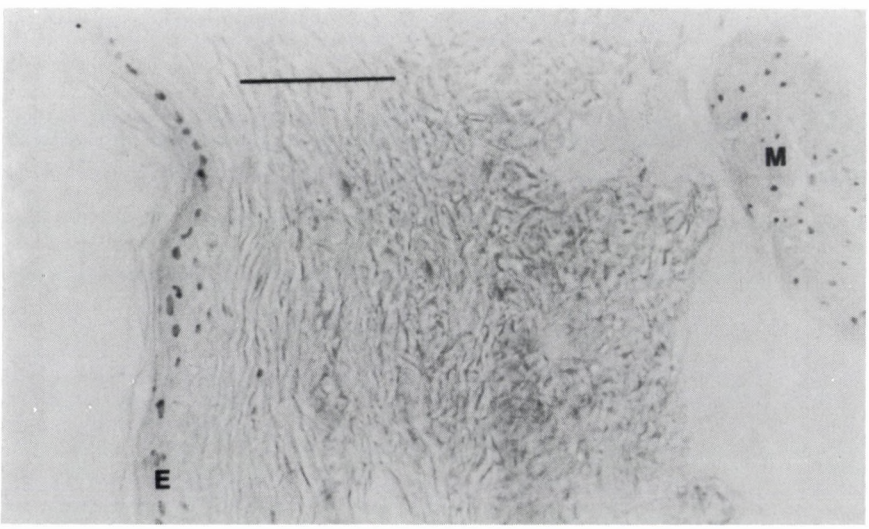

Fig. 10. Immunocytochemical staining of nuclear progesterone receptors in skin of the adult hen (Glutaraldehyde fixation). E = epidermis; M = smooth muscles (bar = 50 μ)

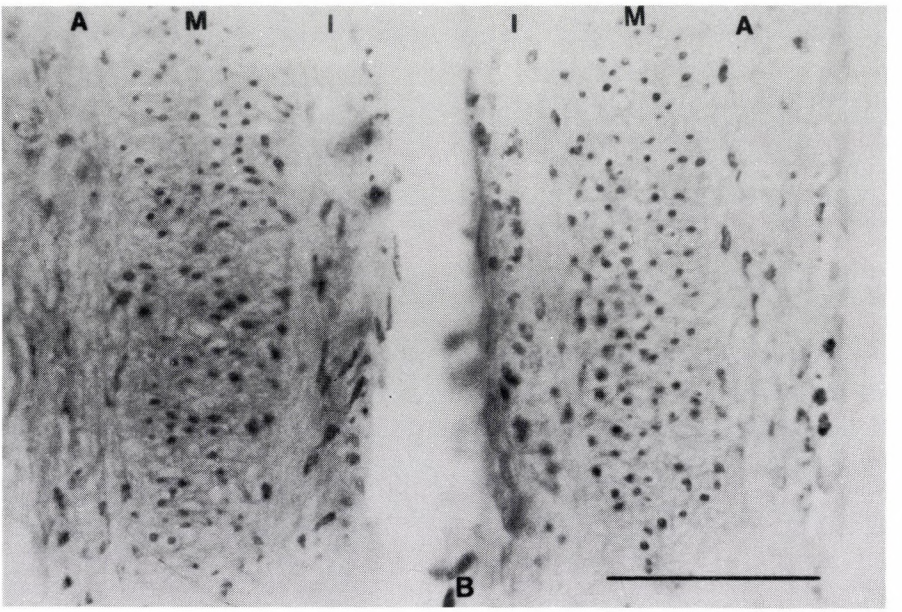

Fig. 11. Immunocytochemical staining of nuclear progesterone receptors in an artery adjacent to a nearly fullgrown secondary (S4) in the adult hen (Glutaraldehyde fixation). I = Tunica intima; M = Tunica media; A = Tunica adventitia; B = Blood cells (bar = 50 μ)

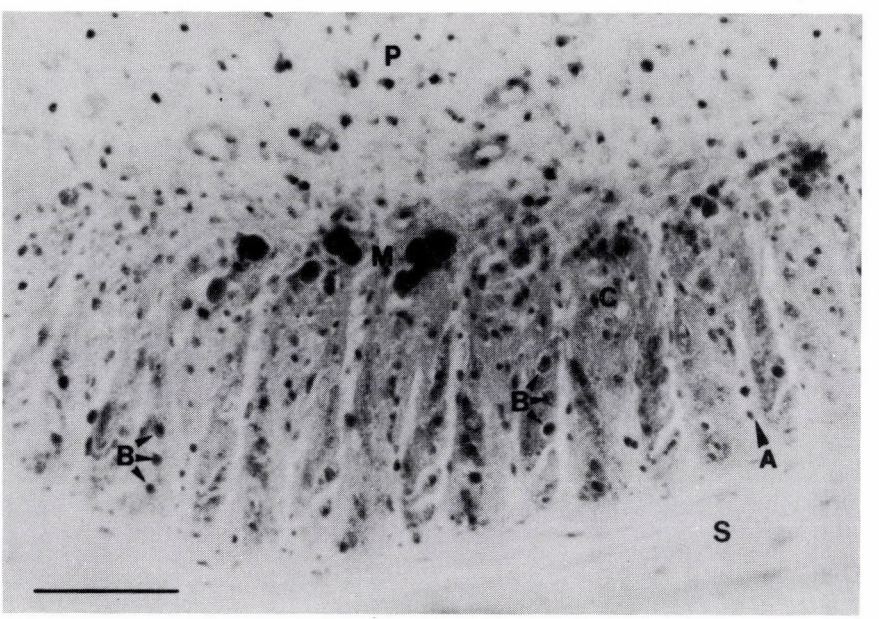

Fig. 12. Immunocytochemical staining of nuclear estrogen receptors in a cross-section of an early growth stage ("pin") of a crural contour feather of the adult hen (BHS fixation). P = dermal pulp; M = melanocytes; S = feather sheath; B = barb cells; A = axial plate; C = ramogenic column (bar = 50 μ)

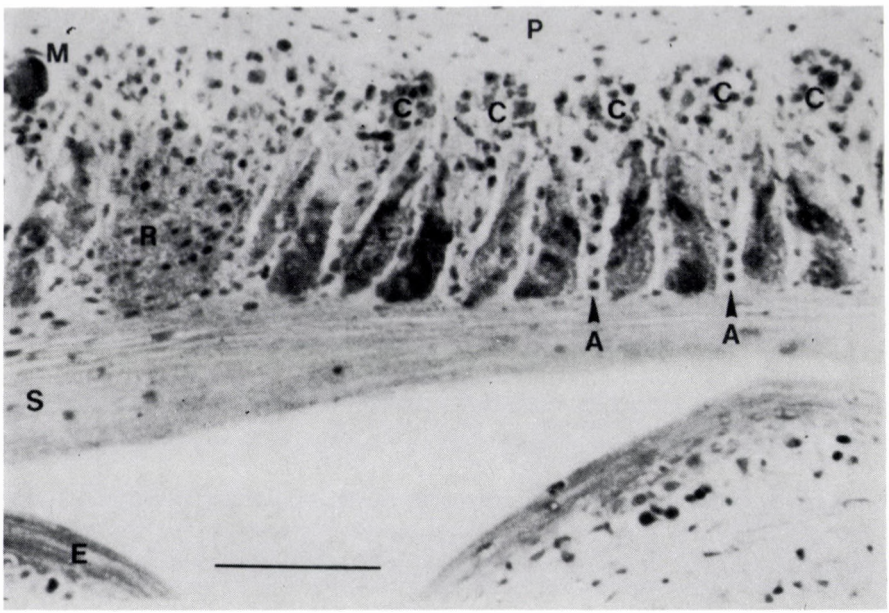

Fig. 13. Immunocytochemical staining of nuclear estrogen receptors in a cross-section of an early growth stage ("pin") of a crural contour feather of the adult hen (BHS fixation). P = dermal pulp; M = melanocyte; S = feather sheath; A = axial plate; C = ramogenic column; R = rachis; E = epidermis of follicle (bar = 50 μ)

melanocytes, it appeared that these cells also have estrogen receptors. Immunostaining was furthermore present in the epidermis of the follicle (Fig. 13), and more weakly in smooth muscles, in the epidermis of the skin and in some parts of the dermal connective tissue. Further stages of the differentiation of the feather become so much keratinized that we did not find a method combining good histological quality with preservation of sufficient epitopic accessibility. Negative controls did not show any labeling. However, care had to be taken in interpreting colouring by pigmentation of endogenous compounds such as melanine.

Discussion

Our experiences with fixatives do not parallel those of Andersen et al. /1/. These authors found cross-linking fixatives (e.g. glutaraldehyde) able to preserve antigenic sites in estrogen receptors (in contrast to coagulating reagents (e.g. Bouin) which precluded immunostaining). On the

other hand, the great enhancement of trypsinisation on the accessibility of the estrogen receptor epitopes confirms the findings of Andersen et al. /1/. Although the antibody against the progesterone receptor of the hen, used in this study (PR13), did not cross-react with the human or rabbit progesterone receptors in biochemical studies /20/, its immunohistological cross-reaction with bovine progesterone receptor, as demonstrated in this study, is in accordance with the cross-reaction of antibodies against the chick progesterone receptor with other mammalian species as reported by Renoir et al. /19/. Cross-reaction of antibodies towards mammalian estrogen receptors with receptors of the hen has also been reported /18/.

Recently Péczely /15, 16/ demonstrated the presence of nuclear glucocorticoid, androgen, oestrogen and progestin receptors in isolated follicles of resting and growing feathers. In these radioreceptor assay studies the moulting regulating role of these receptors was shown.

Although we report here the first immunocytochemical evidence that feather papillae and growing feathers actually are target tissues for ovarian steroids, the mechanistic interpretation of the role of these hormones in the process of moulting remains obscure. The most significant aspect seems that both progesterone and estrogen receptors are apparently present in the same tissues, but that only the estrogen receptor remains well represented, in the second part of feather development, during the differentiation of the epidermal collar into barbs. This fits with the visual evidence for the role of estradiol in the differentiation into sex-specific patterns of plumages and features of feathers (see /3/). In this context, it is very likely, indeed, that particularly also the melanocytes have estrogen receptors. How the decrease in estradiol, but especially progesterone should result in moult initiation is, however, not clear.

Progesterone induces a protein that destroys estrogen receptor /8/ and it also depresses nuclear estrogen receptor binding /13/. As a consequence, the tenfold reduction of plasma progesterone levels observed during egg laying pause /5, 22/ could allow a much greater endocrine efficiency of the "only halved" plasma estrogen levels. It has, indeed, been shown that progesterone inhibits estradiol dependent tissue growth and differentiation in the oviduct (Schimke et al., 1975, quoted by Mester and Baulieu /10/), but to invoke a direct effect of estradiol, it remains to be demonstrated that physiological doses of estradiol can induce mitosis in dormant feather follicles and/or can enhance it in growing feathers. Anyway, the downregulation of progesterone on estrogen receptors at cellular level may account

for its moult-inhibiting effect during egg laying, and for the presence of both receptors in the same tissues, as demonstrated.

The presence of steroid receptors in several structures in the skin adjacent to feather tracts is not very surprising, because the process of moulting also affects parts of the skin other than the feathers: e.g. the epidermis also sloughs off, and vascularization increases remarkably. We have no direct explanation for the function of steroid receptors in smooth muscles in the dermis, but the finding is parallel with the presence of progesterone receptors in smooth muscles in the reproductive tract of the hen /2, 17/. The changes of serum levels of both steroid hormones and possible interactions at the receptor level in target tissues, such as the skin, do, however, not exclude interaction with thyroid hormones as far as moult initiation is concerned. The increase of thyroxine and decrease of T3 is indeed preceeding the moult process /5/, while blocking the T4 to T3 conversion enhances the process once started (Decuypere, unpublished observations).

Acknowledgements

It is a pleasure to acknowledge here the generous co-operation of Dr. D.O. Toft (Mayo Clinic, Minnesota) and Prof. B. Moncharmont (Universitá di Napoli) who provided us with aliquots of monoclonal antibodies to progesterone and estrogen receptors, respectively. M. Herremans and L. Berghman were supported by the National Fund for Scientific Research (Belgium) as senior research assistants.

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ROUTINE OXYGEN CONSUMPTION IN DIFFERENT SIZES OF A TILAPIA, OREOCHROMIS NILOTICUS (TREWAVAS) USING THE CLOSED CHAMBER RESPIRATORY METHOD

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Routine oxygen consumption ($\dot{V}o_2$) measurements on 54 specimens (0.055-190.4 g) of a tilapia, Oreochromis niloticus (Trewavas) were carried out using two different types of closed respirometers: a modified cuvette for fish weighing 0.055-0.91 g and ordinary closed chamber respirometer for fish weighing more than 1 g. $\dot{V}o_2$ values over the weight range studied had a scaling value of 0.743 which relates closely to the values for the gill respiratory surface area and morphometric oxygen diffusing capacity of O. niloticus in a previous study /13/. This shows that a close relationship exists between changes in structural parameters involved in oxygen uptake and the routine metabolism of O. niloticus with development. The values for routine $\dot{V}o_2$ of 1.38 and 7.65 ml/h for 10 g and 100 g fish, respectively (calculated from the regression equation) show that O. niloticus is a moderately active fish.

Keywords: Tilapia — oxygen consumption — body weight

Introduction

Routine oxygen consumption ($\dot{V}o_2$) has been the most commonly measured rate of oxygen consumption /8/. It has been used in determining the amount of oxygen consumed by fish whose only movements are spontaneous /2, 3/. Such movements are seen in fish with restricted movements. Similar work has been done on $\dot{V}o_2$ in relation to fish body weight in cichlids /16/. This has shown variation in the amount of oxygen consumed due to anatomical, physiological, organismic and environmental factors. Fish have tended to show a daily cycle

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of activity /8/ even after all usual precautions to protect them from disturbance which shows the response to directive effect of the environment. Most of the studies on routine $\dot{V}o_2$ have been done on temperate fish. Some studies on routine $\dot{V}o_2$ measurements in larval and post-larval stages of fish have been on the herring, Clupea harengus L. /10, 14/, the rainbow trout, Salmo gairdneri R. /15/, the cod, Gadus morhua L. /6/, the flouder, Platichthys flesus L. /1/ and the tilapia, Oreochromis niloticus L. /7/.

The purpose of the present study was to investigate how routine $\dot{V}o_2$ measurements in Oreochromis niloticus change during development since routine $\dot{V}o_2$ represents the energy requirements of fish under normal conditions in life. An attempt was made to relate these changes to changes in gill respiratory surface area and morphometric oxygen diffusing capacity in the same fish /13/.

Materials and Methods

The fish used in determining routine $\dot{V}o_2$ were obtained from the Institute of Aquaculture, University of Stirling (Scotland). They were kept at the Research Unit for Comparative Animal Respiration, University of Bristol. Fish fry were fed on body fish food 'E' for egglayers (Tetramin^R) and bigger fish were fed on Mainstream expanded trout diet pellets (BP nutrition). The fish were kept at 25 ^oC.

Routine $\dot{V}o_2$ determination

Fish weighing up to 1 g body weight

38 fish weighing 0.055-0.91 g (wet weight) were used in estimating routine $\dot{V}o_2$. The experimental set up consisted of a cuvette (Fig. 1) modified from that described by Hughes et al. /11/ for the volumetric method of measurement of oxygen content of small samples of fish blood. The cuvette contained a 16.5 ml capacity chamber for the fish. The tip of a Po_2 Radiometer electrode type E5046-0 was in contact with the cuvette chamber. The electrode was connected to a Strathkelvin oxygen meter model 381. The water in the cuvette was maintained at 25 ^oC.

Fish used in the experiment had been without food for about 20 hours. Fish were allowed to acclimatize in the chamber for at least 5 hours before air supply was switched off and $\dot{V}o_2$ measured for up to 30 minutes.

Fish weighing more than 1 g

16 fish weighing 2.7-190.4 g (wet weight) were used in estimating routine $\dot{V}o_2$ using the closed chamber respirometer (Fig. 2). It had a capacity of 972 ml. The water temperature was maintained at 25 ^oC. A small tube from the top of the respirometer box circulated water through a cuvette chamber (had a Radiometer electrode type E 5046-0) to another tube which led to a pump and back to the respirometer box.

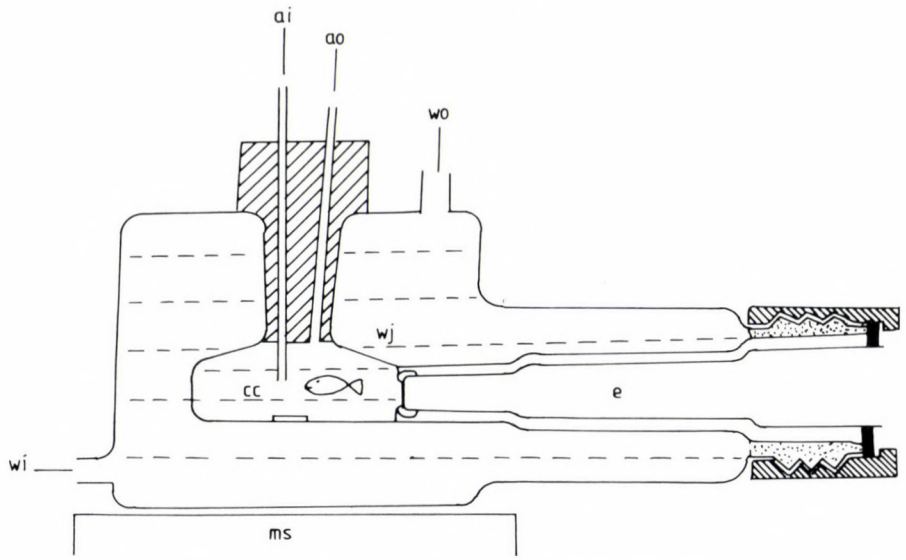

Fig. 1. Diagram of the cuvette used in measuring oxygen consumption in fish less than 1 g body weight. cc: cuvette chamber; wj: water jacket; e: electrode; ai: air inlet, ao: air outlet; wi: water inlet; wo: water outlet; ms: magnetic stirrer

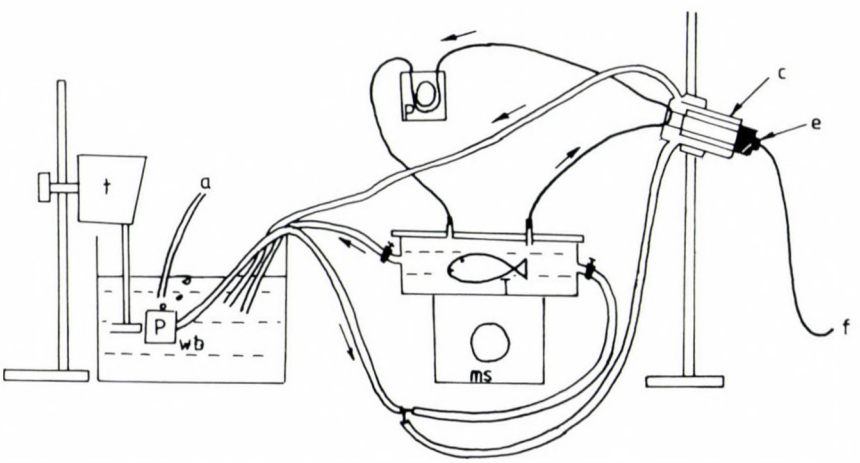

Fig. 2. Diagram of experimental set-up for measuring oxygen consumption in fish more than 2 g body weight. T: respirometer; ms: magnetic stirrer; c: cuvette; e: electrode; p: pump; wb: water bath; a: air supply; t: thermostat

Fish used in routine $\dot{V}o_2$ measurements were not fed for about 20 hours. They were allowed 20-24 hours to acclimatize before $\dot{V}o_2$ was measured. Water circulation through the respirometer box from the water bath was stopped before $\dot{V}o_2$ was measured. There was continuous circulation of water through the cuvette. $\dot{V}o_2$ was measured for 20-30 minutes.

Results

The smaller fish took a shorter time to acclimatize to the respirometer chamber than the larger fish which was taken into consideration before starting measurements on routine $\dot{V}o_2$. No detectable fall in respirometer water temperature was observed at end of measurements after water circulation through the respirometer box from the water bath was stopped (before $\dot{V}o_2$ was measured) for fish weighing more than 1 g.

Routine $\dot{V}o_2$ in relation to body weight was analysed according to the relationship:

$$\dot{V}o_2 = aW^b,$$

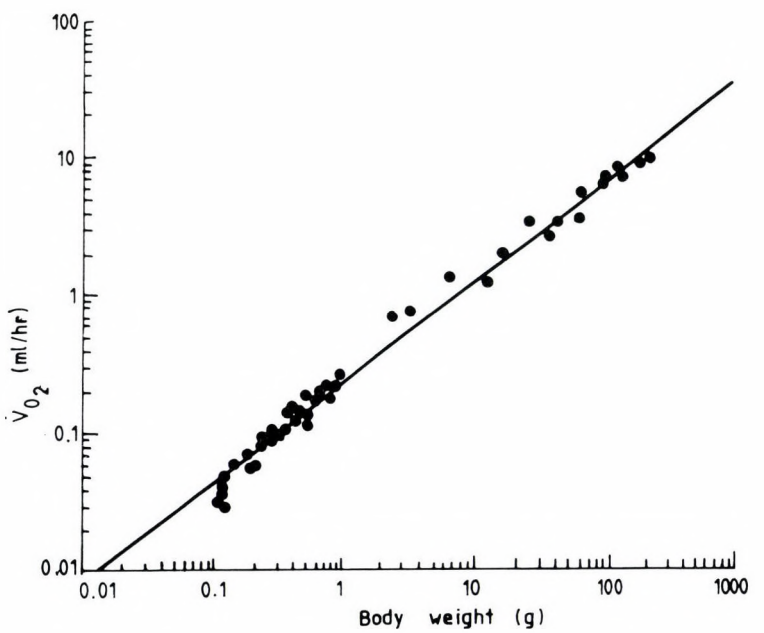

Fig. 3. Bilogarithmic plot of routine oxygen consumption ($\dot{V}o_2$) against body weight (using values of individual fish in present study). $\dot{V}o_2 = 0.23W^{0.743}$

Table 1

Mean routine $\dot{V}o_2$ values (± S.D.) of different weight groups of 54 *O. niloticus* at 25 °C

Weight group (g)	No. of fish in group	ml/h
0.055—0.15 0.0114 Average: 0.11 ± 0.03	8	0.0404 ± 0.0114
0.16—0.3 0.021 Average: 0.24 ± 0.05	9	0.0902 ± 0.021
0.31—0.5 Average: 0.39 ± 0.05	10	0.134 ± 0.021
0.51—1.0 Average: 0.67 ± 0.14	11	0.194 ± 0.043
1.1—10 Average: 3.9 ± 2.02	3	0.96 ± 0.39
11—50 Average: 23.6 ± 10.6	5	2.59 ± 0.95
51—100 Average: 70.5 ± 17.9	4	5.81 ± 1.64
101—200 Average: 147.1 ± 38.4	4	8.76 ± 1.29

where a is the $\dot{V}o_2$ value at W = 1 g, W is the fish weight and b is the slope of the line. Table 1 shows a summary of the $\dot{V}o_2$ values of different weight groups in the study. $\dot{V}o_2$ increased with body weight by a scaling value of 0.743 (Fig. 3). The increase in body weight and $\dot{V}o_2$ in present study were 3460-fold and 366-fold respectively which shows a decline in $\dot{V}o_2$ per unit fish body weight with increase in fish size.

Discussion

Routine $\dot{V}o_2$ is reflection of normal body metabolism (but does not indicate all the detailed metabolic processes occurring in fish) though some fish, for example crucian carp /4/ and goldfish /9/ are not obligate aerobes. Table 2 compares the scaling values for routine $\dot{V}o_2$ of several fish with that of *O. niloticus* in the present study. In a study of the same tilapia species as in present study, De Silva et al. /7/ obtained a weight exponent for metabolic rate of 0.419 and a $\dot{V}o_2$ value for 1 g fish of

Table 2

Routine $\dot{V}o_2$ regression equation values of some fish for comparison with those of *O. niloticus* in the present study. W value is in grams. The 'a' units (y value for w = 1 g) have been converted into ml/h for 1, 5, 7 and 15)

Species	Range in body weight (g)	Temp. (^{o}C)	Regression equation	References
The flounder (*Platichthys flesus*)	0.0095—0.56	20	$0.209W^{0.801}$	Al-Kadhomiy /1/
Rainbow trout (*Salmo gairdneri*)	0.1—20	15	$0.189W^{0.65}$	Morgan /15/
Tilapia (*Sarotherodon mossambicus*)	10—150	25	$0.378W^{0.646}$	Caulton /5/
Tilapia (*O. niloticus*)	up to 1 month old fry	30	$9.68W^{0.419}$	De Silva et al. /7/
Tilapia (*O. niloticus*)	0.055—190.4	25	$0.250W^{0.743}$	Present study

9.68 ml/g dry weight (for fry up to 30 days old). The scaling value is lower than that of 0.743 for routine metabolism and the $\dot{V}o_2$ value for 1 g fish is much higher than that obtained in present study. This could be due to the higher energy demand of organogenesis in tilapia larvae as compared to larger tilapia and also the higher temperature (30 ^{o}C) at which the study /7/ was carried out as compared to that at 25 ^{o}C in present study. $\dot{V}o_2$ values are also expressed in dry weight in the study /7/ but as wet weight in present study.

The role played by cataneous respiration in tilapia larvae needs to be investigated as well though this might not be significant as scales develop in the first few days of life and gills develop very early in tilapia as is seen in a 0.011 g *O. niloticus* which had a gill secondary lamellar area of 21.3 mm^2 or 1936.4 mm^2 per gram of body weight /12/.

The scaling value of 0.74 for routine $\dot{V}o_2$ in present study relates closely to that of gill surface area of 0.78 and morphometric oxygen diffusing capacity of 0.70 in *O. niloticus* /13/ which shows a close relationship in scaling exists between changes in gill respiratory dimensions affecting diffusion of oxygen into the gills and routine $\dot{V}o_2$ (which is a measure of overall routine metabolism) of *O. niloticus*. Since the gills are

the main site of oxygen uptake in tilapia (as the fish lacks accessory air breathing structures and its body is covered entirely by scales) changes in branchial circulation with increase in fish size might scale closely to the value for routine $\dot{V}o_2$ of 0.743.

A study of red muscle in O. niloticus has shown that the scaling values for its total mitochondrial volume, capillary length and capillary area in contact with fibres are 1.13, 1.02 and 1.07, respectively /12/. Body systems are likely to have different scaling values for parameters such as capillary transit time, haematocrit and oxygen permeation coefficient than that for routine $\dot{V}o_2$.

Routine $\dot{V}o_2$ measurements in O. niloticus in the present study show the routine energy demands of O. niloticus but do not tell us the detailed metabolic processes occurring in the fish. The energy requirements of each of these processes as a fraction of the overall energy amount needed by fish are difficult to determine and are likely to vary according to species, age, sex, activity and environmental factors.

From the regression equation for routine $\dot{V}o_2$ in O. niloticus of $0.25W^{0.743}$, a 10 g and 100 g fish will have values of 1.38 and 7.65 ml/h, respectively. These values are close to those of another related tilapia, Sarotherodon mossambicus of 1.67 and 7.40 ml/h for 10 g and 100 g fish /5/, respectively. These values show that both tilapia are moderately active fish. The higher scaling value of 0.743 obtained for $\dot{V}o_2$ in present study as compared to that of 0.646 /5/ could be due to the large number of smaller fish used in the present study as compared to the number in later study. A scaling value of 0.63 was obtained for O. niloticus greater than 1 g body weight in present study.

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EFFECT OF SUBLETHAL CONCENTRATION OF MERCURY AND ZINC ON THE ENERGETICS OF A FRESHWATER FISH CYPRINUS CARPIO (LINNAEUS)

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Exposure of a freshwater fish Cyprinus carpio to the sublethal concentration of mercury (0.1 mg L^{-1}) and zinc (6.0 mg L^{-1}) resulted in distinct changes in the energy metabolism of gill, liver and muscle at 1, 15 and 30 days. The changes were:

(a) The rate of oxygen consumption and SDH activity decreased in the organs of mercury-exposed fish at all the three exposure periods in the order $1 > 15 < 30$ days. Whereas, an increase was observed in these parameters in the organs of zinc-exposed fish in the order $1 > 15 > 30$ days.

(b) The activity of LDH and the levels of pyruvate and lactate increased in all the three organs of the fish at the three exposure periods studied in both the metal media. But, this increase was also in the order $1 > 15 < 30$ days and $1 > 15 > 30$ days in the organs of the fish exposed to mercury and zinc, respectively.

(c) The results indicated greater reliance of mercury exposed fish on the energetically less efficient anaerobic glycolysis as the oxidative metabolism suppressed, and the dependency of zinc-exposed fish on both the oxidative and anaerobic glycolytic pathways in order to meet the energy requirements. On prolonged exposure zinc-exposed fish could adapt to sublethal toxic stress, such type of adaptive-response was not observed in mercury-exposed fish.

Keywords: Mercury — zinc — energy metabolism — fish, Cyprinus carpio L.

Introduction

Nowadays freshwater bodies have become the repositories of pollutants released from a number of industries in the form of effluents, of which heavy metals are predominant. Mercury and zinc, the divalent group IIB

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metals, are among the few which are most prevalent toxic metals concentrated in freshwater bodies beyond to the permissible levels. The production of mercury, a nonessential element to any biological activity, greatly increased during this century with the rapid industrialization /21/. Effluents from the industries manufacturing paints, pesticides, fertilizers etc., consisting of large quantities of mercury are entering into the freshwater environment. It is also released from the manufacture of electrical equipment and electrolytic production of chlorine and caustic soda /19/. Zinc though essential for certain biological functions in minute quantities, beyond the requirement it is highly toxic to freshwater fauna /37/. The global production of zinc has also been increasing steadily during this century and has almost doubled during the last decade through mine and smelters. The largest use is in galvanizing iron and steel products, brass products, and zinc-based alloys. It is also used in synthetic rubber, paints, cosmetics, dyeing of textiles and purification of fats /19/.

Freshwater fishes hold a strategic position in the food-web as they serve as a staple food for human beings. Reports on the effects of mercury and zinc on the energetics of freshwater fishes revealed hyperglycemia, glycogenolysis /11/, gluconeogenesis /28/, inhibition of TCA cycle enzymes and elevation of glycolysis /35/. But these studies are mostly on acute toxicity which have significant limitations such as the occurrence of adaptation. Hence sublethal toxicity studies gained more importance /22/. The survival and productivity of freshwater fishes in the medium containing pollutants depend on their adaptive ability which in turn relies on their energetic efficiency. Thus a measure of energetics is the sensitive parameter since it is the factor responsible for the physiological response of these animals to pollutants /7/. As no published information is available comparing the effects of mercury and zinc at sublethal concentration on the energetics of freshwater fish, it is felt worthwhile to study the effects of these metals on the rate of oxygen consumption, activities of succinate and lactate dehydrogenase and the levels of pyruvate and lactate in the gill, liver and muscle of the commercially important freshwater fish Cyprinus carpio. Since the period of exposure of an animal to any stress is known to have great influence over its metabolism /26/, the present study was carried out at three different exposure periods, 1, 15 and 30 days, to analyse the short- and long-term changes in the energetics of the said organs of the fish subjected to sublethal mercury and zinc stress.

Materials and Methods

Cyprinus carpio (Linnaeus) weighing 20 ± 1 g were procured from the Andhra Pradesh State Fisheries Department and were maintained in the laboratory in 5' x 3' x 3' cement tanks, fifty in each. Water from the local wells was used for their maintenance. It had pH 7.6 ± 0.2, total hardness 100 ± 5 mg/l $CaCO_3$, temperature 28 ± 1 ^{o}C and oxygen content 5.79 ± 0.4 mg/l. The fish were fed daily with commercial food pellets having around 40% protein content. The fish consumed an average of 3 mg/g wet wt/day which is almost the standard consumption as reported by Hastings /13/. Water was changed once a day. The animals were allowed to adapt themselves to laboratory conditions for ten days prior to experimentation. Later, groups of thirty fish were exposed separately to different concentrations, ranging from 0.2 to 0.8 mg/l and 15 to 40 mg/l, of mercury and zinc, respectively. LC_{50}s for 96 h of both the metals were derived from the percent and probit mortality versus 1/Log concentration curves /9/ and were subsequently verified by Dragstedt and Behren's method /6/. The 96 h LC_{50}s for mercury and zinc were 0.5 mg/l and 30 mg/l, respectively. One fifth of these LC_{50}s, i.e. 0.1 mg/l for mercury and 6 mg/l for zinc, were taken as sublethal concentrations to carry out the further experimentation. Groups of ten fish were exposed to the respective sublethal concentration and at the end of each exposure period, 1, 15 and 30 days, gill, liver and muscle were isolated from the fish survived, 10, 8 and 5 at the respective periods in mercury and 10, 9 and 10 in zinc, and were transferred to cold fish ringer solution prepared as per the composition given by Ekberg /8/. The rate of oxygen uptake of these was measured in a Gilson 5/6 oxygraph, and the activities of succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH) and the levels of pyruvate and lactate were estimated using the standard experimental procedures as described by Nachlas et al. /20/, Srikanthan and Krishnamoorthi /31/ modified by Govindappa and Swami /12/, Friedman and Hangen /10/ and Barker and Summerson /5/ modified by Huckabee /15/, respectively.

Estimation of SDH activity

Reaction mixture: 0.2 ml of 0.4 M phosphate buffer (pH 7.7), 0.2 ml of 0.2 M sodium succinate, 1.0 ml of 0.004 M 2-(P-indophenol)-3-P-nitrophenyl-5-phenyl tetrazolium chloride (INT), 0.1 ml of 0.005 M phenazine methosulphate and 0.5 ml of 5% enzyme extract. After extracting the formozan formed into 6.0 ml toluene overnight at 0 ^{o}C, the extraction was measured at 495 nm.

Estimation of LDH activity

Reaction mixture: 1.0 ml of 0.4 M phosphate buffer (pH 7.4), 0.5 ml of 0.1 M lithium lactate, 0.1 ml of 0.0001 M nicotinamide dinucleotide (NAD), 1.0 ml of 0.004 M 2-(P-indophenol)-3-P-nitrophenyl-5-phenyltetrazolium chloride (INT) and 0.5 ml of 5% enzyme extraction. After extracting the formozan formed into 6.0 ml toluene overnight at 0 ^{o}C, the extraction was measured at 495 nm.

Similar measurements/estimations in the organs of fish maintained in freshwater with 0.1 ml of hydrochloric acid per liter, to nullify the chloride effect, served as controls. The data were statistically computed with mean, standard deviation and student 't'-test.

Results

Relative to controls, a significant ($P < 0.05$) decrease was observed in the rate of oxygen consumption and SDH activity of gill, liver and muscle of the fish C. carpio at 1, 15 and 30 days on exposure to sublethal concentration of mercury. The decrease was in the order $1 > 15 < 30$ days, with significant ($P < 0.05$) differences between 1 and 15, 15 and 30 days

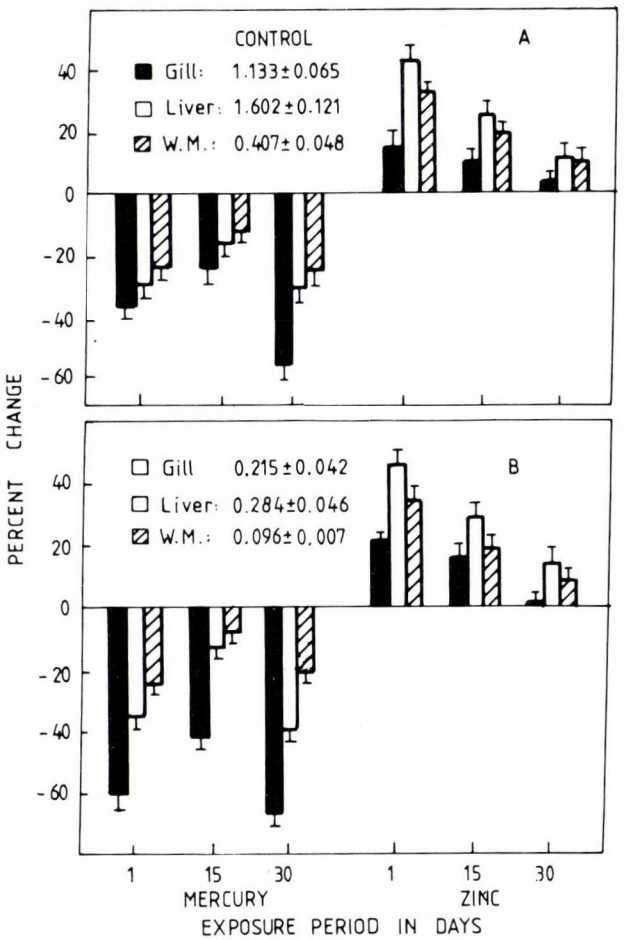

Fig. 1. Percent change over control in oxygen consumption (A) and SDH activity (B). Vertical bars represent standard deviation.
W.M.: White muscle. Controls: Oxygen consumption O_2 µM/g/5 minutes, SDH activity, µM formozan/mg protein/h

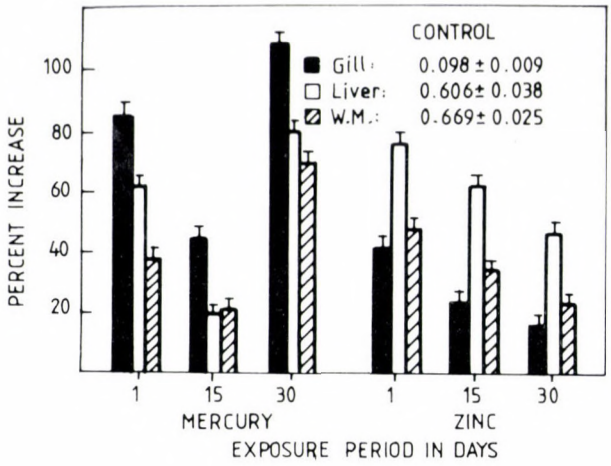

Fig. 2. Percent increase over control in LDH activity. Vertical bars represent standard deviation.
W.M.: White muscle. Controls: μM formozan/mg protein/h

(Figs 1A and B). Interestingly, in the organs of the fish exposed to sublethal concentration of zinc a significant $P < 0.05$) increase was observed in the rate of oxygen consumption and SDH activity at 1, 15 and 30 days compared to their controls. This increase was in the order $1 > 15 > 30$ days with significant ($P < 0.05$) differences between 15 and 30, 30 and 1 day (Figs 1A and B). An increase was observed in LDH activity and pyruvate and lactate levels in the gill, liver and muscle of the fish at 1, 15 and 30 days of exposures to sublethal concentrations of both mercury and zinc. However, in any organ at any corresponding period of exposure this increase was greater in the fish exposed to mercury than zinc, the difference of which was mostly significant ($P < 0.05$). Further, the increase was in the order $1 > 15 < 30$ days, with significant ($P < 0.05$) differences in between, in mercury-exposed fish, whereas the order was $1 > 15 > 30$ days in those exposed to zinc with significant ($P < 0.05$) differences between 30 and day (Figs 2 and 3).

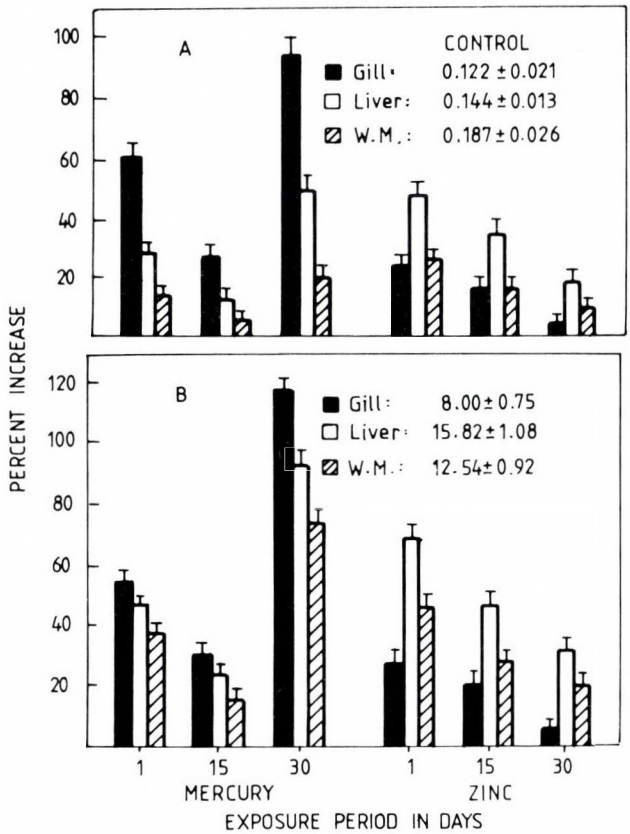

Fig. 3. Percent increase over control in pyruvate (A) and lactate (B) levels. Vertical bars represent standard deviation.
W.M.: White muscle. Controls: Pyruvate and lactate mg/g wet wt

Discussion

Oxidation of pyruvate in Kreb's cycle under aerobic conditions produces more energy but it requires oxygen. If required oxygen is available, pyruvate enters the TCA cycle after decarboxylation to acetyl Co-A. As pollutants gain entry largely through the gills of fishes, any change in their normal respiratory epithelium would affect the rate of oxygen consumption /18/. SDH, a flavin linked enzyme of Kreb's cycle, catalyzes the reversible oxidation of succinate to fumarate and serves as a link between electron transport system and oxidative phosphorylation. Hence the activity of it

can be taken to reflect the rate of operation of TCA cycle in different organs to the fish under the situations of stress /23/.

The findings in the present study reveal the suppression of oxidative metabolic pathway in the gill, liver and muscle of the fish by the interference of mercury ions /2/. The decrease in the oxygen consumption might be due to the damage caused to the normal respiratory area of the gill surface by the intimate contact of the gill with the metal /30/. It could lead the lowering of diffusion of oxygen through the gills /16, 17/, thereby the transport of it to the liver and muscle also decreased. The decrease in oxygen consumption can even be due to the impairment of the structural integrity of other organs /4/ and decreased hemoglobin content /32, 33/. The mercury induced drop in SDH activity can be correlated to the binding of the metal ions with the active sites of the enzyme, specifically at -SH group, and derangement in architectural integrity and permeability of mitochondria /14, 36/. Greater suppression of oxygen consumption and SDH activity at 1 day could be due to the speedy diffusion of metal ions on sudden exposure of the fish to toxic medium. Whereas, less suppression at 15 days than at 1 day indicates the efforts taken by the fish to bring its organ energetics from dropping further to depressingly low level, which could be possible by the activation of some metal detoxication mechanisms. But on further exposure high decrease in oxygen consumption and SDH activity at 30 days indicates the domination of mercury accumulation over its detoxification on prolonged exposure.

The elevation in oxidative cycle in the organs of zinc-exposed fish suggests that this metal at sublethal concentrations is less harmful to fish. Probably, for the elimination/detoxication of the rapidly intruded metal ions the animal elevated its oxidative cycle by increasing its oxygen uptake and SDH activity. It seems that the zinc ions might have not interfered with SDH activity or the structural organization of mitochondria. High increase in oxidative metabolism at 1 day could be to resist the metal influx, as there will be intrusion of more ions during initial period of exposure. The gradual fall in the increased oxidative metabolism, and its restoring to normality on 30 days indicate the fish is able to adapt to lower concentrations of zinc on prolonged exposure.

If the availability of oxygen to the fish decreases, the hypoxic stress results the conversion of pyruvate to lactate, mediated by the enzyme LDH, to meet the energy demands /27/. In the present study the increase observed in LDH activity in the organs of fish exposed to mercury revealed the

reliance of the fish on anaerobic glycolysis in meeting the energy demands during toxic stress. This type of metabolic shift is strategic. Asztalos and Nemcsok /3/ correlated the magnitude of increase in LDH activity in the carp tissues to the tissue damage and organ necrosis. Due to the suppression of oxidative metabolic pathway, pyruvate accumulation increased in the organs, a part of it is converted to lactate by the elevation of LDH activity. The degree of elevation in LDH activity appears to be proportional to the magnitude of suppression of oxidative cycle. Hence there is a more elevation at 30 days. Increase in pyruvate and lactate levels could interfere with acid-base balance and decreases the survival ability of the fish /25/.

The significant increase observed in LDH activity and lactate level in the organs of the fish at 1 day of exposure to zinc suggests the enhanced anaerobic glycolysis in addition to the oxidative pathway, to meet the energy demands for metal detoxication/elimination. But, the hike in pyruvate in the organs is paradoxical as the operation of both the metabolic pathways increased. The probable reason could be due to increased glycogenolysis and gluconeogenesis /24/. However, all these shifts restored to normalcy on 30 days of exposure. These findings thus strengthen that C. carpio can resist the sublethal zinc stress by suitable modulation in its energetics, and can adapt to such stress on a prolonged exposure. This type of adaptation is not noticed in the fish exposed to mercury stress, as the fish is continuously under the pressure of respiratory distress. Probably the permeable rate of mercury into the organs of fish might be significantly more than zinc /29/.

More suppression in oxygen consumption and SDH activity and elevation in LDH activity and levels of pyruvate and lactate in the gill of fish exposed to mercury could be due to the clogging of mucus over the gill surface and the damage caused to gill lamellae /1/. Hence, the anaerobic glycolytic pathway greatly enhanced. Minimal changes in oxidative and glycolytic pathways in the gill of fish exposed to zinc indicate the non-interference of this metal either with the structural or functional status of this respiratory organ. Decreased oxidative cycle in liver, could become an obstacle in deriving the required energy for various metal detoxication mechanisms in mercury exposed fish. The accumulation of pyruvate and lactate could cause hepatic cirrhosis /34/. More increase in oxygen consumption and SDH and LDH activities in the liver than other two organs of the fish exposed to zinc suggests that this organ is playing an active role in detoxication/elimination of the excess metal accumulated in it. Minimal changes observed in oxidative and glycolytic pathways in the muscle of fish exposed to mercury

indicate that this metal is mainly affecting the highly active organs like gill and liver than the muscle. But, in zinc-exposed fish the participation of this effector organ is relatively more than gill in supplementing the required energy.

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METAL TRANSFER IN MARINE FOOD CHAINS: BIOACCUMULATION AND TOXICITY

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Metal transfer in marine food chains may be examined at different scales from field studies to cellular level. Some years ago, the major question was to verify the existence of metal biomagnification, a phenomenon which seems in fact to be limited to mercury and the radionuclide cesium-137. In numerous cases, metal incorporation is not determined chiefly by the trophic level in the food chain. It is also controlled by both the metabolic characteristics of species or populations and the biological role of each element. The most accurate assessment of trophic transfer may be by determining the physico-chemical state of metals in marine organisms, a methology which can be used for both field and experimental samples. Some species exhibiting high detoxication ability may be interpreted as highly contamined links in food chains but according to the nature of the detoxifying ligands — in outline, mineral granules or metalloproteins — the bioavailability to the next trophic level may vary widely.

Keywords: Heavy metals — accumulation — toxicity — marine environment

Introduction

Different approaches may be envisaged with a view to examine the metal transfer in food chain. In field studies, marine species belonging to successive trophic levels are sampled. Metal levels are determined in their tissues and the whole body burdens are compared at the different steps of

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the food chain in order to precise the potential risk of biomagnification. This type of studies takes into account all the environmental factors thus its representativeness is very high but it does not allow to understand the processes which are responsible of metal incorporation.

Thus it has been necessary to develop experimental models of transfer, the representativeness of which is lower but since a large number of ecological and contamination factors may be controlled, this type of studies allows a better approach of processes involved in transfer.

A last approach is through the determination of the physico-chemical forms of pollutants in the food source, a factor which control the bioavailability to the next trophic level. This type of methodology may be applied to both environmental samples and artificially contaminated organisms.

1. Field studies of trophic transfer

Micropollutants introduced into the marine environment as a consequence of human activities can be accumulated at the lowest level of the food web, namely primary producers. The passage of contaminants through food webs has caused much concern in industrialized societies as the Minamata disease have revealed first the existence of a phenomenon of biomagnification of mercury in food chains and some other pollutants have shown appreciable signs of being biologically magnified: mainly insecticides, and among radionuclides cesium-137 /2, 17, 31/.

Some authors have considered the trophic transfer of contaminants from the point of view of specific predator-prey relationship (e.g. /6, 21, 36/), but more generally average trophic levels are taken into account as proposed by Amiard and Amiard-Triquet /2/ or Mearns et al. (1981, in /37/). The latter lead to an oversimplification of the food web structure. More recently, Young /37/ has proposed a chemical index, the cesium potassium ratio, in order to determine more accurately the trophic position of aquatic organisms. This technique is based upon the known biomagnification of cesium normalized to the essential metal potassium. In a food chain of Los Angeles Harbor, the trophic level assignment (TLA) was determined using the frequency, numerical abundance and weight or volume of prey items in organisms' diets. A significant relationship was shown between the Cs/K ratio and TLA (Fig. 1). Using the same approach for Ag, As, Cd, Cr, Cu, Fe, Hg, Mn, Sn and

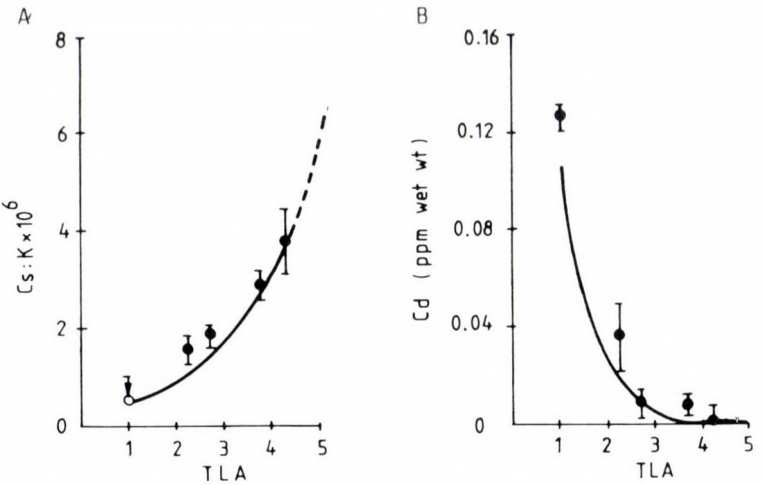

Fig. 1. The Cs/K ratio shows a statistically significant increase with the trophic level in a food web from Los Angeles Harbor (A) whereas Cd tissue concentration (and most pollutant trace elements except Hg) shows a decrease (B) (After Young et al., 1987)

Zn, it was demonstrated that most pollutant trace elements (such as Cd shown in Fig. 1) do not biomagnify in marine food webs with the exception of Hg /38/.

This approach is based upon the assumption that the major source of uptake is the organisms' food but in fact, feral organisms are exposed to metals present in different sources (water, sediment, food). Thus it is often difficult to decide if increased metal concentrations at different levels in the food webs result from a real biomagnification or from a specific ability to concentrate trace metals. From this point of view, the distribution of metals in coastal and estuarine biota provides an interesting basis to examine the limitations of field studies.

In the well-known food web of the Loire estuary, copper levels were determined in numerous species /4, 5, 8, 27/. As shown in Fig. 2, copper concentrations depend on the zoological group (highest levels in Crustaceans, the respiratory pigment of which is a haemacyanin) rather than on their feeding mode.

The distribution of metals in various compartments and organisms sampled from the bay of Bourgneuf (France) seems typical of Cd, Cu and Zn biomagnification (Fig. 3). However, it has been demonstrated, that metal incorporation is mainly governed by specific metabolism. Thus when oysters were experimentally exposed to soluble copper, they exhibited concentration

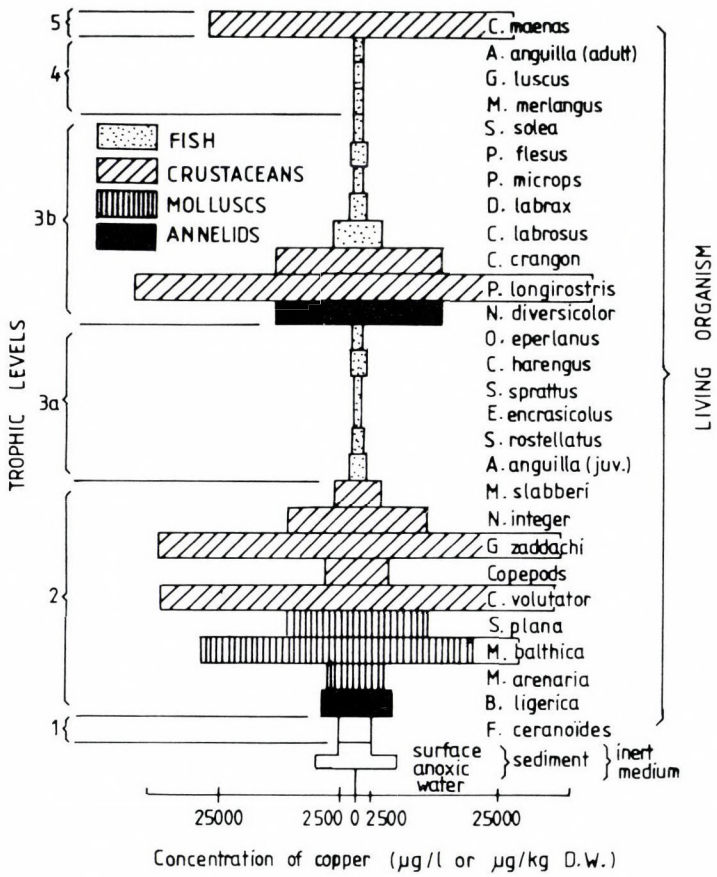

Fig. 2. Copper concentrations in different organisms from the Loire estuary according to their food habits (1: primary producers; 2: primary consumers; 3a: mainly plankton-feeders; 3b: omnivorous bottom-feeders; 4: supercarnivorous fish; 5: necrophagous). Copper bioaccumulation depends on the zoological group (highest levels in crustacean) rather than on their feeding mode (After Amiard et al., 1982)

factors which were considerably higher than those recorded for phytoplankton. Similarly, it has been shown that the level of Cu (and also Cd and Zn) in drills did not depend mainly on the food source as no changes occurred in individuals purged or receiving a diet with low copper content /6/. In Neogastropods, haemocyanin catabolism leads to the storage of Cu as sulphide in conjonctive cells, the number of which increases with age (Bouquegneau and Martoja, 1982 cited by Amiard-Triquet et al. /6/), and such a phenomenon is probably responsible of Cu accumulation in drills. Cadmium and zinc

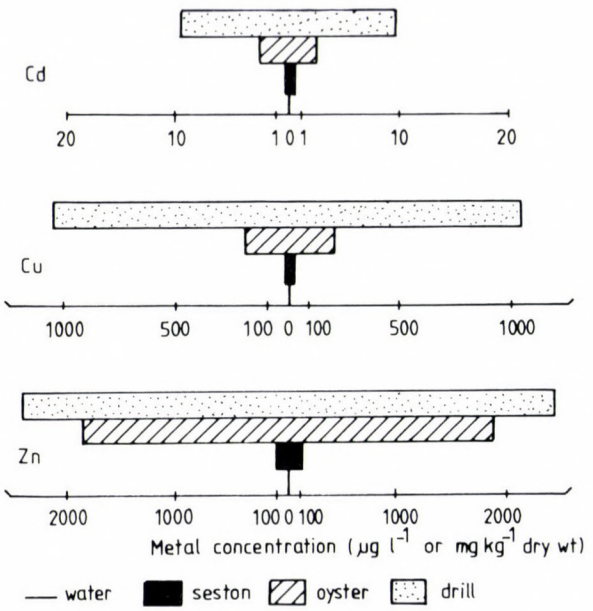

Fig. 3. Metal concentrations in various compartments and organisms sampled from an area devoted to oyster culture. After Amiard-Triquet et al., 1988. (With the permission of the publishers, Pergamon Press and the copyright holders, IAQW)

levels have been determined in different bivalve species collected within the same area (Chesapeake bay studied by Frazier, 1979; Bay of Bourgneuf by Berthet, 1986; cited by Amiard-Triquet et al. /6/): under similar conditions of exposure, the highest levels were always shown in oysters and at least in the case of zinc, the mechanism has been elucidated /29/.

Thus it is necessary to develop comparative methodologies in order to evaluate the respective roles of food (and sediment associated with food) and water in bioaccumulation of metals.

2. Studies of metal transfer in experimental models of food chains including filter-feeders

The bulk of the literature consists in studies about direct contamination of organisms by soluble metals. Only few studies were devoted to the particulate phase (microalgae and bacteria) as a potential source of micropollutants (see in /7/), especially to filter-feeder bivalves which serve as

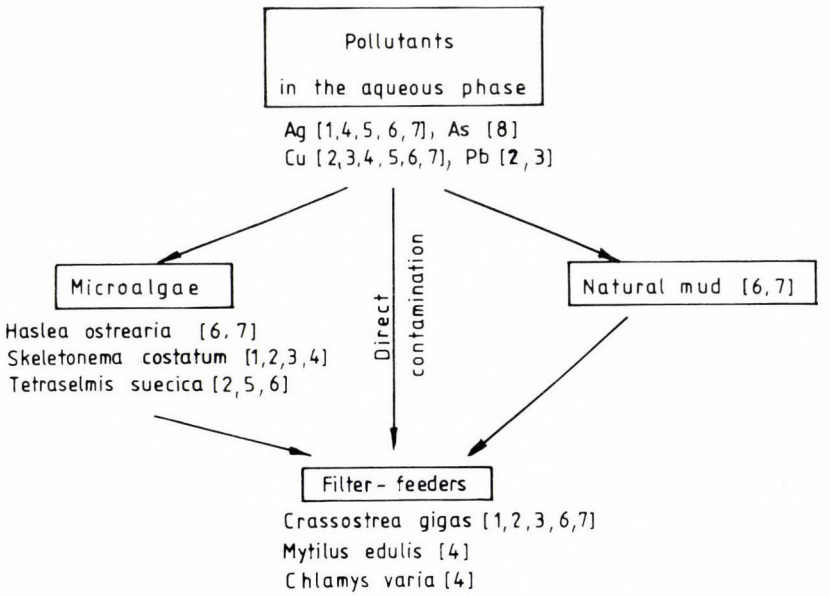

Fig. 4. Study of metal transfer in an experimental model of food chain including filter-feeders: different pollutants studies and various species introduced at each trophic level (1 — Martoja et al., 1988; 2 — Amiard-Triquet et al., 1988; 3 — Amiard et al., 1989; 4 — Métayer et al., 1990; 5 — Ballan-Dufrançais et al., 1991; 6 — Amiard-Triquet et al., 1992; 7 — Ettajani et al., 1992; 8 — non publ.)

international bioindicators of pollution, represent a significant economic resource in coastal regions (mussel, oyster, scallop) or play an important role as a food source in estuarine and coastal food webs. Although indirect evidence of pollutant exchanges between sediments and organisms has been brought /14, 20, 33/, the toxicity that contaminated sediment may introduce through metal transfer in the food web has not been examined extensively. In addition to the release of sediment-bound metals in the water column or pore water /14, 19/, sedimentary particles ingested by a benthic organism are submitted to digestion processes (pH changes, enzymes) leading to transformations of the crystal structure of some inorganic particles /9, 30, 32/ and a partial digestion of organic particles, with a potential release of associated pollutants in the gut.

The laboratory model here-proposed has been run for studying different pollutants. Different types of particles were examined including natural sediment and several species of microalgae. Different bivalves were included in the experimental model as filter-feeders (Fig. 4).

Mass cultures (several hundred liters) of algae were produced by using underground salt-water (Bouin, Vendée, France) naturally enriched with nutrients. Mud has been sampled in a coastal area devoted to oyster culture and the finest particles were selected in order to meet the feeding requirements of filter-feeders. For both types of particles, the levels of exposure to soluble metals were chosen in order to comply with three aims 1) to be consistent with levels which may be encountered in polluted environments; 2) to be consistent with a normal growth of algae; 3) to induce sufficient metal levels in particles so that a potential transfer to consumer may be measurable.

The detailed procedures have been described in previous papers /10, 15/. Cell harvesting was achieved by low shear crossflow microfiltration or centrifugation and algal food was stored by using a cryopreservation process /7/. Inert particles were collected by centrifugation and preserved by freeze-drying.

Bivalves exposed to metals, dissolved in seawater or loaded to particles, were distributed into experimental units with 10 specimens in 10 l of seawater. Controls were reared in uncontaminated seawater and received about 3 mg of uncontamined mud or phytoplankton several times a day. Identical quantities of contaminated particles were provided to groups tested for trophic transfer. In these conditions, the concentrations of suspended particles remained low enough to avoid the production of pseudo-faeces. In the case of direct contamination the overloads of metals introduced in the rearing medium of bivalves were generally similar to those used to load particles. Seawater and contaminant were renewed daily. One hour before renewal, all the oysters were provided with freeze-dried Spirulina.

Metal levels were determined in aliquots of phytoplankton, sediment and in the soft tissues of individual bivalves. The parameters of transfer were calculated for each experimental condition as exemplified in the case of copper in oysters (Table 1). For the groups exposed to copper dissolved in water, the metal overloads available daily for each oyster was 30 µg Cu since the concentration in seawater was 30 µg Cu/l, renewed every day, and the experimental density was one oyster per litre of breeding medium. The quantitiy of Cu available from suspended particles was calculated from amount of particles supplied and concentration of Cu in these particles. For these different sources, the quantity of metal available was determined per unit weight of bivalves over a finite period after natural levels

Table 1

Experimental contamination of oysters Crassostrea gigas by copper introduced in water (Group B) or associated with microalgae Haslea ostrearia (Group C) or natural mud (Group D). Data for a standard oyster (soft tissues: 1 g, dry weight) after Ettajani et al. /15/

Way of exposure	Length of exposure 8 days	14 days	21 days
Copper fluxes (µg) in soft tissue of a standard oyster (1 g dry weight)			
Group B	392	713	992
Group C	4	41	90
Group D	20	38	72
Copper overload (µg) available to a standard oyster from seawater			
Group B	960	1680	2520
Copper overload (µg) ingested with contaminated microalgae			
Group C	29	52	97
Copper overload (µg) ingested with contaminated sediment			
Group D	25	46	74
Copper overload (µg) released from sediment and available to a standard oyster from seawater			
Group D	16	31	49
Percentage of lead at disposal accumulated in soft tissues			
Group B	41	42	39
Group C	13	79	93
Group D	52	54	71

(measured in controls) were deducted. Using the concentrations of Cu accumulated by the oysters for each exposure period, it was possible to calculate the percentage of Cu retained and thus to evaluate the transfer to oysters. In the case of sediment, a part of the Cu initially bound to particles was released into the rearing medium of oysters and thus was available from water. In order to separate this pathway of uptake, we have therefore taken into account the concentration of Cu accumulated by the oysters form water.

Oysters exposed to metals dissolved in water exhibit generally the highest rates of accumulation as illustrated in the case of Cu (Fig. 5). The importance of metal accumulation is due at least partly to the fact that several pathways of input are involved. Oysters are able to accumulate

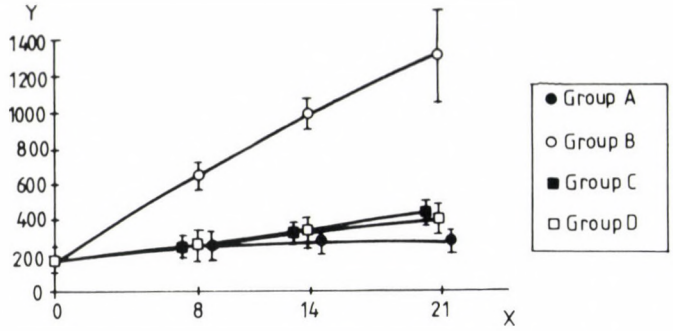

Fig. 5. Accumulation of copper in soft tissues of oysters after direct contamination (B) or exposure through phytoplanktonic food (C) or sediment (D). Group A: Controls. X: Length of exposure (days); Y: Concentration (mg Cu/kg D.W.) (After Ettajani et al., 1992). (Reprinted with the permission of Kluwer Academic Publishers)

metals through the mantle epithelium, the gill surface and the gut. On the other hand, in contamination via food or sediment, the ingestion of particles is the only pathway involved.

Table 2 allows to compare the retention of Ag, As, Cu and Pb, according to the source of metals. Generally, bivalves retained a higher percentage of metals from particles — microalgae or sediment — than from water. Thus the food pathway could play a significant role in metal accumulation as a consequence of long-term exposure. In the case of oysters, among the four studied metals, Cu is the most easily transferred whereas no transfer occurs in the case of As. Considering the results of direct uptake from water, the same pattern may be observed: an important accumulation of Cu, whereas As accumulation was undetectable. This reflects the fact that metal absorption is controlled partly by the biological role of each metal. Moreover, it must be duly noted that even if no incorporation has been demonstrated, the temporary uptake of arsenic induced cytological effects such as an increase of the number of lysosomes and structural alterations of mitochondria and endoplasmic reticulum, suggesting a disturbance of the cellular respiratory and energetic metabolism.

Since the percentage of available silver retained by each species has been determined per unit weight of soft tissues over a finite period, Table 2 allows to compare the specific ability to concentrate silver. Independently of the way of exposure, scallops (Chlamys varia) exhibit always the highest percentages of retention. Thus the characteristic metabolisms of bivalve species also influence greatly metal absorption but even different

Table 2

Percentage of metals dissolved in seawater or associated with particles accumulated in soft tissues of bivalves (14 d exposure)

Species	Contaminant	Direct uptake	Exposure to the particulate phase		
			Haslea ostrearia	Skeletonema costatum	Sediment
Mussel	Ag 20 µg/l	1.1		11.8	
Scallop	Ag 20 µg/l	37		83	
Oyster	Ag 20 µg/l	8.6		12.4	
Oyster	Ag 10 µg/l	0.4	8.3		
Oyster	As 10 µg/l	ND			ND
Oyster	Cu 30 µg/l	42.4	79		53.7
Oyster	Pb 70 µg/l	5.4			1.0

ND: not detectable

populations in the same species can show highly variable metal absorption as exemplified by silver accumulation in oysters /12/.

The major difficulty to overcome in order to generalize the methodology of contamination here-proposed is to determine the alimentary demands of filter-feeders since responses to the presence of particles may be completely different between nearly-allied species. Filtering organisms select the size of the particles they consume and the selection is characteristic of each species. Moreover, the efficiency of ingestion may vary depending on the nature of the particle. Thus oysters are able to consume largest particles when they are rich in organic mater. It is obvious that certain species, at least, are capable of sorting among particles to select organic matter and living organisms such as bacteria /1/. Once again, the response of filter-feeders to bacterial food may vary widely from species to species. Thus a bacterial strain which had been used successfully as a vector of transfer of Cd from water to mussels /16/ was assayed as food for oysters. Individual oysters received bacteria, five times a day over a period of two weeks. Seawater was renewed daily. As soon as the third food intake had been provided, seawater turned milky although bacteria have been filtered by oysters as verified by their enumeration. This is probably due to mucus which has been discharged by oysters exposed to bacteria /7/.

In addition, the quantity of seston present in the water column controls the ingestion, through two phenomena: filtration ratio and digestive

transit time. This is due to the fact that many filter-feeders cease to filtrate when turbidity is excessive; when it is moderately high, filter-feeding bivalves retain many particles on their gills but a (sometimes large) proportion bypasses the digestive tract to form pseudo-faeces. In cases of such overload, the digestive transit is considerably speeded up and digestion occurs only on a small portion of the ingested food /1/.

This type of methodology is heavy to bring into operation. It is impossible to be applied when large animal species are concerned and it does not give any information about the metal compounds bioaccumulated and then on their potential toxicity. Thus an alternative methodology have been proposed based upon the study of the physico-chemical forms of storage of trace elements at different levels of the food chain /7/.

3. Determination of the physico-chemical state of metals in marine organisms: contribution to the assessment of trophic transfer

The bioavailability of a metal to consumers does not depend only on its gross concentration in preys but also on its physico-chemical state. In some species exhibiting high detoxication ability, important quantities of metals can be accumulated. The role of these species as vectors of transfer in food webs is questionable.

Detoxication is achieved according to different kinds of processes. In vertebrates the major way of detoxication is through the binding of metals to peculiar metalloproteins, namely metallothioneins. In invertebrates, numerous processes are involved in detoxication but the preferential way is through insolubilization of metals as mineral concretions.

A tentative methodology has been developed in our laboratory in order to determine the distribution of metals in different fractions of animal tissues (Fig. 6). The first step consists in the separation of the insoluble fraction including mineral compounds responsible of metal-binding and the cytosol, through homogenization and ultracentrifugation of organisms or organs. The second step is based upon the heat-stable character of metallothionein-like proteins (MTLP) and it is carried out according to the method described by Thompson and Cosson /34/. Thus the heat-stable fraction is composed at least partly of MTLP whereas the thermolabile compounds include enzymes, the metabolic role of which is disturbed by toxic metals. In these three compartments, metal levels are determined by using atomic ab-

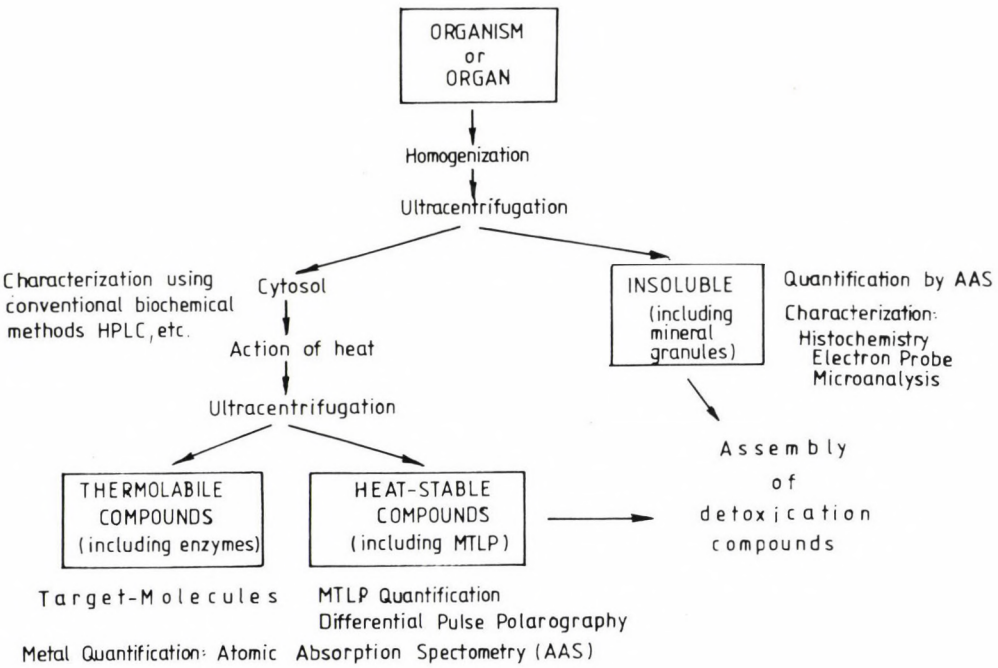

Fig. 6. Methodology for the compartmentation of metals in marine organisms

sorption spectrophotometry (AAS). To go further in the matter, it is necessary to characterize these fractions by using mainly histochemistry and electron probe microanalysis in the case of mineral concretions, biochemical techniques for cytosolic compounds.

The methodology here-proposed may be exemplified by case studies such as bioaccumulation of trace metals in oysters.

In young oysters exposed simultaneously to copper and lead /3/ a diffuse contamination of all tissues was observed by using ionic analysis. The chemical forms of storage of these metals were examined in cells insuring an

Fig. 7. A. Digestive tubules of control oysters. The Von Kossa's method (used for the localization of anions) is entirely negative (x 500) (dt = digestive tubules)
B. In oysters exposed to Pb, microanalysis indicate a storage of this metal and a corresponding increase of P amounts in the lysosomes of digestive cells. Here are shown digestive tubules in which the Von Kossa's method gives a positive reaction which colours the digestive cells brownish (arrows). These facts suggest a storage of Pb as phosphate (x 1000) (Photographs R. Martoja)

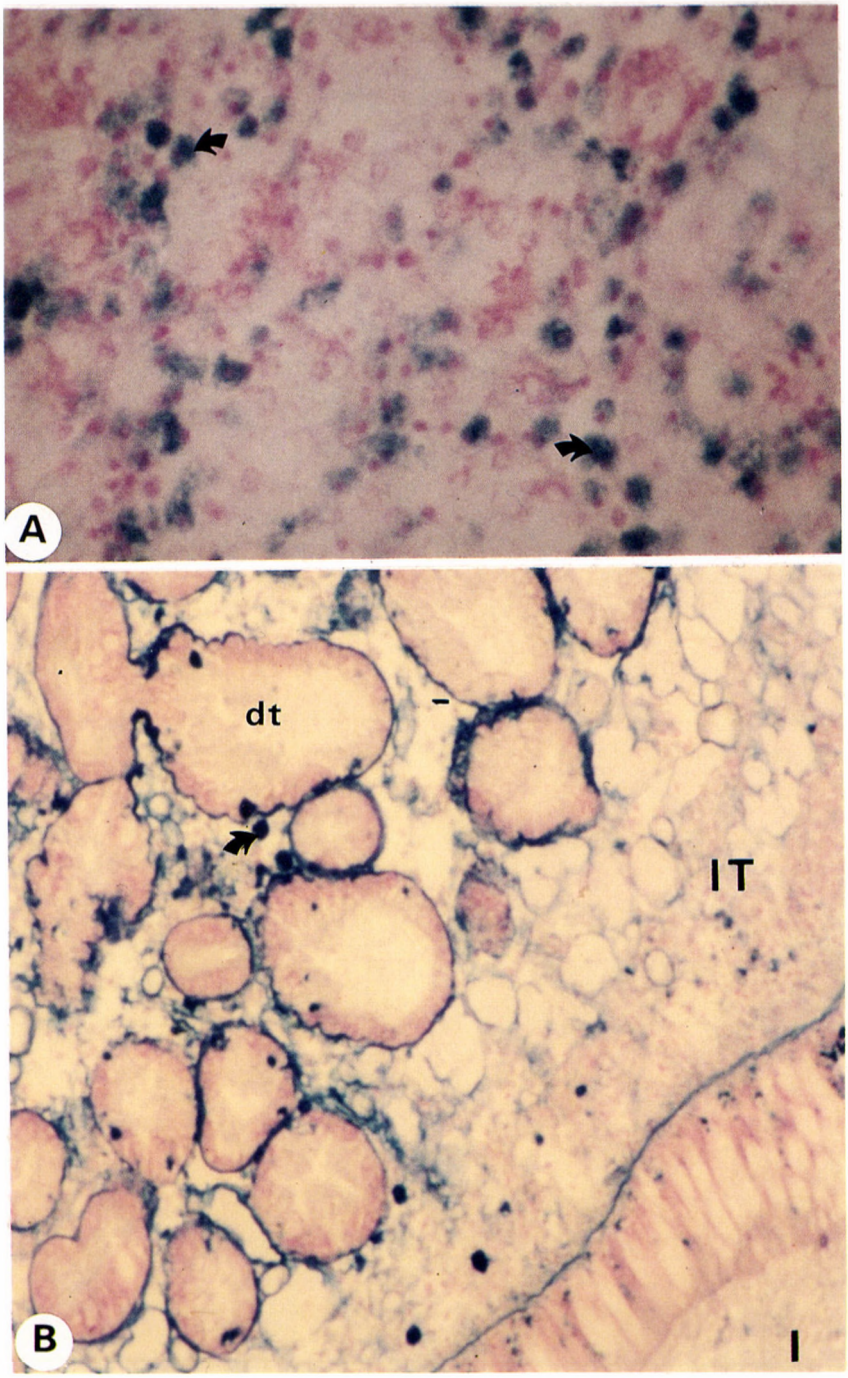

Fig. 7

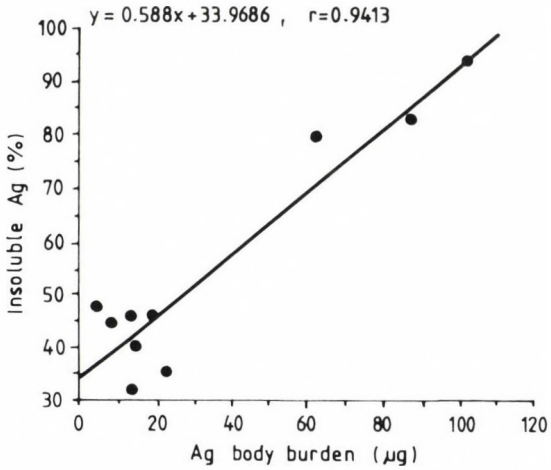

Fig. 8. Relationship between body burden and insoluble fraction of silver in soft tissues of oysters exposed to this metal

important storage of lead or accumulating high overloads of copper, namely hemocytes, branchial and digestive cells. The results of histochemistry are shown in Figs 7B and 9A, in comparison with controls (Fig. 7A). In hemocytes, the correlation between intensities of X-ray signals for sulphur and copper suggests a chelation of copper by a sulphur ligand. The determination of the solubility characters (total elimination of Cu at pH_2 without any decrease of X-ray signals for S) shows that copper is bound to a protein with a high level of sulphur, as it has been demonstrated after /25/ cadmium exposure. The chemical form of copper agrees here with the presence of a metallothionein-like protein. In the case of lead, the solubility tests (elimination with ammonia-water) indicated that the metal was stored in a mineral form, probably in spherocrystals. Microanalysis indicated a storage of lead and a correlated increase of P amounts, a fact which suggests the

Fig. 9. A. The ferric-ferrocyanide method (Schmorl's procedure) gives a positive reaction which colours the hemocytes blue in oysters exposed to Cu. Microanalysis shows that these hemocytes (arrows) have numerous dense inclusions which concentrate elements (Zn, Cu, Al, Fe, P, S). In oysters exposed to Cu, Cu and S amounts increase concomitantly (x 1000)
B. In oysters exposed to silver, Ag-sulphide has been revealed (in blue) by using the ferric-ferrocyanid method. The major part of silver as Ag_2S is sequestered in hemocytes (arrow) and basement membranes of all the tissues. (x 200) (dt = digestive tubules; I = intestine; IT = interticial tissue) (Photographs R. Martoja)

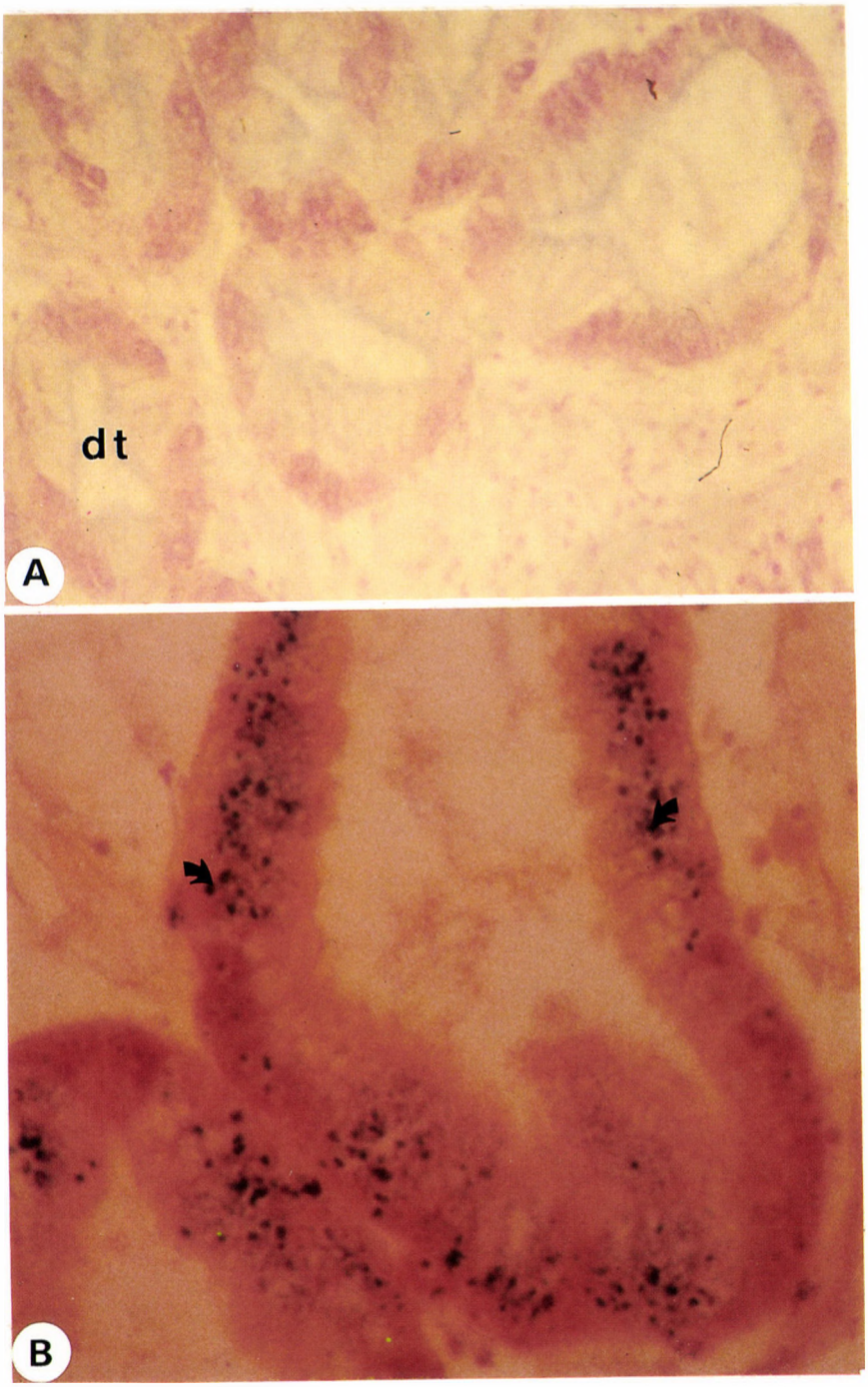

Fig. 9

storage of lead as phosphate, as it has been demonstrated in the case of a natural Zn phosphate in hemocytes /18, 25/.

The hypothesis of a general accumulation of Cu as MTLP and Pb as phosphate is reinforced by the fact that natural sulphur proteins and phosphates occur in cellular structures involved in the storage of both metals. Thus storage cells would not have to synthesize ligands different from those normally present.

In oysters exposed to silver, the percentage of insoluble silver increased with increasing levels of the total metal in soft tissues and it could reach 95% in the most heavily contaminated specimens (Fig. 8). By using histochemistry and electron probe microanalysis, it has been demonstrated that silver was mainly stored as silver sulphide /22/. The major part of silver as Ag_2S is sequestered in hemocytes and basement membranes of all tissues (Fig. 9B). The ultrastructural aspects of Ag_2S and precise localization are shown in Fig. 10.

The distribution of silver in the cytosol was determined by using gel permeation chromatography. Ag is associated with high molecular mass compounds α whereas a second peak β corresponds to a range of lower molecular mass compounds (Figs 8 and 9). The profiles for thiols and optical densities and the similarity of absorption spectra in Ag-exposed oysters and controls suggest that Ag-ligands are present by nature in oysters. Absorption spectra and their derivatives (not represented here) suggest a proteinic nature of Ag-binding compounds. The relative importance of silver compounds varies according to the mode of exposure /11/: α seems to depend mainly on food (Fig. 11B), whereas β is enhanced as a consequence of direct exposure (Fig. 12C). The distribution is more equilibrated after a mixed contamination (Fig. 12D).

Bioavailability of metals associated with mineral compounds depends on their nature and chemical stability. Variations in composition of metal-containing granules have been reported /13/ according to species and organs. No organism in the food chain is able to metabolize again silver stored in bivalves as silver sulphide (or mercury bound to selenium as mercuric selenide in marine mammals /23/). In these conditions there is no hazard to

Fig. 10. Oysters exposed to silver. Digestive gland.
A. Ultrastructural aspect of a digestive cell. The metal is stored as Ag-sulphide (Ag_2S) in the basement membranes (arrows). (x 20 000) (N = nucleus, L = lipidic inclusions).
B. The deposit of Ag_2S consists of microgranules (2 to 3 nm) jointed in long needles (100 to 200 nm) which radiated from the center (x 230 000)

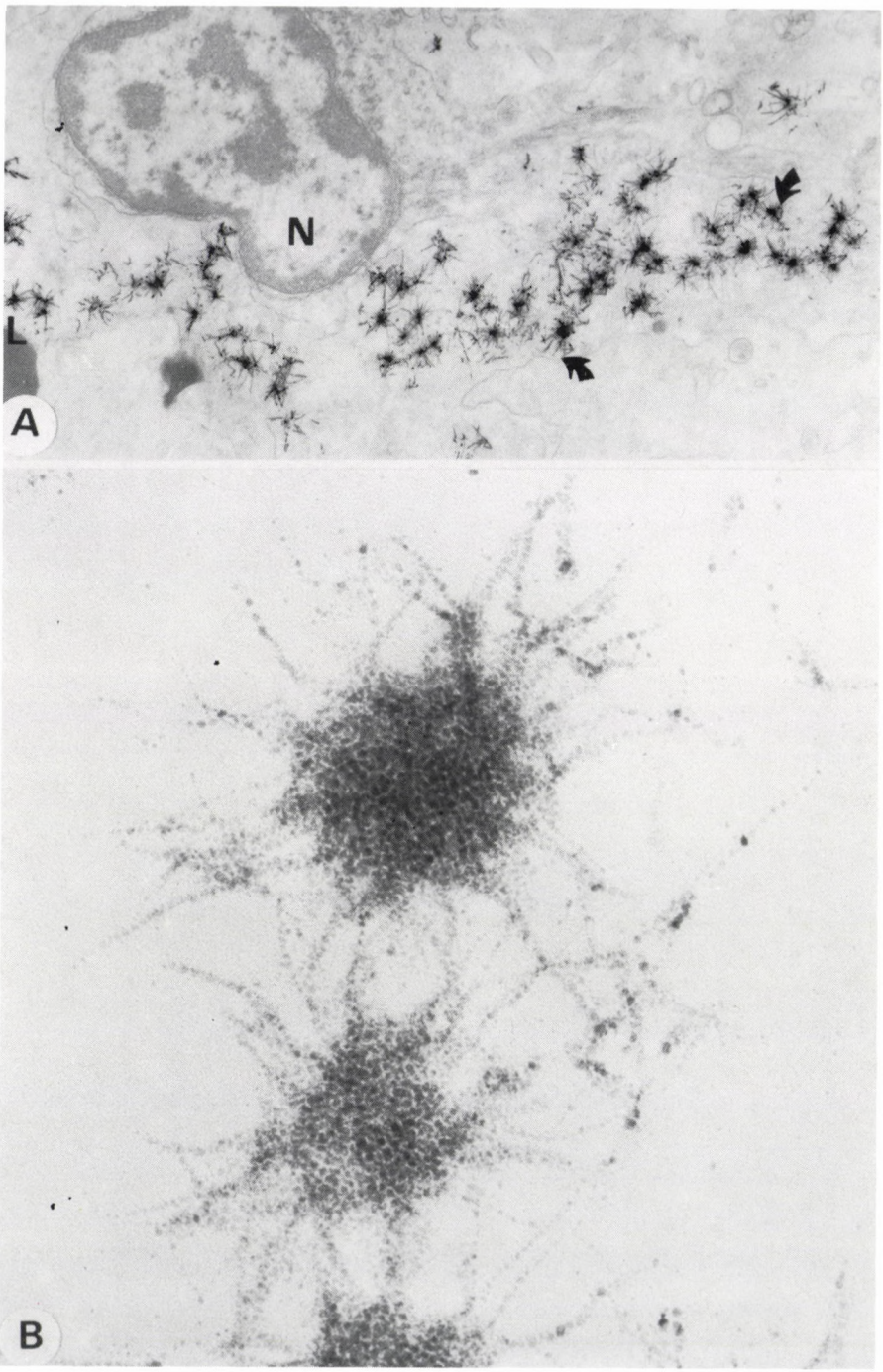

Fig. 10

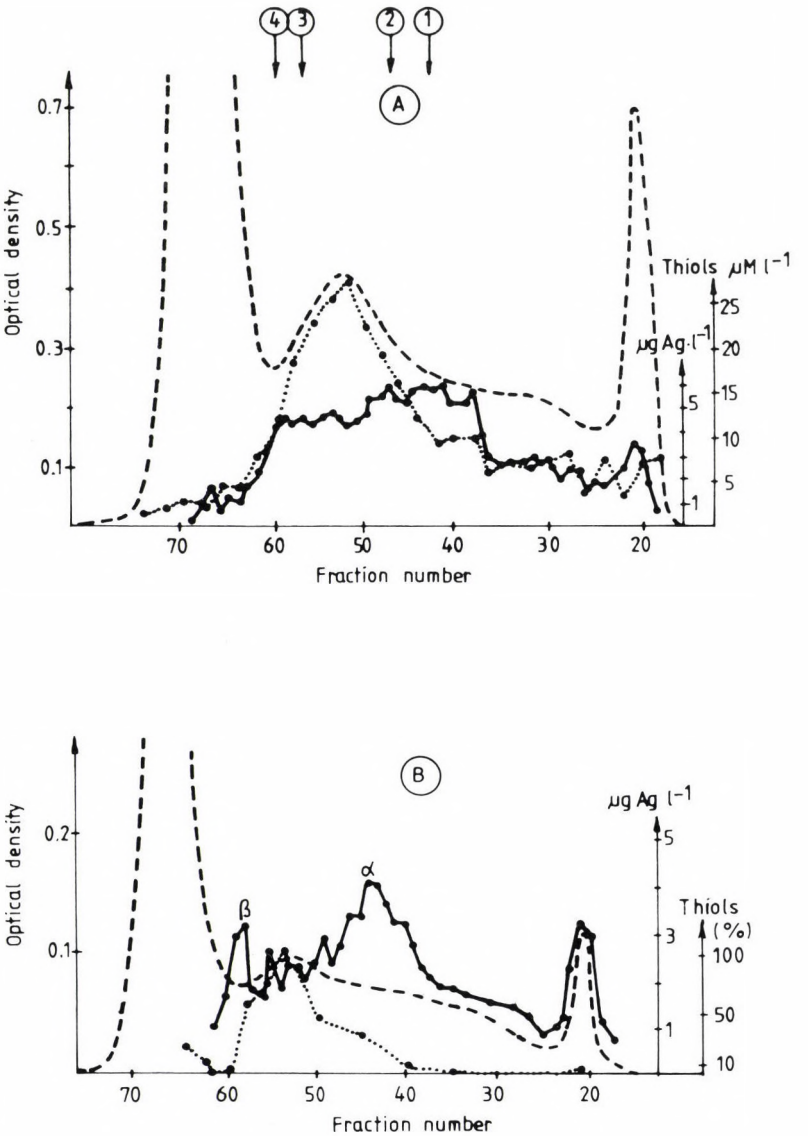

Fig. 11. Distribution of cytosolic silver in oysters. Concentrations of silver (continuous line), optical density at 280 nm (pecked line) and thiols (dotted line). Fraction 20 corresponds to void volume, fraction 68 to total bed volume. The column was calibrated for molecular mass estimations using standard markers (1 — Ferritin, 440 KDa; 2 — Catalase, 232 KDa; 3— Chimotrypsinogen, 25 KDa; 4 — Ribonuclease, 13.7 KDa). A: Controls, B: oysters contaminated through food (S. costatum) (After Martoja et al., 1988; Berthet, 1990)

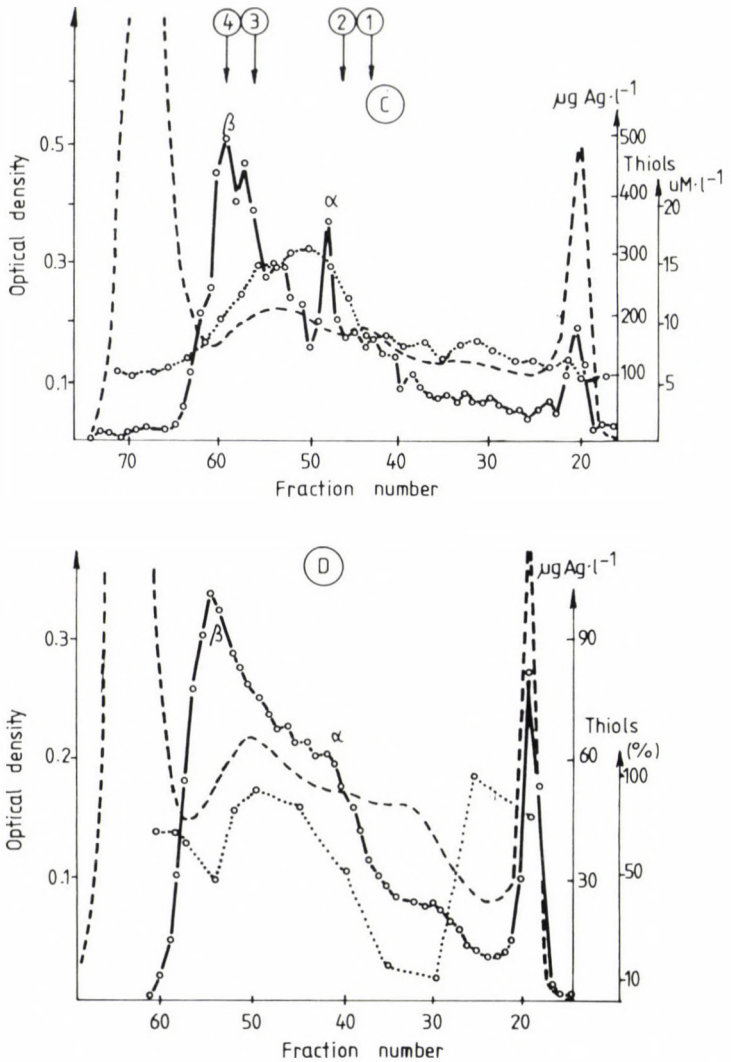

Fig. 12. Distribution of cytosolic silver in oysters (see caption of Fig. 11). C: oysters contaminated from water; D: oysters contaminated via both food and water (After Martoja et al., 1988; Berthet, 1990)

the consumer whereas the risk due to metals associated with phosphates needs on accurate assessment.

The potential bioavailability of phosphate-bound metals has been tested by Nott and Nicolaidou /28/. Carnivorous gastropod molluscs were fed on the tissues of different preys (Fig. 13): kidney of C. opercularis,

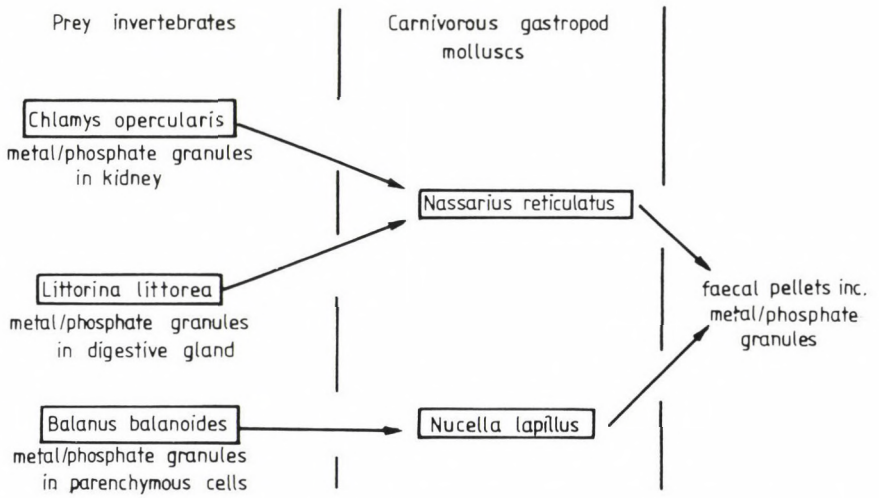

Fig. 13. Transfer of metals detoxified in phosphate granules in a marine food chain (modified after Nott and Nicolaidou, 1990)

digestive gland of L. littorea, soft tissues of B. balanoides in which metals such as zinc and manganese are incorporated in intracellular phosphate granules. After digestion, the residues including granules are egested in the faecal pellets. Comparative X-ray micro-analysis of granules in preys and faeces of the predators demonstrate that metals detoxified in phosphate granules are unavailable to carnivores.

The biosynthesis of such mineral compounds is a process leading to the "fossilization" of some trace elements according to the concept proposed by Martoja and Berry /23/. This constitutes a protective effect against the toxic action of such pollutants and moreover these strategies of survival are also efficient as they reduce metal availability along food chains. Nott and Nicolaidou /28/ have proposed the concept of "transfer of metal detoxication along marine food chain" to describe this phenomenon.

On the other hand, in the stomach content of predators, both thermolabile and heat-stable compounds are exposed to low pHs and to the action of digestive enzymes which seem able to break metal linkages. Thus the binding of metals to detoxication proteins has a protective effect at the level of individual species but it may be considered as responsible of a high availability of metals for transfer in the food webs.

Conclusions

In order to assess metal transfer in food chains, field and experimental studies can be brought into operation, the representativeness and explanatory degree of which vary conversely. An alternative methodology has been proposed with a view to determine the physico-chemical forms of storage of metals in aquatic organisms, data which constitute generally a reliable basis in order to evaluate the bioavailability of trace metals in food chains. It may be applied to both field and experimental samples provided that detection limits may be sufficient, a situation which is often met in situ as demonstrated for Ag, Cu, Fe, Mn, Pb and Zn in gastropods and bivalves /24, 28, 35/. Sediment is often associated with food ingested by benthic organisms, thus the assessment of bioavailability of sediment-bound metals is an interesting complement with a view to elucidate the fate of metallic pollutants in food webs and different methodologies have been proposed /1/.

However, the potential bioavailability of metals in food sources, both living and inert, is often concealed due to the peculiar biological role of the metal or to the biological control characteristic of individual species.

In order to apply the cellular-compartmentation and tissue-specific partitioning approach to field conditions, a transfer model in food webs could be proposed. It would be built on the determination of physico-chemical forms of metals in the most representative species at each trophic level of an ecosystem. Such an approach has to be developed first in estuaries or coastal confined areas. Their fauna living under severe ecological conditions consists in a limited number of species, a fact which make easier the operation of the proposed methodology. Moreover, these areas are particularly at risk for metal contamination.

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INDUCTION OF IN VITRO TUBERIZATION BY SHORT DAY PERIOD AND DARK TREATMENT OF POTATO SHOOTS GROWN ON HORMONE-FREE MEDIUM

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Effects of an environmental factor, the photoperiod, on tuberization were analyzed in vitro on 7 different potato cultivars from various maturity groups and genetic origin. No growth regulators were added to the culture medium, to preserve natural endogenous equilibrium of hormones.

After culturing of shoots for 4 weeks under long days, tuberization was induced with pouring of 8% sucrose solution onto the cultures and five different photoperiodic treatments consisting of different combinations of short days and total darkness.

Light (8 h) during tuber induction delayed tuberization, while the dark treatment (0 h) after short days (8 h) promoted a rapid tuber inititation. The beginning of tuberization could not be correlated with maturity groups.

In vitro tuber formation has occurred at a rate of at least one tuber per plantlet and this tuberization rate were at least as high as any method previously described based on the addition of growth regulators. This would indicate, that photoperiod controlling tuberization processes in vivo, trigger a general state of induction in plantlets cultured in vitro.

Keywords: Solanum tuberosum L. — microtubers — photoperiod — in vitro induction and development

Introduction

Tuberization of potato plants is a complex developmental process. Morphological and physiological changes during tuberization are considerably influenced by environmental factors such as photoperiod, temperature or light intensity.

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Potato plants in vivo are able to sense changes in environmental conditions that are required to trigger tuber production. The leaves of potato plants play an important role in the perception of environmental stimuli and in the synthesis of endogenous growth regulators and a specific tuber-inducing substance /5, 8, 19, 21, 24/. Therefore the adequate state of development of the leaves is of great importance in tuberization processes. It is well documented that the development of foliage of potato plants in vivo is promoted by long days, whereas the short day conditions are required to induce tuberization by alteration in balance of endogenous growth regulators and stimulating the transport of the tuber-inducing substance from the leaves to the other plant parts /1, 10, 11, 21, 24/.

Majority of the published attempts to induce tubers in in vitro cultures has been based on the use of exogenous regulators (phytohormones, hormone analogues /3, 7, 12, 13, 15, 16, 18, 20, 25/. However the application of growth regulators reduced the expression of tuberization capacity determined by the genotype of potato plants. Consequently less than 1 microtuber per plantlet can be produced on Murashige and Skoog medium and the number of tubers per shoot varied between 0.27-1.0 depending on cultivars. Jasmonic acid is known as related compound to a specific tuber-inducing substance /23/ that was found in the leaves of potato plants /9/.

Application of jasmonic acid can induce 100 per cent tuberization (number of tubers per stolon x 100) on potato stolons cultured in vitro by triggering a general state of induction throughout the stolon /17/.

However already Hussey et al. /6/ observed in vitro tuberization without using growth regulators, but little attention has been paid to this possibility. The first detailed investigation was made by Garner et al. /4/. They concluded that reliable microtuber formation could be obtained using a medium containing 8% sucrose and Murashige-Skoog salts /14/.

Microplants responded to short photoperiods by the rapid formation of microtubers, particularly if first given at least four weeks under long days. Exposure to complete darkness stimulated rapid microtuber formation when preceded by short days, but there was a little effect when applied immediately after long days /4/.

The aims of present studies were to analyze the tuberization responses of in vitro plants from different maturity groups and genetic origin of potato to the environmental stimuli, particularly daylenght, that is known as inductive factor under in vivo conditions. In the described experiments growth regulators were omitted from the medium to preserve

natural endogeneous equilibrium of hormone balance and to avoid the potential effect(s) of hormones on the response to photoperiod.

Materials and Methods

Plant material

Solanum tuberosum cv. Cleopatra (early), Gülbaba and Nyírségi rózsa (mid-early), Desiree and Beáta (mid-late), Boró and *Solanum tuberosum* x *Solanum brevidens* hybrid line TB 38/10/A (late) were used. Each genotype will be referred to as a clone. The *Solanum tuberosum* x *S. brevidens* somatic hybrids were obtained from the Institute of Plant Physiology, Biological Research Center, Hungarian Academy of Sciences, Szeged.

Clones were maintained as shoot cultures on a medium containing the salt mixture of Murashige and Skoog /14/ supplemented with 0.8% agar-agar and 3% sucrose.

The cultures were grown in a culture room at 24/15 ^{o}C day/night temperature and 16 h photoperiod and 8000 lux light intensity. Nodal cuttings of *in vitro* plants growing under the above-mentioned conditions were used to the experiments. Initial explants were placed in jars (Kilner-jars, 400 ml, 75 mm inside diameter and 85 mm long) covered with plastic caps. The experiments were carried out with jars containing 30 nodal explants on 40 ml of medium.

Tuber induction

Cultures were grown for four weeks at 16 h photoperiod of the same light intensity and temperature used for the maintenance of cultures. Tuber induction treatments were applied four weeks later when the jars filled with

Table 1

The applied tuber induction treatments after four weeks culture period under long days

Treatment	Weeks		
	SD (8 h)	D (0 h)	SD (8 h)
Fp-1	13	—	—
Fp-2	2	2	9
Fp-3	2	11	—
Fp-4	1	12	—
Fp-5^{+}	1	12	—

$^{+}$Cultures were kept for 3 days at 8 h photoperiod prior to exposure to total darkness (0 h)
SD: short days (8 h), D: darkness (0 h)

plantlets. Tuberization was induced with pouring of 8% sucrose solution onto the cultures and five different photoperiodic treatments, which are constructed from different combinations of short days (8 h) and total darkness (0 h) (Table 1). Temperature and light intensity under short day period was the same as that used for long days (16 h) and temperature during total darkness was constant 15 ^{O}C.

Measurements and data analysis

The tuberization in vitro was evaluated by recording the time of the appearance of first microtuber in the jar (= time of the tuber initiation) and also by counting the number of jars with tubers of at least 2 mm in diameter and the number of tubers per jar and plantlet, respectively. Only jars free from visible microbial contamination were included. At least 10 jars per clone per treatment were evaluated for the effect of treatments.

The tuberization response was analyzed using two different equation: (1) tuberization (%) = jars with tubers per total number of jars; (2) tuberization = (number of microtubers per jar) per (number of fully developed plantlets per jar).

We considered at least the four cm long in vitro plants with large leaf surfaces and developed roots as fully developed plantlets. The statistical evaluation for the time of tuber initiation and the number of tubers per jar was made by variance analysis /22/.

Results and Discussion

It is widely known, that photoperiod is one of the key environmental factors controlling the development of potato plants also in vitro culture. At least four weeks of culture period under long days applied at the beginning of plant development encourages vigorous leaf and stem formation in vitro /4/. Charles et al. /2/ concluded, that a 16 h photoperiod rapidly produced vigorous plants with large leaves and two peaks of leaf surfaces could be observed after 4 and 7 weeks and the second peak was lower than the first one due to a more intensive aging. Kahn et al. /8/ proved, that there is a positive correlation between the leaf surface and the tuberization capacity of potato plants and the younger leaves are more effective in the perception of photoperiodic stimuli than the older ones. Considering these date we have applied the 16 h photoperiod for four weeks prior to the induction of tuberization. On average 27 fully developed plantlets per jar could be grown after the long day treatment of four weeks (Fig. 1).

Fig. 1. In vitro potato plants after 4 weeks under long days (16 h), removed from jar

Tuber initiation

The initiation of in vitro tubers was recorded for the first visible sign of tuber formation over 17 weeks (Table 2). Exposure of cultures to light (8 h) during the experiments (Fp-1 treatment) caused delays of 1-2 weeks in tuberization in the case of cv. Cleopatra and Desiree (1 week), cv. Beáta and Boró (2 weeks), but there was not significant effect by cv. Gülbaba and Nyírségi rózsa. Delaying effect of light during tuber induction was observed by others /13/, too. Exposure to darkness after short days stimulated rapid tuber formation and the effect of dark was more clearly detectable when it was not followed by short days or reduced short days was applied before darkness (Fp-4, Fp-5 treatments). The delaying effect of light (8 h) was the strongest in the case of the hybrid line Solanum tuberosum x S. brevidens, TB 38/10/A. Replacement of the short-day period by Fp-2 treatment caused total inhibition of tuber initiation. The tuber initiation was started significantly earlier when the short-day periods were only applied for 3 days prior to darkness (Fp-5 treatment) at this clone. So the dark treatment (0 h) after short-day periods promoted an initial acceleration in the rate of in vitro tuber formation particularly after 3 days in short periods. Similar results were observed by others /4/ regarding to the accelerating effect of darkness on the tuber formation.

Table 2

Effect of different treatments on the time of the tuber initiation of different potato clones. The number of days required for the appearance of first tuber in a jar after induction

Treatment / Clone	Fp-1	Fp-2	Fp-3	Fp-4	Fp-5
Cleopátra	33 ± 7	c 24 ± 3	c 24 ± 3	c 18 ± 4	c 16 ± 4
Gülbaba	15 ± 5	13 ± 4	a 12 ± 4	b 12 ± 3	c 9 ± 2
Nyírségi rózsa	17 ± 5	15 ± 5	b 12 ± 4	c 12 ± 3	c 10 ± 2
Desiree	29 ± 4	c 21 ± 3	c 20 ± 3	c 14 ± 3	c 13 ± 4
Beáta	33 ± 5	c 20 ± 2	c 21 ± 2	c 21 ± 6	c 17 ± 7
Boró	36 ± 5	c 22 ± 3	c 22 ± 2	c 20 ± 5	c 16 ± 6
TB 38/10/A	35 ± 8	29 ± 2	34 ± 7	36 ± 8	b 26 ± 6
Average	28 ± 6	21 ± 3	21 ± 4	19 ± 5	15 ± 4

(a: P = 0.05, b: P = 0.01, c: P = 0.001)

Tuberization as calculated in equation (1) was 37.5% by Fp-1 treatment and 88% by Fp-2 treatment, respectively, at the clone TB 38/10/A.

Other treatments caused 100% tuberization with this clone. The per cent of tuberization according to equation (1) was 100% with the other examined clones by every treatment.

The clones from the early-maturity group would be expected to produce tubers first in the field. In our *in vitro* systems the beginning of tuberization was significantly (P = 0.001) earlier in the mid-early clones (cv. Gülbaba and cv. Nyírségi rózsa) than it was in the early clone (cv. Cleopátra). It was followed by the tuber initiation of the mid-late clones (cv. Desiree and cv. Beáta), the early clone (cv. Cleopátra) and the late clones (cv. Boró and hybrid TB 38/10/A) by the Fp-1 treatment. As a result of the other treatments the tuber initiation of mid-early clones was significantly (P = 0.001) the earliest, too and that of the hybrid TB 38/10/A was significantly (P = 0.001) the latest. The early (cv. Cleopátra), the late (cv. Boró) and the mid-late (cv. Beáta and cv. Desieree) clones have taken a middle position in the order of time necessary for tuber initiation between the mid-early clones and the hybrid from late maturity group. It may proposed, that this *in vitro* response is inconsistent with the normal open-field reaction.

This observation is similar to findings of Garner et al. /4/. They found, that the outset of tuberization was earlier in the maincrop than it was in the early cultivars. Nevertheless some authors /12, 13/ persume, that in vitro tuberization is suitable for distinction of clones from different maturity groups. These authors were only able to distinguish between early and late lines.

Number of in vitro tubers

The number of in vitro tubers per jar was counted at the end of the experiments (17 weeks) (Fig. 2). The tubers with smaller than 2 mm in diameter were discorded from the analysis and only the number of tubers with 2 or larger than 2 mm in diameter were counted and evaluated by analysis of variance (Table 3).

Table 3 includes the average number of microtubers per jar and, in parentheses, that per average plantlet per jar (according to equation /2/).

The TB 38/10/A clone failed to form normal (sessile or nonsessile) tubers, only swollen leafy shoots with many small leaflets on their tops by Fp-1 and Fp-2 treatments, and they were not classified as tubers. The Fp-1 and Fp-2 treatments caused nonsessile tuber development by the other clones. That means, stolons were formed and subsequently swelled at the tip

Fig. 2. In vitro tubers on potato plants after 17 weeks (Fp-3 treatment), removed from jar

Table 3

Effect of different treatments on the average number of in vitro tubers per jar and per fully developed plantlet (in parenthesis) at the end of the experiments[+]

Treatment / Clone	Fp-1	Fp-2	Fp-3	Fp-4	Fp-5
Cleopátra	32 (1.19)	30 (1.11)	34 (1.26)	34 (1.26)	32 (1.19)
Gülbaba	29 (1.07)	b 35 (1.30)	a 34 (1.26)	c 36 (1.33)	c 40 (1.48)
Nyírségi rózsa	30 (1.11)	32 (1.19)	31 (1.15)	33 (1.22)	a 36 (1.33)
Desiree	34 (1.26)	36 (1.33)	35 (1.30)	35 (1.30)	34 (1.26)
Beáta	30 (1.11)	32 (1.19)	33 (1.22)	32 (1.19)	31 (1.15)
Boró	28 (1.04)	27 (1.00)	c 34 (1.26)	a 32 (1.19)	a 33 (1.22)
TB 38/10/A	0	0	17 (0.63)	17 (0.63)	c 32 (1.19)
Average	26 (0.96)	27 (1.00)	31 (1.15)	31 (1.15)	34 (1.26)

[+]Only the tubers with size larger than 2 mm were counted and evaluated.
(a: P = 0.05, b: P = 0.01, c: P = 0.001)

to form tubers. Exposure to darkness after short days untill the end of the experiments (Fp-3, Fp-4, Fp-5 treatments) caused the formation of sessile tubers, which were formed by swelling of the axillary buds. In vitro tuber formation has consistently and reliably occurred at a rate of at least one microtuber per fully developed plantlet with the exception of the Solanum tuberosum x S. brevidens hybrid line (the TB 38/10/A), by which alone the Fp-5 treatment allowed a high tuberization rate.

This reaction of the TB 38/10/A clone as well as its reaction to Fp-1 and Fp-2 treatments, by which the cultures were exposured to short days during the experiments or after darkness they were replaced by short days, with the absence of formation of normal tubers probably due to the effect of the genome of non-tuberbearing Solanum brevidens.

In conclusion, the investigation of an environmental factor, the photoperiod controlling plant development and tuberization process of potato under in vivo conditions has made possible to reach at least as high, if not higher, tuberization rate without using growth regulators for 7 different cultivars as any method previously described and based on the addition of growth regulators. This would indicate, that the here applied treatments consisting of different combinations of short days (8 h) and darkness (0 h), applied after four weeks under long days, trigger a general state of induction throughout the in vitro plantlets similarly to potato plants under

openfield conditions. Although the most important environmental factor, which control the tuberization, is the photoperiod, other environmental factors such as light intensity, wavelengths, temperature interact with it and could be modified its effect /2, 10, 24/.

In this paper we have examined only the effect of photoperiod, but with the optimalization of the other, above-mentioned factors supposedly further improvement may be possible.

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HIGH-EFFICIENCY PLANT REGENERATION FROM AN EMBRYOGENIC CELL SUSPENSION CULTURE OF WINTER WHEAT (TRITICUM AESTIVUM L.)

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Highly embryogenic cell suspension cultures were established from immature embryo-derived embryogenic calli of winter wheat (Triticum aestivum L., cv. GK Ságvári). Weekly subcultures were made in liquid MS medium supplemented with 2 mg l^{-1} 2,4-D. An average of twenty-two compact, organized calli were obtained from each 1 ml suspension cells when plated on solid MS medium containing IAA and zeatin under a 16/8 h light/dark cycle, while only 9 calli were produced in the dark. Variation in the callus inducing ability was correlated to the time elapsed after subculture. Plated cells responded best 9 days after the subculture and 59% of the calli were regenerable, retaining their embryogenic capacity over 6 months. Several hundred green shoots and plants were regenerated on differentiation media supplemented with IAA and BAP, and 300 plants were transferred to the greenhouse. The majority of plants had an abnormal chromosome number and a low viability.

Keywords: Cell suspension — plant regeneration — Triticum aestivum — wheat

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Abbreviations: MS: Murashige and Skoog basal medium; IAA: indole-3-acetic acid; BAP: 6-benzylaminopurine; IBA: indole-3-butyric acid; 2,4-D: 2,4-dichlorophenoxyacetic acid; NAA: 1-naphthaleneacetic acid.

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Introduction

Cereals are the most important staple food and feed crops in the world, subjected to continuous improvement by breeding and in vitro methods. However, for along time, they seemed to be recalcitrant to in vitro manipulations, especially to cell suspension and protoplast culture /15, 23, 31, 34/. Lack of reproducible plant regeneration from cultured cereal cells and protoplasts questioned the success of genetic interventions, such as somatic hybridization or direct gene transfer /15, 23, 31, 34/. Regenerable embryogenic cell suspension culture are very suitable for mass propagation and for in vitro selection /5, 34/, they are good sources of totipotent protoplasts /1, 15, 23, 31, 34/ and can serve as targets of direct gene transfer via fibre-mediated DNA delivery system /13/ or by biolistic methods /30, 32/ to produce regenerated fertile transgenic plants /30/.

As reviewed by Lazzeri and Lörz /15/ efficient cell suspension cultures with plant regeneration capability were obtained from some cereal species, e.g. rice /3, 4, 5, 12/, maize /20, 21, 24, 29/ and barley /11/. Root, shoot or plantlet recovery from callus-derived cell suspension cultures of wheat has been reported by a number of authors /2, 7, 8, 10, 19, 25, 27, 36, 37, 39/. However, these cultures were in part not true cell suspensions since they contained large tissue pieces /2, 7, 8, 10, 36/ or had a very low rate of regeneration /19/. Morphogenic cell suspensions of wheat were obtained from immature inflorescences /16/ and immature embryo-derived, embryogenic calli /17, 25, 27, 35, 37, 39/. Harris et al. /9/ established a ture and morphogenesis-competent cell suspension culture from a single wheat anther, from which regenerable protoplasts were isolated.

Earlier we reported the regeneration of the first fertile plant from regenerable embryogenic suspension cell-derived protoplasts of wheat /1/. In this paper, we describe protocols for initiation and maintenance of the cell suspension cultures with morphogenic potential.

Materials and Methods

Callus induction and subculture

Immature embryos, 10-12 days after anthesis, immature inflorescences (1-2 cm long) and mesocotyls of mature embryos were isolated aseptically from 14 winter and spring wheats (GK Ságvári, GK Kincső, GK Bence, GK Mini Manó, 84-82, 74-2; Sakha 8, Sakha 69, Giza 155, Giza 157, Giza 160, Siete Cerros, Lerma Rojo, Tobari 66). The explants were cultured on solid MS

medium (22), containing 1-2 mg l^{-1} 2,4-D at 26 °C in the dark. Calli were subcultured monthly on solid MS medium supplemented with 1 mg l^{-1} 2,4-D. The first three subcultures were incubated at 26 °C in the dark and the following four-five subcultures were kept in light (16 h photoperiods; intensity: blue 1.1 $\mu W/cm^2$, red 1.2 $\mu W/cm^2$).

Initiation and maintenance of cell suspensions

Highly embryogenic calli were selected visually for initiation of the primary suspension cultures. One g fresh weight of callus was placed into 150 ml Erlenmeyer flask containing 50 ml of liquid MS medium, supplemented with 0.5 mg l^{-1} each of nicotinic acid, pyridoxine.HCl, thiamine.HCl, 2 mg l^{-1} glycine, 150 mg l^{-1} asparagine, 100 mg l^{-1} myo-inositol, 2 mg l^{-1} 2,4-D and 3% sucrose. The flasks were placed on a rotary shaker at 26 °C under a 16 h/8 light/dark cycle and agitated at 130 rpm. Subcultures were made weekly by replacing all the old medium by equal volume of fresh medium. Only the small aggregates were decanted at every subculture. Ratio of cells to medium was 1:3-5 (v/v) in culture flasks. For measuring increase of cell mass and change of pH in the culture medium, one ml of settled cells was cultured in 40 ml of liquid MS medium plus 2 mg l^{-1} 2,4-D in a 100 ml Erlenmeyer flask on a gyrotory shaker at 130 rpm in 16 h photoperiods at 26 °C. Fresh and dry weights of the cells and pH of the culture medium were measured 0, 1, 2, 5, 7 and 9 days after culture initiation /24/.

Regeneration from cell suspensions

For plant regeneration, 5-12-month-old suspension cultures were used. One ml cell suspension, 7-22 days after subculture, was pipetted onto solid MS medium containing 0.5 or 1 mg l^{-1} IAA and zeatin, 10 mg l^{-1} $AgNO_3$, 2% sucrose and 0.2% Gelrite in a 50x15 mm Petri dish. Other hormone combinations (IAA + BAP, IAA + kinetin, IBA, NAA, no hormones) were also tested in liquid media under low-speed (15 rpm) shaking /5/ at 26 °C in the dark or at 16 h/8 h light/dark cycle for 4-6 weeks. Organized compact calli developed from plated suspension cells were transferred to solid MS regenerating medium, supplemented with 0.5 mg l^{-1} each of IAA and zeatin or IAA and BAP or 0.1-0.2 mg l^{-1} 2,4-D. Sucrose concentration was 2% throughout. The callus cultures were kept at 26 °C on 16 h photoperiods for 4 weeks. The developed plantlets were transferred to the same regeneration medium and shoots to rooting media, i.e. half or full-strength MS, liquid or solid, without hormones or with 2,4-D (0.1-0.2 mg l^{-1}), NAA (0.3 mg l^{-1}) and IAA (0.5 mg l^{-1}) plus kinetin (0.02 mg l^{-1}), respectively. At every removal of the regenerants, embryogenic callus pieces were transferred to fresh regenerating medium (half-strength MS supplemented with 0.5 mg l^{-1} each of IAA and BAP). Well-developed regenerants were then potted and brought into the greenhouse. The established 4 week-old plants were moved to vernalization for 7 weeks at 5 °C in a cold chamber, then the plants were grown under greenhouse conditions (16-20 °C/12-16 °C in 12 h light). The pH of all media used in this study was adjusted to 5.8 before autoclaving at 121 °C for 18 min. Zeatin was added to the cold autoclaved media after filter-sterilization. The experiments were repeated five times with one month intervals each.

Cytogenetial studies

Chromosome counts is suspension cells were carried out according to the method of Karp et al. /14/ with minor modifications. Suspension cells (20 month-old), 2 days after subculture were incubated with 0.1% (w/v) colchicine solution for 2 h at 26 °C, and fixed in acetic acid : ethanol (1:3) mixture overnight at 25 °C. The fixed cells were incubated in an enzyme solution (1% cellulase R10, 0.5% macerozyme R10, 0.05% pectolyase Y-23, pH 4.5) for 1-2 h at 26 °C. After each treatment the suspension cells were washed with 0.1 N sodium acetate buffer and centrifuged at 1000 rpm for 5 min. The cells were fixed again in acetic acid : ethanol (1:3) or in acetic acid 45% alone, dropped onto a clean slide and stained with 0.5% acetocarmine. Metaphase chromosomes were counted in one-hundred well-spread cells.

Root tips of several regenerated plants were immersed in 0.1% colchicine (w/v) for 2 h at room temperature, fixed overnight with acetic acid : ethanol (1:3) and stored in 70% ethanol. The root tips were rinsed with water and hydrolysed for 12 min in 1 N HCl at 60 °C. Tips were stained with 0.5% acetocarmine and metaphase chromosomes were counted. Total number of studied plants was 80, and good-spread chromosomes in at least five cells from at least two roots each were counted. Variation of the chromosome number was detected among the independent regenerants and within the individual root tips also.

Results

Establishment of cell cultures

Of the different explants from the winter and spring wheat genotypes studied by us, only the immature embryos of the cv. GK Ságvári produced a friable, nodular, non-mucilagineous and rapidly growing,embryogenic callus suitable for establishment of true suspension culture. Six to 8 month-old calli of this type formed a finely dispersed, embryogenic cell suspension within two months. Younger calli or calli derived from immature inflorescences or mesocotyls of GK Ságvári were poor sources of fine, embryogenic cell suspension.

Early in the establishment of suspension cultures of GK Ságvári, large, elongated and highly vacuolated cells with thick cell walls emerged. They were stepwise eliminated during the weekly subcultures by systematic renewal of the medium and continuous selection for finer aggregates. By this means, six stable cell lines were established, which produced fine, small, yellowish aggregates (approx. 0.5 mm in diameter, consisting of isodiametric, densely cytoplasmic and thin-walled cells, suitable for protoplast isolation /1/).

Table 1

Change in fresh mass, dry mass and medium-pH of KSV-90A GK Ságvári wheat cell line in suspension culture during 9 days. One ml settled cells was cultured in 40 ml of liquid MScs medium in 100 ml Erlenmeyer flasks. Figures are means and standard deviations of 5 replicates

Days	Fresh mass g	%	Dry mass g	%	pH of the medium
0	0.554 ± 0.016	100	0.028 ± 0.001	100	5.80
1	0.695 ± 0.099	125	0.035 ± 0.007	125	5.79
2	0.790 ± 0.019	142	0.036 ± 0.010	128	5.67
5	1.089 ± 0.087	196	0.045 ± 0.009	161	5.29
7	1.995 ± 0.519	360	0.075 ± 0.019	268	4.90
9	2.084 ± 0.630	376	0.103 ± 0.033	368	4.88

Growth rate in the suspension culture was determined with the KSC-90A cell line. Its cells grew and multiplied rapidly with a doubling time of 4.7 days. Fresh and dry weight increase of the cells during a 9-day growth period was nearly fourfold. The pH of the culture medium dropped from 5.80 to 4.88 within this period (Table 1).

Regeneration from suspension-cultured cells

No organized calli were formed when the suspension cells were cultured in full- or half-strength liquid MS medium without hormones or supplemented with IAA + BAP, IAA + kinetin, 0.5 mg l^{-1} each, and IBA, 1 or 5 mg l^{-1}, respectively. However, embryogenic, division-competent suspension cells plated onto solid MS medium containing 0.5 or 1.0 mg l^{-1} IAA and zeatin developed white, compact, organized and nodular calli of about 3 mm in size within 4 weeks, while their larger portion formed a soft, lawn-like nurse layer. Best callus induction was obtained with MS medium supplemented with IAA plus zeatin (1 mg l^{-1}).

Cell cultures kept under a 16 h/8 h light/dark cycle produced a higher number of organized callus (22.2 calli per ml suspension in average, Table 2) than those kept in the dark (9.3 calli per ml suspension, Fig. 1). The callus-inducing ability of the plated cells gradually decreased with time after subculture due to ageing of the subcultured suspensions. However, on

Table 2

Callus induction and number of regenerating calli from RECS of GK Ságvári wheat at 26 °C under 16 h photoperiods. Induction medium: solid MS + IAA and zeatin, 0.5 or 1 mg/l each. Regenerating medium: solid MS + IAA and zeatin or IAA and BAP, 0.5 mg/l each

Age of the suspension (days after subculture)	No. of compact calli/ml suspension cells*	Regenerating calli	
		no./ml suspension	% of compact calli
7	24.0	15.0	62.5
9	41.5	29.0	69.9
11	33.3	20.0	60.1
12	22.0	13.0	59.1
13	19.8	13.0	65.7
14	17.0	7.0	41.2
15	15.0	8.0	53.3
17	16.0	7.0	43.8
22	11.3	6.0	53.1
mean	**22.2**	**13.1**	**59.0**

*Average of three experiments.
Note: All regenerating calli maintained their morphogenic capacity over 6 months.

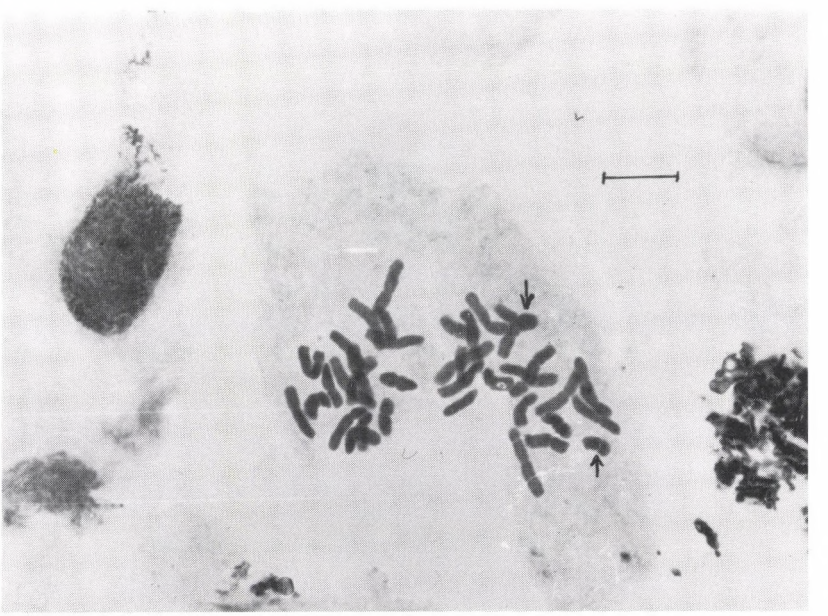

Fig. 1. Aneuploid chromosome number (2n = 36) and fragments (arrows) of a regenerated wheat plant (x 1000). Bar = 10 μm

the 22nd day, under 16 h photoperiods more calli were produced than after 13 days in darkness (11.3 vs. 9.3 per ml suspension, Table 2).

Ten days after culturing on callus induction medium, green areas appeared on the compact calli. They were then transferred to MS regeneration medium containing 0.5 mg l^{-1} each of IAA and zeatin or IAA and BAP or 0.1-0.2 mg l^{-1} of 2,4-D. Under 16 h photoperiods and at 26 ^{o}C, the majority of them rapidly increased in size and became similar to the calli used to initiate the suspensions. Some organized calli turned brown, fail to grow and died, but in the average 59% of them was regenerable. Nevertheless, the regeneration response depended on the time elapsed after subculture of the suspension. Similarly to the induction, calli responded best 9 days after starting the subculture (Table 2). Shoots/plants arose from green areas after two weeks on regeneration media. Some calli produced more than 5 shoots/callus, which were transferred to fresh regeneration medium or to the rooting medium. For rooting, full- or half-strength, hormone-free liquid MS medium containing 1% sucrose proved best.

After removal of the regenerants, the growth rate of the callus pieces and their shoot/plant regeneration frequency did not change during 5-6 subcultures on the same (half-strength) regeneration medium, and 6 month-old calli derived from the suspension cultures still maintained their growth and regenerating ability, resulting in an increased total number of shoots/plants from the multiple subcultures. On the other hand, new cell suspension cultures similar to the original ones could be established from the suspension-derived calli within 6 weeks. By means of this system, several hundred green shoots and plants have been produced and 300 plants transplanted into pots in the greenhouse. A few plants had poor roots but they could be kept living in vitro. Some regenerated shoots did not develop roots, but remained alive on the rooting medium. All six selected cell lines were regenerable and neither albinos, nor only roots were regenerated in this study.

Numerical chromosome variation was present in cell suspension cultures (KSV-90A, 20 month-old) and their regenerated plants. Chromosome number of suspension cells ranged from 13 to 56. The majority of cells (52%) had 30-35 chromosomes. Root tip cells of several regenerated plants had 14-42 chromosomes (Fig. 2). In the majority (80%) of root tip cells, the chromosome number was 26-42, and only 27% of the cells had the normal bread wheat chromosome complement (2n = 6x = 42). Mortality rate of the potted plants was high after vernalization, as only 30% of them survived and reached an

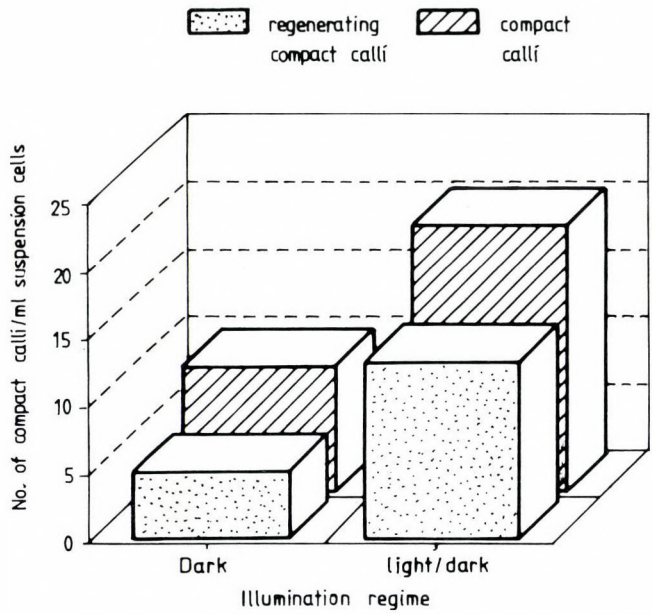

Fig. 2. Effect of illumination on the callus induction and number of regenerating calli of GK Ságvári cell suspension. One ml of suspension cells, 13 days after the subculture were plated on solid MS + IAA and zeatin (1 mg l^{-1} each) in the dark or under a 16 h/8 h light/dark cycle at 26 °C. Regeneration was carried out on solid MS + IAA and BAP (0.5 mg l^{-1} each) on 16 h photoperiods at 26 C

age of 4 months under greenhouse conditions, but no one plant grew to maturity.

Discussion

The critical step for the initiation of embryogenic suspension cultures is the selection of compact or friable, nodular, non-mucilagineous and rapidly growing, highly embryogenic callus as has been shown on a number of cereal species /rice: 3, 4, 5, 12; maize: 20, 21, 24, 29; barley: 11; wheat: 9, 16, 17, 19, 25, 26, 27, 35, 37, 39/. However, according to Redway et al. /26, 27/, cell suspensions of aged, friable callus-origin from the FLA 302 wheat formed an unorganized, soft lawn of friable callus after plating, and only those prepared from aged, compact calli were able to develop into somatic embryos or shoots. It was shown in our experiments that suspension-

cultured cells derived from friable, embryogenic calli of winter wheat were also regenerable.

It can be supposed that friable, embryogenic calli of the cv. GK Ságvári formed spontaneously, as mentioned by Lowe et al. /18/ in maize. This change in callus type probably did not occur in the other tested varieties. Since our plated cells never developed into roots and some of the regenerants had poor roots or no roots at all, it is also probable that the root-competent cells have been selected out automatically during the prolonged callus stage and the following suspension culture without any directed selection /37/.

Cells of the KSV-90A GK Ságvári cell line had a doubling time of 4.7 days. This is within the 3-5 days period observed by Maddock /19/, but is considerably longer than the doubling time (2.3-2.5 days) experienced by Redway et al. /27/ on MS medium. During the 9 days period of active cell growth of the KSV-90A cell line, pH of the nutrient medium changed from 5.80 to 4.88. This pH shift has been also mentioned by prioli and Söndahl /24/ in a maize suspension culture.

After plating, organized calli from the GK Ságvári cell suspension developed on solid regeneration medium only. This observation is in agreement with that of Maddock /19/ and Jähne et al. /11/ made on wheat and barley suspension cultures respectively, but is in contrast with the statement of Biswas and Zapata /5/, who reported the development of embryos from rice suspension cells cultured in a liquid medium. Use of solid callus induction medium similar to the suspension culture medium at the beginning of regeneration is probably advantageous for cellular adaptation /11/, although some of the calli formed after transferring the suspension to the solid regeneration medium stopped growing and died. This was also found by Chang et al. /6/ with protoplast-derived calli of wheat.

MS solid medium supplemented with IAA and BAP at 0.5 mg l^{-1} each proved to be efficient for regeneration of shoots/plants from suspension-derived calli of wheat, as pointed out by Redway et al. /27/ also. BAP seems to be essential for this process since media containing IAA and zeatin resulted in a lower regeneration percentage in our study.

In the experiments of Redway et al. /27/, one ml of suspension cells delivered 0 organized calli in darkness, which is comparable to our results, i.e. 9.3 calli per ml under similar conditions, but significantly less compared to the 22.2 calli obtained under 16 h photoperiods (Fig. 2 and Table 2). Consequently, a light/dark regime is more efficient than darkness in

inducing organized calli from suspension cells of GK Ságvári wheat, plated onto solid media.

It is known that cell suspension cultures are cytologically instable and chromosomal aberrations frequently occur in them. Wheat (Triticum aestivum L.) is not an exception in this respect /1, 6, 14, 28, 37, 38/. In contrast to the results of Yang et al. /39/, the mitotic chromosome number of suspension cells (20 month-old) and their regenerants was only exceptionally normal (2n = 6x = 42). Chromosomal aberrations may be the main cause of restricted or physiologically defective roots as well as low viability in wheat plants regenerated from suspension cells.

Since protoplasts could be isolated from the GK Ságvári cell suspension cultures and a fertile wheat plant could be regenerated from them /1/, the regenerable embryogenic cell suspensions described above seem to be suitable targets of gene transfer by biolistic or other methods. During the in vitro processes from protoplast isolation through protocallus formation up to plant regeneration, some cells probably regained their capability to regenerate fertile plants. It is possible that besides chromosomal defects, the protoplast — plant in vitro phase can induce some restorations, since the protocalli were highly embryogenic and maintained their regeneration capacity longer than their counterparts from suspension cultures. The weaker adaptability of the suspension cell-derived regenerants to the greenhouse or growth chamber environments also cannot be excluded. It is clear that causes of the low viability and chromosomal instability of the regenerants should be clarified.

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FUNCTIONAL CONNECTION BETWEEN INTRACELLULAR AND EXTRACELLULAR SECRETION IN SPECIES OF EUPHORBIA GENUS

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In species of the Euphorbia genus the intracellular latex-secretion and the extracellular nectar-secretion are anatomically connected. The functional connection of the secretional systems was proved to be probable by TLC and GC-MSD. The chemical composition of Euphorbia latex and honey was examined with these analytical methods. In our research, the comparative chemical analysis of the latex and the honey of two Euphorbia species (Euphorbia cyparissias L., Euphorbia seguieriana Necker.) was discharged. These species are unusually good melliferous plants in Hungary.

Four chemical components that can be found both in the latex and in the honey were detected with ultraviolet light (254 nm), and three general alkaloid reagents (Dragendorff, Meyer and 1% $Ce(SO_4)_2$ in 2n H_2SO_4) were detected by TLC.

By means of mass spectrum generated by GC-MSD, the following compounds of Euphorbia honey were identified: butyl-2-methylpropyl phtalat, hexadecane acid, diheptyl phtalat, bis (2-ethyl-hexyl) phtalat, benzenedicarboxylic acid decyl-hexyl ester, benzenedicarboxylic acid isodecyl-octyl ester.

The first compounds can also be found in the latex of both examined Euphorbia species.

This partial correspondence in the composition of the latex and the honey led to results that suggest a further, functional connection between the two, anatomically connected secretional systems.

Keywords: Euphorbia — honey — laticifers — nectar — nectary — secretion

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Introduction

It is commonly known that all species of the Euphorbia genus have a latex system. This secretional system consists of non-articulated laticifers that are longitudinally expanded unicellular growths /6/. Latex is a substance consisting of a liquid matrix with minute organic partical in suspension. The matrix can be regarded as the cell sap of the laticifer /7, 8/. Euphorbia plants are very important in medicine, and in the Ayurvedic system of medicine for curing different diseases. The latex contains various substances in solution and in colloidal suspension: carbohydrates, organic acids /3, 5, 20, 25, 26/, alkaloids /16, 18, 28/, terpentes /4, 9, 10, 11, 12, 13, 23/ and fatty alcohols /1, 2/.

Hegnauer /17/ wrote comprehensively about the composition of the latex of Euphorbia genus. In the latex of the Jatropa genus of Euphorbiaceae family, protease, amylase and peroxydase activities were also detected /27/. The latex of the plants of Euphorbia genus was found to be toxic and irritant, although, on the other hand, it has much medical value because of its chemical composition. The milky sap is caustic, emetic, purgative, and internally irritant, and it also shows cocarcinogenic activity on mouse skin /32/.

The compotision of the nectar and finally the honey produced by the nectaries as a result of extracellular secretion is unusually varied. Besides different kinds of sugar, which are the main constituents of nectar and honey, it may consist of several other compounds, as well. They can also contain ascorbic acid /33/, thiamine, riboflavin, nicotiamide /34/, and other organic acids /21, 22/. The nectar and the honey of several groups of plants -- like Euphorbia species -- can be toxic because of their chemical composition (containing biologically active substances). In arid zones of South Africa several species of Euphorbia (e.g. E. Ledienii, E. coerulescens, E. triangularis, E. ingens, E. tetragona, E. cooperi) known collectively as "noors doring" or "noors", are highly attractive for honeybees, but noors honey can cause a strong burning sensation in the mouth and in the trhoat /19, 29/. Upadhyay and Hecker isolated compound with the same effect of the latex of these plants /32/. In Hungary E. cyparissias and E. seguieriana have a great importance in bee-farming, their honey is sour-sweet and dark brown, and it has been an additional component in mixed honey /14/. The intracellular and extracellular secretional systems of Euphorbia genus are anatomically connected to each other. The glanular tissue of the nectary is

thoroughly woven through by non-articulated laticifers that often end on the boundary between the glandular tissue and the glandepidermis as the nectar secreting surface /32/.

In our research we attempted to verify the functional connection between the two anatomically connected systems, and give evidence of the possibility of secreting certain constituents of the latex into the nectar, and finally into the honey, either transformed or unchanged.

Materials and Methods

In our research we discharged a comparative analysis of the latex and the honey of two species of Euphorbia (E. cyparissias, E. seguieriana) that play an important role in Hungarian bee-farming. According to plantgeographical data, the honey that we used was derived from the two species mentioned above. It was available for us by favor of the Bee-farming Department of Gödöllő.

Alkaloids and compounds that reacted positively to three general alkaloid reagents were detected by TLC, and other organic compounds were found by GC-MSD.

Four g of both the latex and the honey was mixed with 15 cm^3 of 30% ethanol, and then they were acidified with 15 cm^3 of 10% sulfuric acid. After filtration they were alkalized with 25% solution of ammonia, then they were extracted with 3x15 cm^3 of chloroform, and finally, with 3x15 cm^3 ethyl acetate. The extracts were shaken with vitriolic water in order to get a cleaner extract, and then the water layer was alkalized with solution of ammonia added to it as a surplus. Then the essences were extracted again with 3x15 cm^3 of chloroform and 3x15 cm^3 of ethyl acetate, and finally, they were evaporated. The extracts produced in this way consisted of several constituents but they were solutions enriched in alkaloids /31/.

The components of these extracts were examined by TLC on glass plates of 10x20 cm^3 coated with Kieselgel 60F 254 (MERCK) in a solvent system of benzene : methanol (85:15 v/v). The preliminary examination of the chromatograms was discharged in ultraviolet light (254 nm). The chromatograms were made visible with three general alkaloid reagents: Dragendorff, Meyer and 1% $Ce(SO_4)_2$ in 2n H_2SO_4 /15, 24/. The examination of the extracts were continued with ethyl acetate with Hewlett Packard GC-MSD (HP 5890A GC, HP 5970 MSD, 70 eV EI) using apolar capillary column (12 m HP-1) and He as a carrier gas.

Results

As a result of examinations by TLC, we found that several compounds in Euphorbia honey were also constituents of the latex (Fig. 1). Examining the chromatograms in ultraviolet light (254 nm), we found two components of the latex both in the chloroform and in the ethyl acetate extracts of the honey

Table 1

Rf data of compounds indentified from Euphorbia honey with TLC. Sample 1: Euphorbia honey, sample 2: latex of E. cyparissias, sample 3: chloroform (a) and ethyl acetate (b) extracts of the latex of E. seguieriana

UV_{254}						1% $Ce(SO_4)_2$/2 n H_2SO_4/						Meyer						Dragendorf					
1.a	1.b	2.a	2.b	3.a	3.b	1.a	1.b	2.a	2.b	3.a	3.b	1.a	1.b	2.a	2.b	3.a	3.b	1.a	1.b	2.a	2.b	3.a	3.b
0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95						
0.9	0.9																						
—						0.75		0.75		0.75													
														0.6		0.6							
						0.55		0.55	0	0.55		0.55		0.55		0.55							
0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0.15																							
0.1																0.1							

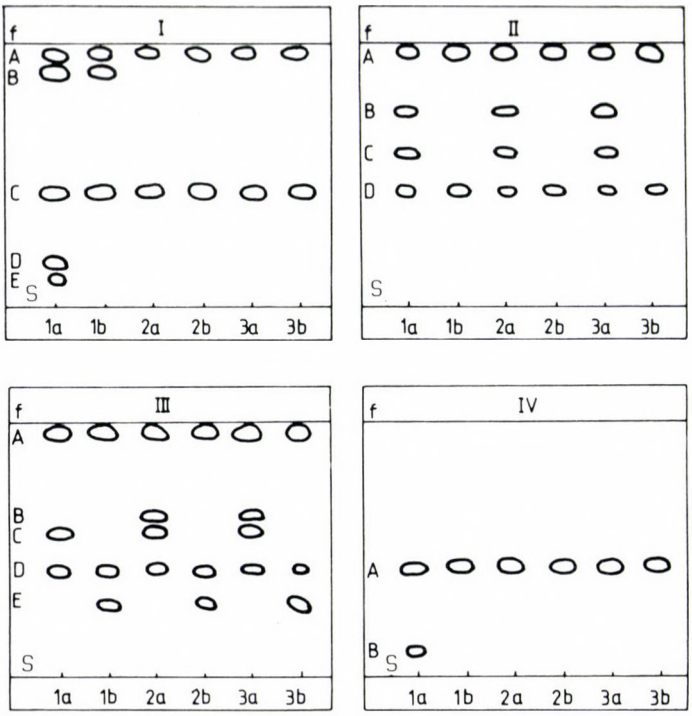

Fig. 1. TLC of chloroform (a) and ethyl acetate extract of Euphorbia honey (1), latex of Euphorbia cyparissias (2) and E. seguieriana (3). Kieselgel 60F_{254} (MERCK), solvent system: benzene : methanol (85:15 v/v).
Developing reagents: plate I: UV_{254}, plate II: 1% solution (105 °C) of $Ce(SO_4)_2$ soluted in 2n H_2SO_4, plate III: Mayer-reagent (105 °C), plate IV: Dragendorff-reagent. s: start, f: front (explanation in the text)

(Fig. 1: plate I, spots A and D of samples 1a and 1b). On the plate which was made visible with $Ce(SO_4)_2$, there were four of such compounds in the extract with chloroform of the honey, and two in the extract with ethyl acetate (Fig. 1: plate II, spots A, B, C and D of sample 1a, and spots A and D of sample 1b). On the plate which was made visible with Meyer reagent, we found three compounds of the latex both in the chloroform and ethyl acetate extracts of the honey (Fig. 1: plate III, spots A, C and D of sample 1a, and spots A, D and E of sample 1b). On the plate which was made visible with Dragendorff reagent, there were two compounds in the chloroform extract of the honey that responded to the reagent, but only one of them was the constituent of the latex (Fig. 1: plate IV, spot A of sample 1a). This compound (probably an alkaloid) shows a positive reaction to both $Ce(SO_4)_2$ and Meyer

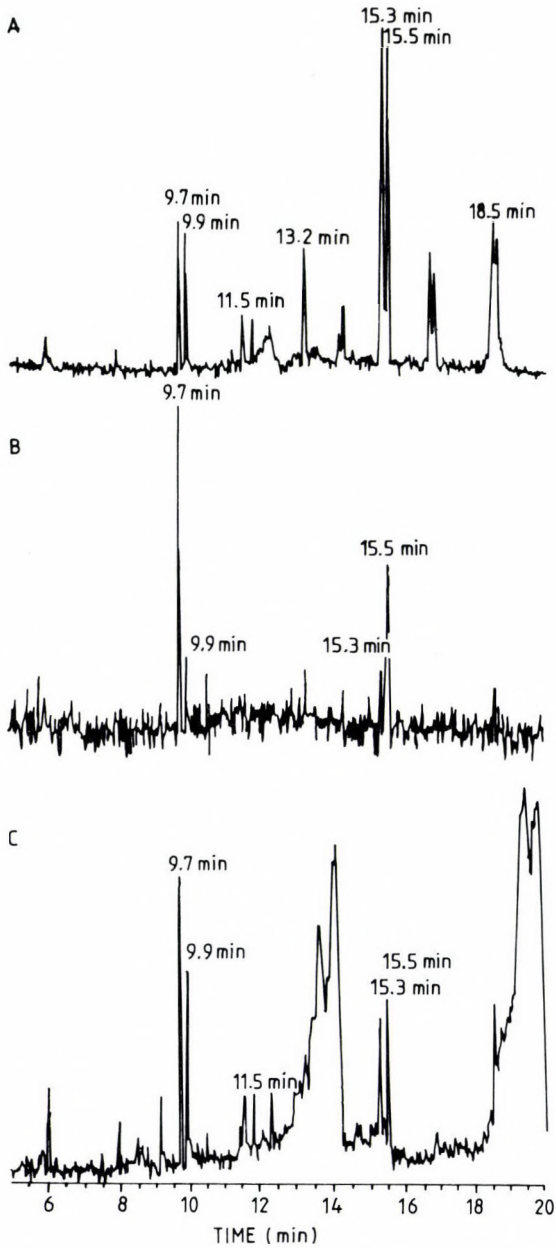

Fig. 2. Gaschromatogram of ethyl acetate extract of Euphorbia honey (A), latex of E. cyparissias (B) and E. seguieriana (C). Peaks with equal retention times and mass spectrums at 9.7, 9.9, 15.3, 15.5 minutes

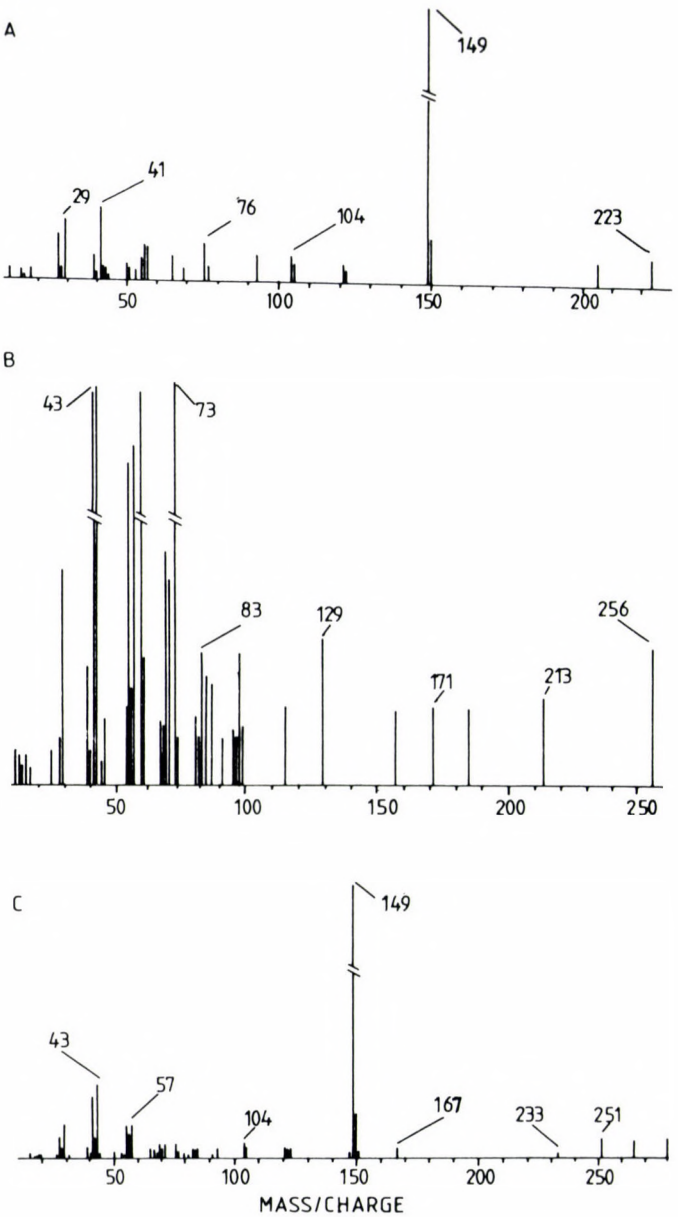

Fig. 3. Mass spectrum of the peak belonging to the 9.7 min (A), 9.9 min (B) and 15.3 min (C) retention time of the Euphorbia honey extract gaschromatogram. Identified compounds: butyl-2-methylpropyl phtalat, hexadecane acid, diheptyl phtalat. (HP 5890A GC, HP 5970 MSD, 70 eV EI)

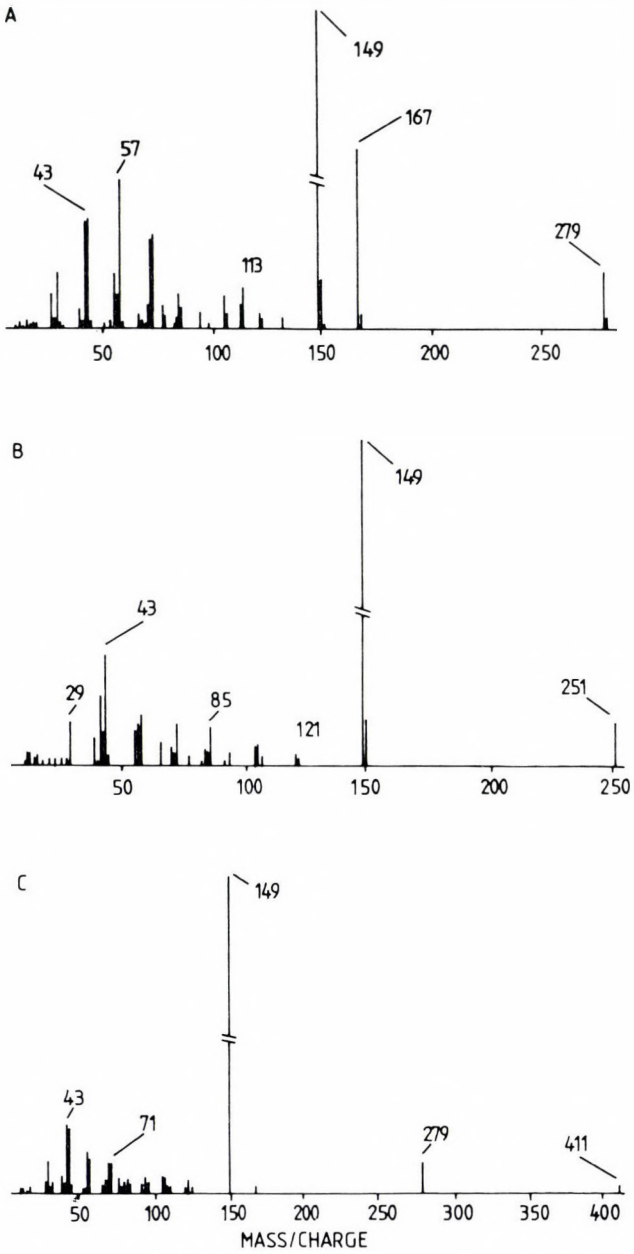

Fig. 4. Mass spectrum of the peak belonging to the 15.5 min (A), 13.2 min (B) and 18.5 min (C) retention time of the Euphorbia honey extract gaschromatogram. Identified compounds: bis (2-ethyl-hexyl) phtalat, benzenedicarboxylic acid decyl-hexyl ester, benzenedicarboxylic acid isodecyl-octyl ester. (HP 5890A GC, HP 5970 MSD, 70 eV EI)

reagents. Due to the lack of TLC standards, we could not strive for accurate determination of these components; we could only establish the correspondences in the composition of the products of the two secreting systems. The presence of such compounds in the extract of the latex that were not found in the extract of the honey (Fig. 1: plate III, spot B) shows that either the functional connection of the two secretional systems is selective or the amount of the compounds is under the lowest detection limit in the honey. Considering also the gaschromatograms of the extracts of the latex and the honey with ethyl acetate in our GC-MSD analyses, we found several identified compounds between them (Fig. 2).

The gaschromatographic peaks of the compounds of the honey can be seen clearly on Fig. 2A. Based on mass-spectrum (Figs 3 and 4) and with the help of the NBS mass-spectrum library, we could identify six of these compounds. They were the following: butyl-2-methylpropyl phtalat (Rt: 9.7 min), hexadecane acid (Rt: 9.9 min), diheptyl phtalat (Rt: 15.3 min), bis (2-ethylhexyl) phtalat (Rt: 15.5 min), benzenedicarboxylic acid decyl-hexyl ester (Rt: 13.2 min), benzenedicarbolyxic acid isodecyl-octyl ester (Rt: 18.5 min). The first four of these compounds above are also constituents of the latex. The peaks of these compounds can also be seen on the gaschromatogram of the latex extract (Fig. 2B, C). As these peaks appeared in both extracts at the same retention time, and their mass-spectrums were also the same, these compounds of the extracts are considered identical.

Conclusion

The functional connection between the intracellular latex-secretion and the extracellular nectar-secretion arises from their anatomical connection as suggested by the results of our TLC and GC-MSD examinations. As the latex of Euphorbia species contains several toxic compounds, the secretion of these compounds into the nectar and finally into the honey may cause medical problems as it is well-known about the honey of tropical "noors".

Considering the facts above, the toxicological and pharmacodinamic examinations of mixed honeys containing Euphorbia honey is suggested.

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BOOK REVIEW

NEUROBIOLOGY OF INVERTEBRATES

Signal Molecules, Networks, Behaviour
Proceedings of the Symposium of the International Society for Invertebrate Neurobiology, Tihany, Hungary, June 23--28, 1991.

Edited by J. Salánki, K. S.-Rózsa and K. Elekes.

Akadémiai Kiadó, Budapest 1993, pp. 1 447. 75.- USD

The situation in the broad field of neurosciences of invertebrate organisms is still in a developing period similar to the historical ages of "roving over see and land", that is in a state of peculiar "migration". Research in this exciting field is still merely an "adventure" in the eyes of some colleagues working on vertebrate organisms.

The above circumstance renders justification to the existence of a separate Society of Neuroscientists working on invertebrates and to the decision of the Editors (J. Salánki, K. S.-Rózsa and K. Elekes) to publish a separate volume containing 44 original papers presented in Tihany in June 1991, and originally printed in Acta Biologica Hungarica in 1992.

The forty-four presentations represent an outstanding selection of recent achievements in Invertebrate Neurobiology including the most important authors and topics in that field. E.g. L. Tauc et al. present their new findings on regulation of transmitter release by presynaptic receptors at a cholinergic neuro-neuronal synapse; K. S.-Rózsa displays her impressive data on reconstruction of neuronal networks guided by signal molecules in Helix neurones; G. A. Horridge summarizes his theory on visual motion perception in insects; N. I. Syed et al. report on their in vitro data of respiratory regulation in Lymnaea neurones; etc. These few arbitrary examples merely demonstrate the abundance and originality of the papers in this important selection.

All in all, Professor Salánki's and his coworkers' volume is a most valuable contribution to recent database in Invertebrate Neuroscience. Although the Reviewer is altogether not an adherent of the publication of isolated Proceeding Volumes (considering such Volumes peculiar "cemeteries of valuable data"), such special cases, as the publishing of findings on Invertebrates, as outlined above, can be justified by the peculiarity and by the still existing "eccentricity" of such important and long-needed topics.

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(Budapest)

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