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Acta Biologica Hungarica 48 (1), pp. 1—8 (1997)

EFFECTS OF DIETHYL-DITHIOCARBAMATE ON ANTIOXIDANT SYSTEM IN CARP TISSUE

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(Received: 1995-05-05; accepted: 1995-09-20)

The influence the pro- and antioxidant values the diethyl-dithiocarbamate (DDTC) in different concentrations in carp tissues was studied.

From antioxidant enzymes superoxide dismutase, catalase and glutathion peroxidase activity changes were studied in tissue homogenates.

Beside enzyme activities tissue lipid peroxidation (LP), reduced glutathione (GSH) and hydroxyl radical loading of the tissues were measured.

Our results indicate that DDTC affects antioxidant parameters by generating GSH excess, not loading the cells thereby with "oxidative stress".

Keywords: Diethyl-dithiocarbamate — pro- and antioxidant influence — hydroxyl radical — carp

Introduction

In our previous work we reported on how an organophosphate influenced tissue antioxidant enzymes and some redox parameters in the different tissues of carp (Cyprinus carpio L.) and catfish (Ictalurus nebulosus). We found that the organophosphate tested affects the fish tissues by developing "oxidative stress" (oxidative dominance) /7/.

We also drew conclusions about the effect of Gramoxon (a Hungarian paraquat (PQ) preparate) on tissues of silver carp (Hypophthalmichthys molitrix) /13/.

DDTC is known as a superoxide dismutase (SOD; EC 1.15.1.1.) inhibitor /10, 17, 21/. On this reason it appeared to be important to know how the DDTC in different concentrations influences the antioxidant systems of carp, the most widespread freshwater fish in Hungary.

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Materials and Methods

Chemicals applied in our studies were purchased from Sigma, Merck and Reanal. They were of highest purity and used without any further purification. DDTC was also purchased from Sigma.

Fishes of both sexes were kept in well-aerated 100 L tanks with free-running tap water. Water temperature was 18 ± 1 °C. The adaptation time was 36 hours. The weight of fishes was between 600-800 g. In every tank 5 fishes were kept and used as control and/or for treatments. "Random" samples were taken from both control and treated fishes /7/.

DDTC was added to the fish keeping tanks in two concentrations, 10^{-3} and 10^{-4} M/L, respectively. Twenty-four h after intoxication fishes were randomly sacrificed and tissue samples were rapidly homogenized at 0 °C. For measurements either the whole homogenate or the aliquots of supernatants were used after 10 min centrifugation at 3000 g.

Determined parameters

(i) Lipid peroxidation (LP)

LP was measured in the aliquots of the whole homogenate with the method of Placer et al. /20/ modified by Matkovics et al. /15/. Generally the amount of total thiobarbituric acid reactive substances (TTBARS) was determined.

(ii) Determination of enzyme activities from homogenates

Superoxide dismutases (SOD; EC 1.15.1.1) were determined on the basis of enzyme content dependent inhibition of epinephrine-adrenochrome autoxidation /13, 19/. Mitochondrial Mn-SOD was measured in the presence of KCN with the method of Beauchamp et al. /2/.

Glutathione peroxidase (GP-ase; EC 1.11.1.9) activity was determined with the method of Chiu et al. /6/ with cumen hydroperoxide and GSH. After the action of enzyme (5 minutes) the rest of the GSH was measured again by the method of Sedlak et al. /22/ using Ellman's reagent /15/.

Catalase (C-ase; EC 1.11.1.6) activity was determined by the spectrophotometric method of Beers et al. /3/.

(iii) Other parameters

GSH measurement: GSH content was measured with the method of Sedlak et al. /22/, using Ellman's reagent.

Hydroxyl radical ($HO^{\cdot}$) was measured with the deoxyribose method /5, 16, 24/.

The quantity of protein in the homogenate supernatants was estimated by the method of Lowry et al. /11/.

The results were subjected to statistical evaluation with Student's t-test. All numerical data are given as mean ± SEM. Student's t-test and significances were calculated after 5 independent measurements and indicated in the Figures. (In enzyme activities and LP measurements the difference between duplicate determinations from the same sample were never higher than 5%.)

Results

The effects of DDTC applied in two concentrations (10^{-3} and 10^{-4} M/L), on antioxidant systems of carp tissues are summarized in the Figures.

The comparative column diagrams and first column shows the control value, while the second and the third ones show the effect of DDTC of 10^{-3} and 10^{-4} M/L concentrations, respectively. The sequence of carp organ homogenates applied for comparison was as follows: liver, kidney, gill, heart, muscle and brain.

Figure 1 shows the effect of DDTC on the change of LP and GSH.

An important feature of DDTC is that it reduces LP in the function of concentration in every organ except in the brain tissue. GSH slightly

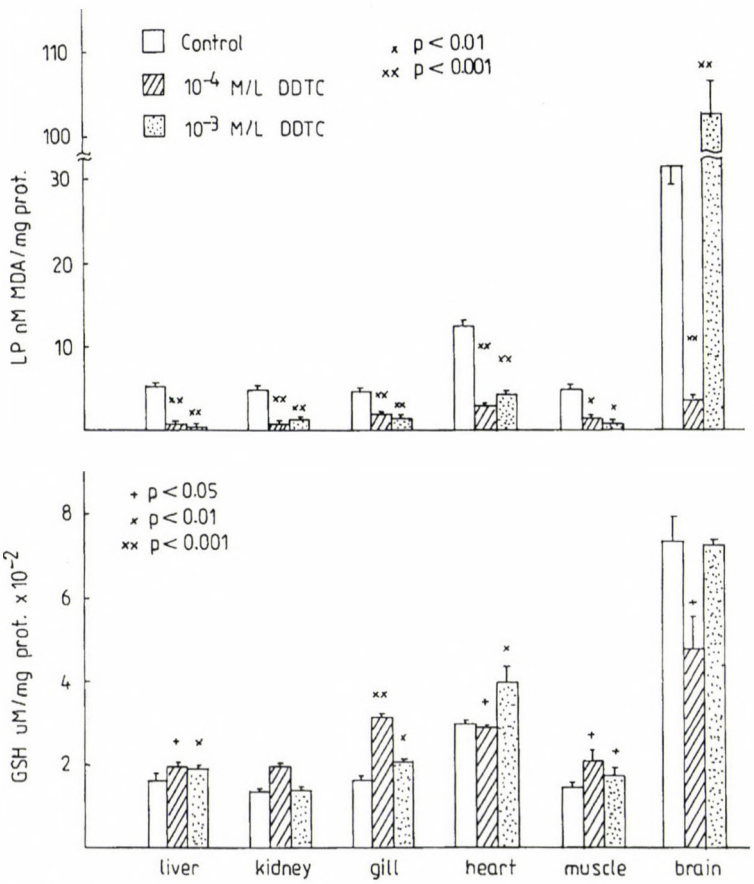


Fig. 1. Lipid peroxidation and reduced glutathione values of tissue homogenates of carp (Cyprinus carpio L.) measured in control after 24 hour treatment with diethyl-dithiocarbamate (DDTC) in two concentrations

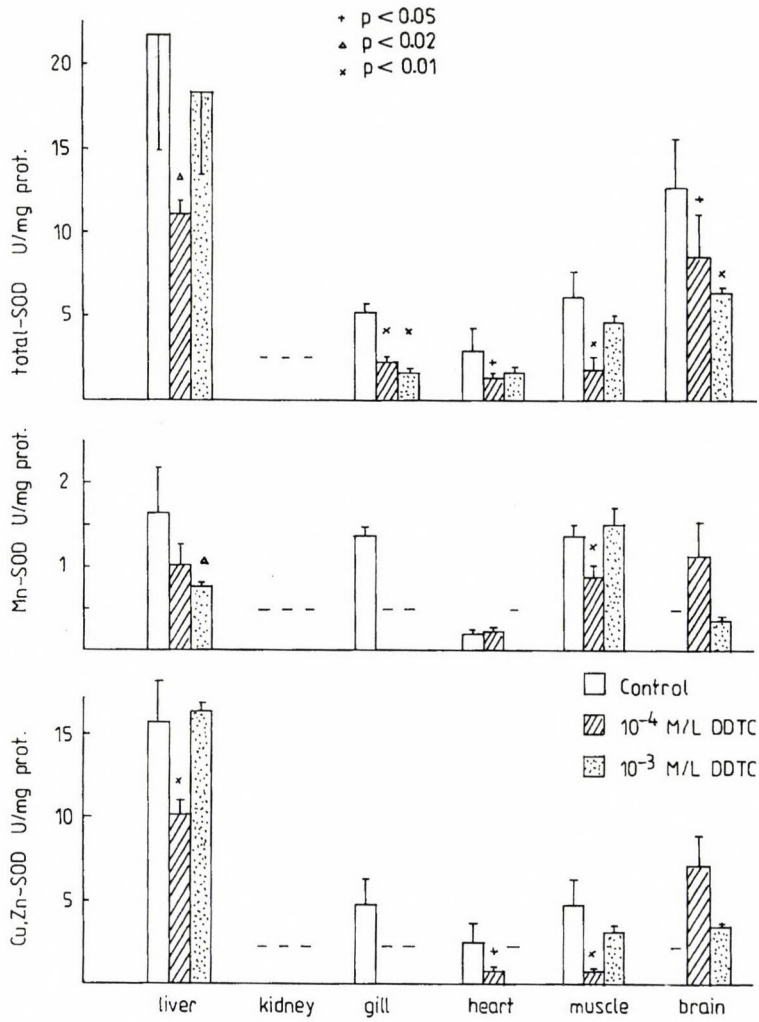


Fig. 2. In the same way as described for Fig. 1, tissue superoxide dismutases activity values were compared

changes, a slight increase in GSH can be observed in tissues on the effect of DDTC concentrations applied.

Regarding column diagrams of Fig. 2 it should be noted that in majority of cases we were not able to measure the SOD activity with the used method by inhibition autocatalytic conversion of epinephrine-adrenochrom. E.g. no SOD activity could be detected at all in kidney homogenate. In cases when SOD activities could be measured, DDTC inhibited Cu-, Zn- and Mn-SOD alike.

In certain cases, e.g. in brain tissue, SOD inhibition showed a concentration dependence.

In Fig. 3 changes in activities of C-ase and GP-ase are compared in the earlier sequence (C, 10^{-3} and 10^{-4} M/L DDTC concentrations). The Figure is worth of attention because. DDTC inhibits C-ase activity in liver in the function of concentration, while it induces an increase of C-ase activity in all the other organ homogenates studied. GP-ase, however, responds with a more or less increase in activity upon DDTC treatment of organs. In brain tissue, a decrease in GP-ase activity occurs upon DDTC treatment.

Figure 4 shows quantitative changes in HO· radical induced by DDTC treatment, and the protein values of homogenates in g prot./100 g wet tissue weight (w.t.w.).

DDTC treatment decreased HO· production concentration dependently in every tissue but in the brain the higher DDTC concentration enhances significantly the HO· radical production.

24-hour-long DDTC treatment hardly influenced tissue protein values.

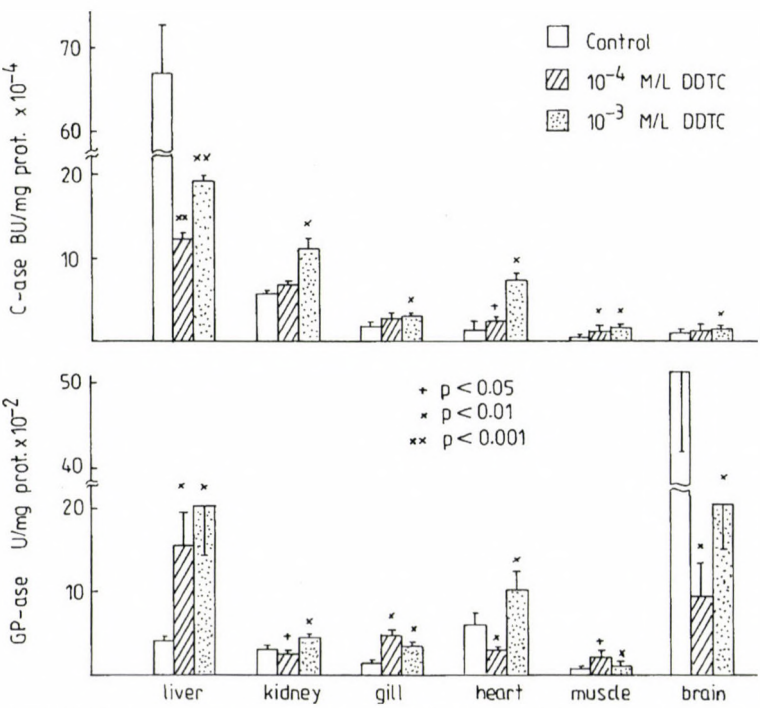


Fig. 3. Peroxide decomposing enzymes activity (glutathione peroxidase and catalase) changes in control and DDTC treated carp tissues

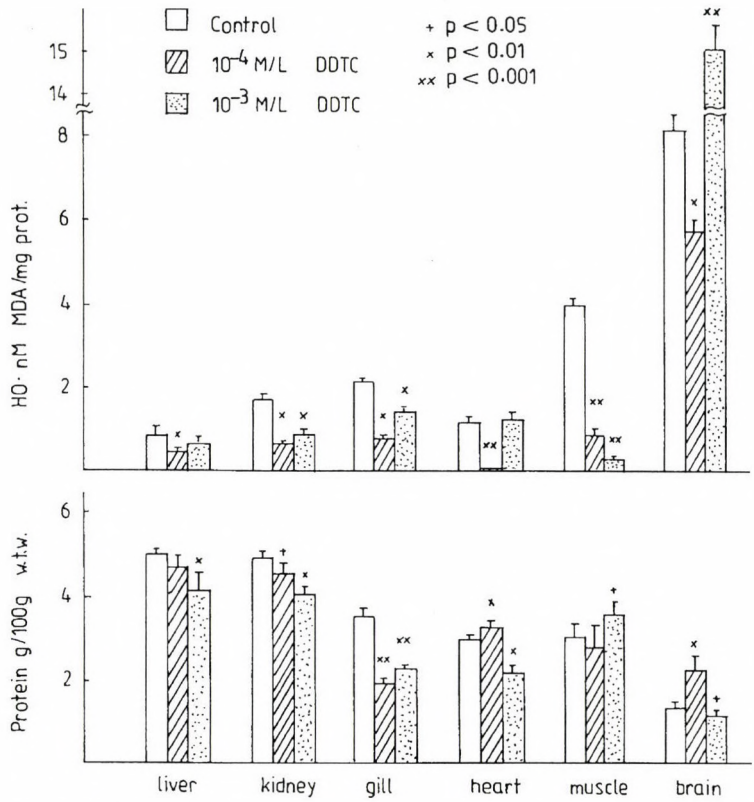


Fig. 4. Changes in the value of HO· radical and protein in carp tissues upon DDTC treatment

Discussion

In several laboratories /1, 9, 18/ including ours /21/ the occurrence of antioxidant enzymes has been studied in fish tissues. No study is known, however, that focuses on the effect of DDTC on antioxidant enzymes, HO· production and on other antioxidant systems.

It has been demonstrated that increase of GSH in rat brain astrocyte cell cultures prevents Cu accumulation in the cell and restores GP-ase activity /23/.

Surveying our studies conducted with fish tissues, it appears that our GSH and GP-ase activity results are in accord with the results of previously used tissue culture experiments. DDTC, however, beside a moderate SOD inhibition, inhibited C-ase, too.

LP inhibition, as our studies have confirmed, can be explained with SOD inhibition (the amount of tissue superoxide is increasing). The hydrogen peroxide and superoxide radicals are similarly dangerous for DDTC, they are oxidatively decomposing it /12/. Another reason of LP decrease the quantitative decrease of HO$^{\cdot}$ radical, the main initiator of LP. The O_2^- radical and H_2O_2 participate in the Haber—Weiss reaction the main producer of HO$^{\cdot}$. The activation of tissue GP-ase decreases the amount of H_2O_2 and that way the amount of HO$^{\cdot}$ radicals, too.

After our results we can suppose that the inhibitory effect of DDTC in different tissues depends on the local concentration of Cu as well as the amount of different oxygen radicals.

Acknowledgement

This work was supported by the National Scientific Research Fund, OTKA, Hungary, No. I/3-64/1993.

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UNCONDITIONAL DISCRIMINATION AS A PARADIGM FOR INVESTIGATING VISUAL PROCESSING IN THE AVIAN BRAINSTEM

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(Received: 1995-03-01; accepted: 1995-07-10)

Visual discrimination between colors and patterns was studied in telencephalectomized chicks of Japanese quail lines that were artificially selected for early approach preferences for particular colors and patterns. Since these preferences are not conditional on prior learning, they provide an effective experimental paradigm to study visual discrimination without relience on acquired stimulus discrimination. The data indicate that the quail's brainstem-thalamus complex is sufficiant for relatively fine discriminations between various colors and achromatic patterns.

Keywords: Japanese quail — decerebration — visual discrimination — unconditional preferences — selected lines

Introduction

Much of the extant knowledge about the avian visual system has been derived from data on the effects of brain lesions on trained visual discriminations. However, this procedure does not permit direct assessment of the visual competence of the brainstem. This is so, because isolating the brainstem by telencephalic lesions may interfere with the memory necessary for acquired stimulus discrimination quite apart from the bird's visual competence /2, 11/. The unconditional discrimination paradigm used in the present experiment did not depend on prior learning. It does permit the elimination of telencephalic influences in the processing of visual information by complete bilateral hemispherectomy.

Naive Japanese quail chicks approach any conspicuous stimuli shortly after hatching, and exhibit distinct preferences for particular patterns and

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colors /9/. Such preferences are not conditional on prior experience with the stimuli, and they can be readily modified by artificial selection /8, 10/. A system of quail lines has been developed in our laboratory by bi-directional selection for unconditional approach choices between particular colors and patterns. Chicks of the "Blue Line" (BL) and "Red Line" (RL) exhibit nearly errorless preferences for blue or red light stimuli. Another selected line exhibits similar though not quite as extreme preferences for an achromatic pattern: vertical grating over circular dots ("Vertical grating Line": VL).

Microspectrophotometric analysis of visual pigments and oil droplets /1/, together with earlier psychophysical studies /9/ indicated that the effects of selection for color preferences were not mediated by peripheral mechanisms of color vision.

The selected preferences were expressed in choices between stimulus combinations not used in the selection. For example, BL chicks reliably preferred the shorter over longer light wavelength in various paired combinations of colored stimuli, whereas RL subjects preferred the longer wavelength /9/. Recently, we found that complete telencephalectomy did not abolish the selected color and pattern preferences in BL, RL and VL chicks /5/. In the present experiment we examined whether more refined visual discrimination indicated by the earlier psychophysical data might be also spared by decerebration.

Materials and Methods

Subjects were newly-hatched Japanese quail chicks drawn from an unselected "Control Line" (CL) and three genetic lines described above (BL, RL and VL). They were either anesthetised only (NON-OP) or were telencephalectomized (TEL-X) under deep anesthesia (Ketamine: xylazine mixture, 3 mg: 0.02 mg/10 g body weight). The cerebral hemispheres were removed by aspiration rostral and dorsal to the ventral portion of the tractus septomesencephalicus (TSM) by a standard procedure worked out previously /6/. Visual discrimination was tested 24-28 hours following treatment by the same mass-screening apparatus that was used in artificial selection /9/.

The apparatus consisted of 28 identical choice compartments arranged hierarchically, so that a single compartment was on top ("starting box"), two compartments were in the second row, three in the third row, and so on, until eight collection boxes on the ground floor. Two light stimuli were presented at the two corners of the back wall of each choice compartment. The stimuli were backlighted by fluorescent light sources and were identical in size (2.5 x 2.5 cm), shape (rectangular), luminance (10.1 lux) and intermittence (3 Hz of equal light-dark cycles), but different in color or pattern.

Color discrimination was tested in CL and BL chicks by their approach choice of blue or green versus red stimuli, and in RL chicks by choice of red or yellow versus blue stimuli. The blue, green, yellow and red stimuli were presented at 480 nm, 540 nm, 580 nm and 630 nm of peak wavelength with spectral characteristics corresponding to No. 45, No. 58, No. 16 and No. 29 Wratten gelatin filters, respectively (see Kodak manual No. B-3). VL chicks were tested for pattern discrimination by approach preference for vertical gratings over circular dots, and for vertical over horizontal gratings. The pattern stimuli were identical in relative black and white areas (approximately 32% white).

The chicks were placed in the starting box individually at about 2 minute intervals. The chicks approaching either of the two flashing light stimuli to within 7.5 cm stepped on a trap door, and slid into one of the compartments in the next row. After completing 7 trials the chicks arrived at one of the 8 collection boxes in the 8th row. The serial number of the collection box (0-7) indicated the number of choices of one over the other stimulus. Each subject was individually scored and returned to the starting box for the completion of a second run of seven trials.

Chicks were allowed 45 minutes to complete 14 approach choices. Preferences were defined as probabilities of choosing one stimulus over the other stimulus. Preferences were compared to random choices by Student's t-test (after arcsine transformation). Contributions to choice variations by genetic background, telencephalectomy and stimulus combination were assessed by 3-way ANOVA.

Approximately half of the chicks were decapitated after the preference test at day two for histological verification of the lesions. Others were used in a subsequent behavioral study for two weeks to reduce the number of subjects undergoing surgical procedures. Lesions were examined on 20 μm coronal sections stained according to Kluver and Barrera /7/ in brains of 3 or 16 day old birds.

Results and Discussion

Histological examination revealed that apart from a small variation in the amount of tissue at the telencephalic-diencephalic juncture the cerebral hemispheres were completely removed in every subject. No traces of higher visual areas (ectostriatum, visual Wulst), and the neostriatum or paleostriatal complex were detected in the lesioned brains.

Decerebration attenuated preferences in the lines tested for discrimination between colors (ANOVA, effects of surgery in RL, BL and CL: $F = 146.8$, $DF = 1$; $P < 0.0001$) as well as patterns (ANOVA, effects of surgery in VL: $F = 14.4$, $DF = 1$; $P < 0.0001$). Low, but statistically reliable green preference in NON-OP CL birds ($t = 4.59$; $DF = 28$; $P < 0.001$) was completely abolished by surgery ($t = 1.59$; $DF = 29$; $P > 0.05$). Interestingly, though preference for blue over red stimuli was not significantly different from random choice in intact CL birds ($t = 1.38$; $DF = 28$; $P > 0.05$), it was highly significant after telencephalectomy ($t = 4.89$; $DF = 32$; $P < 0.001$).

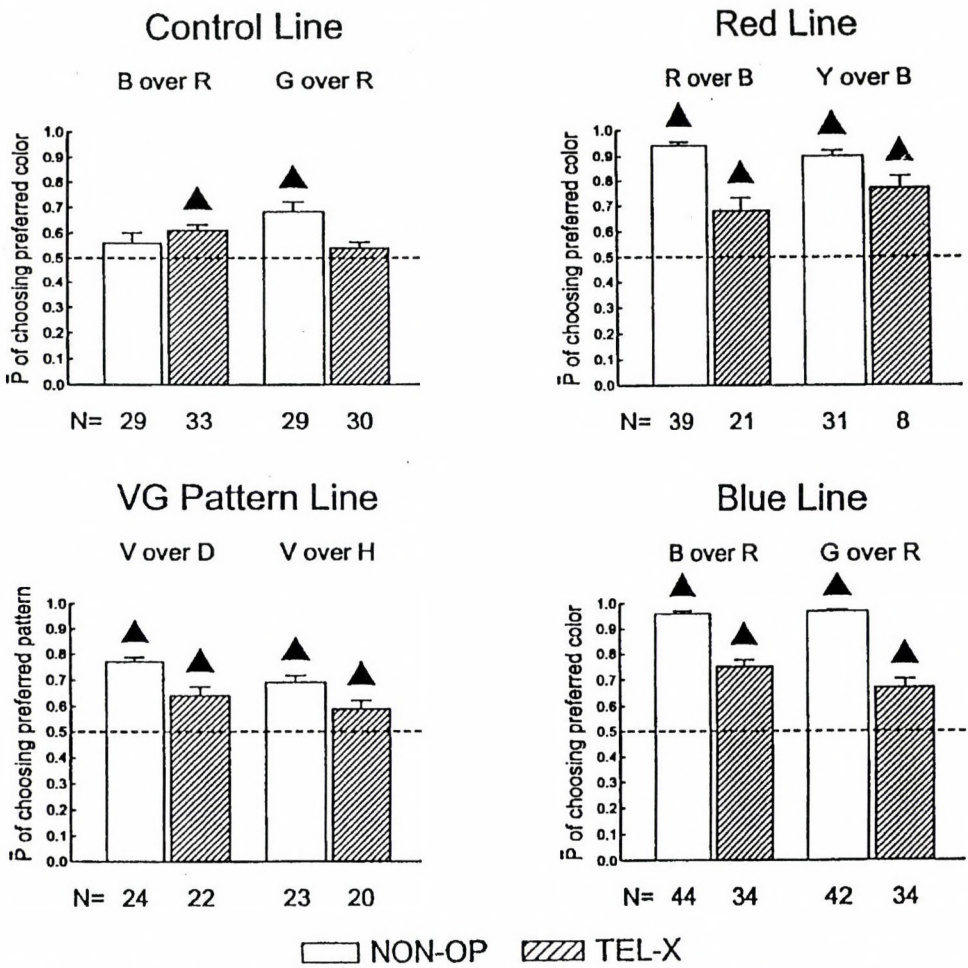


Fig. 1. The expression of short and long wavelength, and vertical grating pattern preference in the unconditional discrimination test. Performances of intact (NON-OP) and telencephalectomized (TEL-X) chicks of the genetically selected lines were significantly different from random choice (by Student's t-test, at $P < 0.02$, depicted by triangles above bars). Number of subjects in each group (N) is indicated under bars (B: blue, G: green, Y: yellow, R: red, V: vertical and H: horizontal grating, D: circular dots stimuli)

In non-operated as well as in telencephalectomized RL birds, preference for long wavelength was expressed both in red versus blue and in yellow versus blue stimulus choices ($P < 0.001$; see Fig. 1). Similarly, short wavelength choices in every BL group were significant, i.e. in blue and red as well as in green and red choices ($P < 0.001$).

Telencephalectomized VL chicks exhibited significant preferences for vertical grating not only over the circular dot pattern ($t = 3.6$; $DF = 21$; $P > 0.01$), but also over horizontal grating ($t = 2.7$; $DF = 19$; $P < 0.02$).

The present data thus corroborated our previous findings that the telencephalon was not critical for the early expression of genetically selected preferences in the Japanese quail /5/. More importantly, preference generalization after telencephalectomy indicated that the avian brainstem-thalamus complex is sufficient for relatively fine visual processing, such as discrimination between green and red, blue and yellow stimuli or between achromatic vertical and horizontal gratings. Thus the present findings together with earlier studies in other species /3, 4, 12/ indicate prominent visual competence of brainstem-thalamic areas in the absence of telencephalic influences. The genetically selected quail lines should provide a hitherto unavailable opportunity to investigate the subtelencephalic mechanism of the avian neural mediation of fine visual discrimination between colors and patterns.

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THE MORPHOLOGY OF THE INTESTINE OF THE ENTOMOPHAGOUS LONGFINGERED BAT, MINIOPTERUS INFLATUS: MUCOSAL TOPOGRAPHY AND POSSIBLE LANDMARKS

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The intestinal tract of the longfingered bat, Miniopterus inflatus, was studied macroscopically, with the light microscope and the scanning electron microscope. The intestine comprised a small mass of coiled loops contained in a rather small abdominal cavity. Macroscopically, the stomach was of the simple type and the intestine was a short convoluted tube whose diameter decreased craniocaudally. A caecum, an appendix and a colon were absent and the only portion of the large intestine observed was a short rectum grossly identifiable only on the account of its greater diameter. Microscopically, a small initial part of the intestine bordering the pylorus was characterized by numerous pits of variable sizes and shapes. This segment preceeded the ridge-like, transversely oriented villi that occupied the rest of the foregut. These villi were tallest in the proximal parts of the foregut and decreased in height caudally, ceasing completely at the junction between the small intestine and the rectum. Goblet cells were few in the cranial part of the intestine and increased caudally, reaching a maximum in the rectum. Intestinal glands were abundant in the region between the villi but Brunner's glands were absent in the submucosa. Generally the intestine of Miniopterus resembles that of the other bats which have been studied but showed structural details suggestive of an increased digestive and absorptive efficiency.

Keywords: Entomophagous bat — intestine — topography — landmarks

Introduction

The chiropteran gut may lack specific external landmarks for identifying the small and large intestines /15, 16, 21, 23/. The colon is reported to be absent in the insectivorous bats, Tadarida nigeriae /23/ and Rhinolophus hildebrandti /18/ while in the African fruit bat, Eidolon helvum /23/, a colon was present but only discernible at histological level. Studies by Ishikawa and others /7/ described an intermediate zone, ileocolon,

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between the ileum and the colon of the entomophagous bat, Myotis frater kaguae.

Investigations on chiropteran gut morphology show a great variation in mucosal topography /27/ and for a long time controversy has existed on what parts of the intestine are actually present in the chiropteran gut. As pointed out elsewhere /18/, the presence of a colon in the intestine of the bat has been mentioned by many workers but it was only on a few occasions that evidence for the existence of such a structure was unequivocal /7, 17, 23, 29/. A rudimentary caecum has been observed in only one species of bats, Rhinopoma hardwickei /15/, and an appendix is generally absent in bats /12, 15, 20/. However, controversy on the presence or absence of the various parts of the large intestine of the bat, especially the colon, has not been resolved as is evident from some recent studies /17, 18/.

The current investigation was prompted by a desire to explore the chiropteran intestinal topography in an attempt to identify the boundary between the small and large intestine and to identify the parts of the large intestine present.

Materials and Methods

This study employed five adult longfingered bats, Miniopterus inflatus (four males and one female), caught in caves in Naivasha (Kenya) by mist-netting (for details on capture methods, the reader is referred elsewhere /19/). The bats were weighed and then killed by intraperitoneal injection of sodium pentobarbitone. The abdominal cavity was opened through a ventromedian incision to reveal the abdominal viscera which were studied in situ. The oesophagus was severed cranial to the diaphragm and the entire gastrointestinal tract separated out by tearing off the mesenteries. Subsequently, the entire tract was transferred to a bath of 0.85% sodium chloride. The intestine was then opened along the mesenteric border, ingesta washed off with fresh saline and measurements of the lengths of the foregut and the hindgut taken. The intestine was then fixed by immersion in 2.5% phosphate buffered glutaraldehyde solution (pH = 7.3) for at least four hours. Three intestinal specimens were prepared for light microscopy (LM) while the rest were prepared for scanning electron microscopy (SEM).

Light Microscopy

For each of the three intestinal specimens set aside for LM, serial segments measuring 1 cm in length were obtained. These segments were rinsed twice with phosphate buffer, post-fixed with 1% osmium tetroxide, rinsed again in phosphate buffer and then serially dehydrated in ascending concentrations of acetone starting from 50% through 60%, 70%, 80%, 90% and 100%. The samples were finally infiltrated with and embedded in Transmit resin

(Taab, UK). Semithin sections measuring 1 µm thick were obtained by use of a Reichert Jung (Austria) ultramicrotome, stained with toluidine blue and viewed under a Carl Zeiss or Nikon light microscope.

Scanning electron microscopy

From the remaining two intestinal specimens, serial segments of 1 cm length were prepared as outlined above under LM. These were dehydrated in ascending concentrations of ethanol starting from 50% through 60%, 70%, 80%, 90%, two changes of 100% and then left in 100% ethanol for 24 hours. These segments were subsequently critical-point dried in liquid carbon dioxide, sputter-coated with gold-palladium complex and viewed on a Jeol (JSM-T100) scanning electron microscope.

Results

The *in situ* disposition of the intestinal loops varied from one individual to the next but the position of the stomach in the cranial left hand corner of the abdominal cavity and that of the rectum in the middle of the pelvic cavity were always the same. The abdominal cavity was very small with the intestinal loops tucked at the caudal end next to the rather small pelvic cavity. The stomach was of the simple type with a short pylorus whose aperture was very close to the entrance of the oesophagus (Fig. 1). The entire intestine was a continuous tube that gradually decreased in diameter in a craniocaudal direction up to the junction between the rectum and the foregut where the diameter again appeared to increase posteriorly (Fig. 1). On average the entire intestine measured 20.4 cm (mean, SD = 1.39) in length with a foregut approximating 19.20 cm (mean, SD = 1.30), while the short

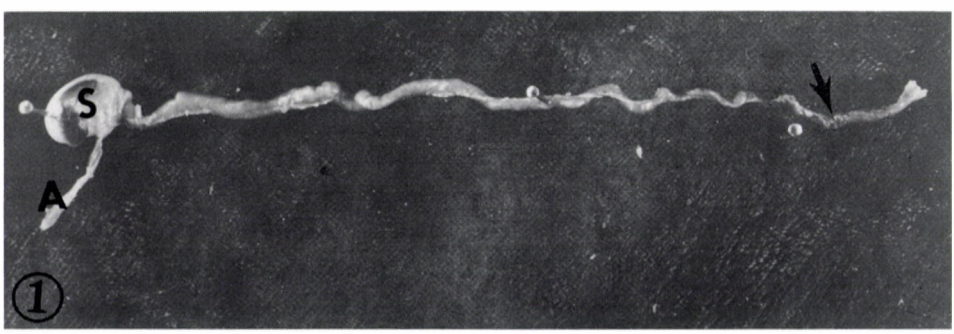


Fig. 1. Photograph of the entire stretched intestine of the adult *Miniopterus inflatus* showing the oesophagus (A), the stomach (S) and the approximate beginning of the rectum (arrow). Grossly, the exact position where the rectum begins may be difficult to pick. Scale: 1 cm represents 1.5 cm

Table 1

Lengths (cm) of parts of the intestines of Miniopterus inflatus. Body weights and the ratio of the intestinal length to body weight are also given

Bat No.	Sex	Body weight	Foregut	Hindgut	Int.	Int.: Bw ratio
1	male	8.79	19	0.9	19.9	2.16
2	male	8.19	21	1.2	22.2	2.56
3	female	9.03	18	1.0	19.0	1.99
4	male	8.16	18	1.5	19.5	2.21
5	male	9.40	20	1.6	21.6	2.13
Mean		8.71	19.20	1.24	20.4	2.21
SD		0.54	1.30	0.31	1.39	0.21

Abbreviations: Bw = Body weight
Int. = Intestinal length

rectum measured about 1.24 cm (mean, SD = 0.31) in length (Table 1). Immediately posterior to the pylorus was a "honeycomb" cranial segment of the intestine (Fig. 2) made of low longitudinal folds and intervening shallow depressions. The mucosal surface of this segment was characterized by numerous pits of variable sizes and shapes (Fig. 3). These pits were separated by

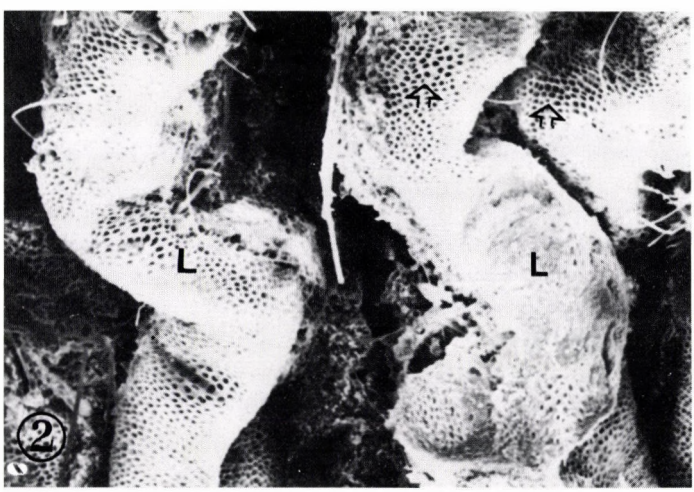


Fig. 2. A scanning electron micrograph showing the proximal honeycomb segment of the intestine of Miniopterus with longitudinal folds (L) and the intervening depressions with the numerous pits (arrows) distributed evenly over the folds and the depressions. x 36

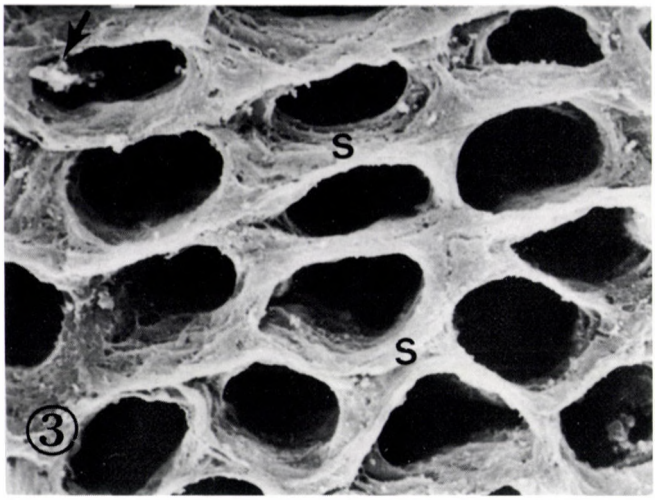


Fig. 3. A close up of the pits in the honeycomb segment showing thick septae (S) and some non-mucus debris in the pits (arrow). This debris is probably part of the ingesta. x 770

thin septae measuring about 5-19 μm thick. The honeycomb segment measured about 10 mm in length and in this segment the mucosa of the entire internal circumference was endowed with the pits. Caudal to the honeycomb segment,

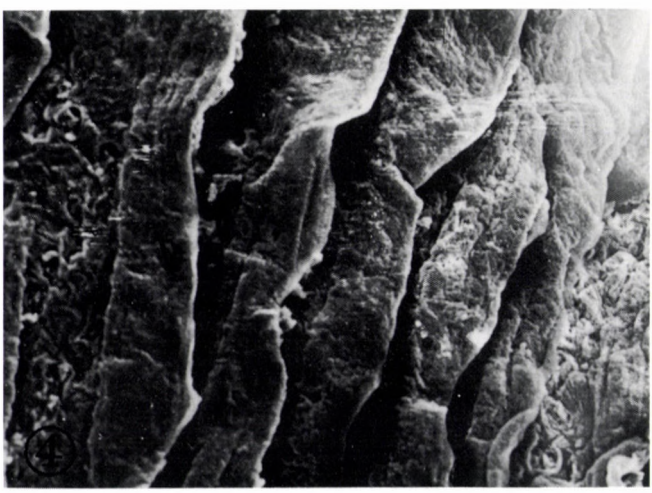


Fig. 4. A low power scanning electron micrograph showing the transverse ridge-like villi (V) found in the greater part of the small intestine of Miniopterus inflatus. This pattern of villi characterizes about 95% of the length of the small intestine. x 150

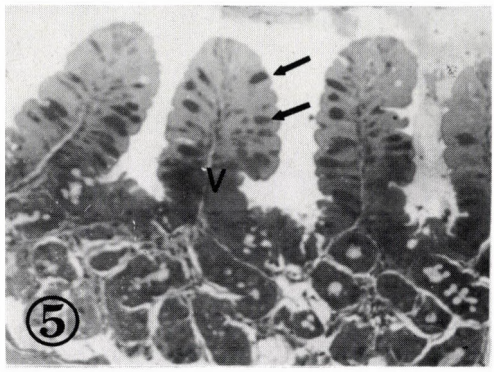


Fig. 5. A histological section to show the type of villi (V) found in the small intestine of Miniopterus inflatus with numerous goblet cells (arrows). This section was obtained from the posterior third of the small intestine (Toluidine Blue). x 400

the luminal surface of the intestine was characterized by transversely oriented ridge-like villi (Fig. 4). The ridge-like villi occupied the entire length of the small intestine and diminished in height craniocaudally (Fig. 5) ceasing completely at the junction between the rectum and the foregut. This region gave way to a short rectum which was distinctly of a

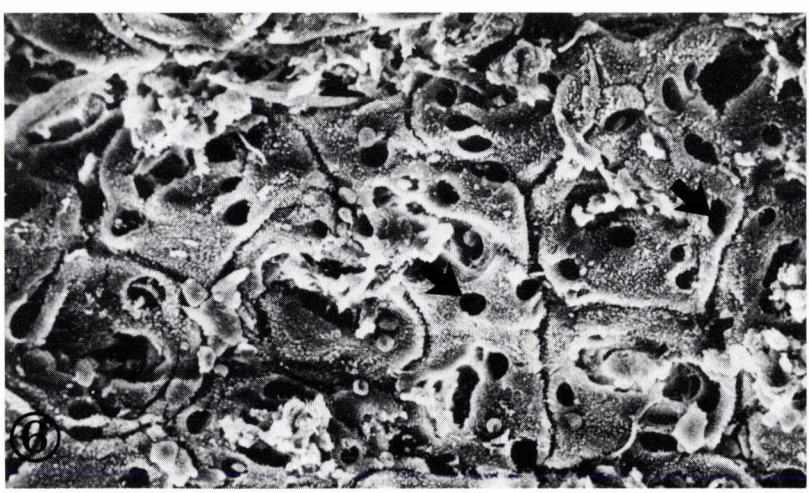


Fig. 6. A low power scanning electron micrograph showing the mucosal surface of the rectum of Miniopterus inflatus with numerous openings to goblet cells (arrows). x 360

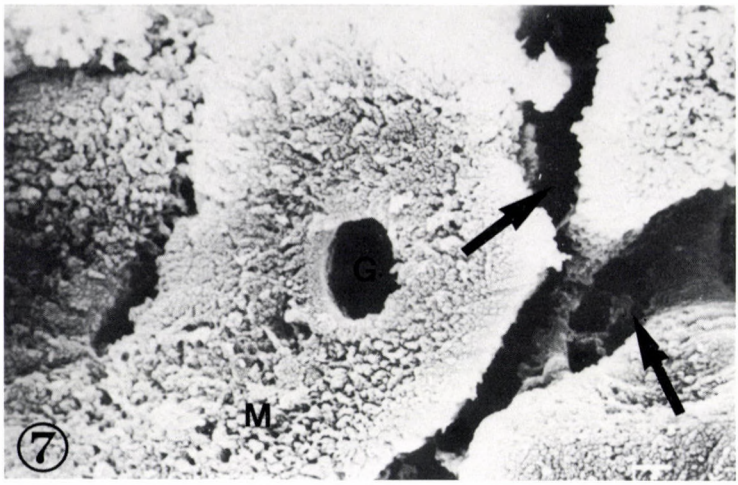


Fig. 7. A higher magnification of the surface of the rectal mucosa showing an outlet of a goblet cell (G), mucosal surface convolutions with a rich microvillous cover (M) and openings to intestinal glands (arrows). x 3800

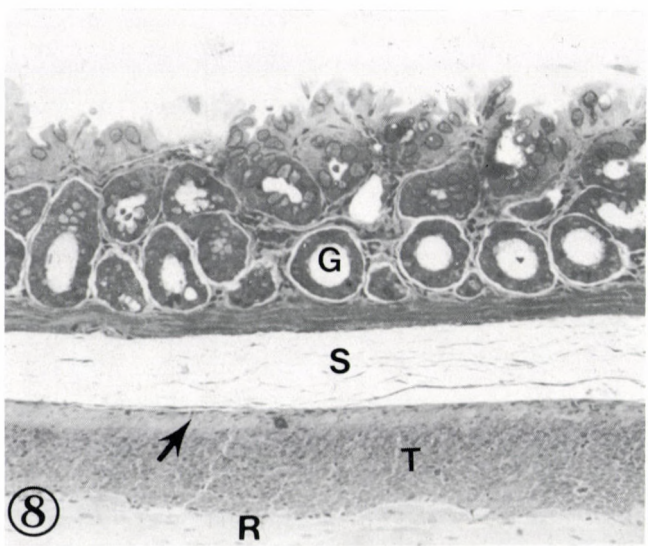


Fig. 8. A light micrograph showing the structure of the rectum with many compact intestinal glands (G). The submucosal layer (S), the inner circular muscle layer (arrow), the outer longitudinal muscle layer (T) and the tunica serosa (R) are also shown. x 900 (Toluidine Blue)

greater diameter than the preceding part of the intestine (Fig. 1) and whose mucosa was devoid of villi but was, nevertheless, thrown into numerous convolutions that were separated by openings to intestinal glands. The convolutions had a rich microvillous cover and very many goblet cells (Figs 6, 7).

The intestinal wall generally comprised the four main layers: the mucosa, the submucosa, tunica muscularis and tunica serosa (Fig. 8). The submucosa of the intestine was notably devoid of Brunner's glands that are normally encountered in the duodenal segment of the mammalian small intestine. In cross section, the villi were finger-like or tongue-like (Fig. 5). Goblet cells were very few in the epithelium of the villi in the cranial part of the intestine but increased gradually reaching a maximum in the rectum. Structures that are normally characteristic of the mammalian large intestine such as haustrae, taenia coli and appendices epiploicae were not observed. The tunica muscularis comprised an inner circular and an outer longitudinal layer (Fig. 8). In general the intestine of the longfingered bat was divisible into a foregut and hindgut only.

Discussion

Except for one reported case /15/, the intestine of the bat is a continuous tube devoid of an appendix or even a caecum and whose diameter is almost uniform. In entomophagous bats, the intestine is noted to be generally short, measuring 12 cm in Myotis frater kaguae /7/, 15.5 cm in Rhinolophus hildebrandti /18/ and 20.4 cm in Miniopterus inflatus (this study). However, as noted elsewhere /18/, comparisons on gross measurements alone may be misleading and body mass weighted values presented here (Table 1) are considered better for such comparisons.

Various parts of the chiropteran intestine may be difficult to discern and in cases where this has been accomplished, special techniques such as histology or scanning electron microscopy have had to be employed /7, 18, 23, 29/. However, these investigations have come up with different findings with some workers reporting the presence of a colon and some unique parts such as an ileocolon in the intestine of the insectivorous bat /7/ while in other cases a colon was reported to be absent /18, 23/. Identification of the various parts of the bat intestine has mainly been based on either mucosal topography /7/ or histology alone /23/ but as has become obvious in this and previous studies /17, 18/, both histological and topographical

(SEM) techniques may be necessary if the various parts of the chiropteran intestine are to be identified. This is in view of the fact that although histologically the villi in the entomophagous bat may appear as discrete finger-like or tongue-like structures (Fig. 5), they are actually disposed as transverse folds spanning the entire internal circumference of the intestine (Fig. 4) or may be interconnected in a reticular formation /18, 27/. The possible significance of such an orientation in withholding less bulky ingesta for efficient digestion and subsequent absorption has been advanced elsewhere /18/ and this probably applies for the bat examined in this study. This seems plausible since anatomically constant folds are capable of reducing digesta transit time /13/ and villi are generally permanent structures.

The use of histology in identification of the various parts of the chiropteran intestine may prove essential since such structures as Brunner's glands that are unique to the duodenum or taenia coli that are specific to the colon or even haustrae and appendices epiploicae that are only found in the large intestine are easily identified at this level. Although such structures have not been encountered in the bat intestine, the increase in number of goblet cells and presence of numerous more compact intestinal glands in the rectum are conspicuously characteristic (Figs 6, 7, 8).

The variation in the chiropteran gastrointestinal tract morphology is related to diet type and has some taxonomic inclinations /25/. Miniopterus inflatus feeds on high flying insects including small beetles /11/. Most insectivorous bats are opportunistic feeders that prey on the available insects rather than selected species /2, 5/. Therefore, the reported variation in patterns of the intestinal villi and general mucosal topography amongst insectivorous bats /27/ shows that the specific type of diet may have little effect on the intestinal morphology. Resemblances observed in a certain feeding group may be related to phylogeny and general type of diet and variations are probably attributable to energy requirements as may be determined by flight ecology and foraging strategies. Unfortunately very little has been studied on the ecology of the tropical entomophagous bats.

The bat intestine is generally remarkably unique both in structure and function. Reports on chiropteran gut physiology indicate that digestion occurs very fast /12, 23, 24/, food transit times are short /8, 12, 14, 29/ and absorption rates for sugars may be three to four times faster than in the rat intestine /9/. Studies on intestinal morphology further indicate that in the bat, villi occur in various shapes including finger-like,

tongue-like, lamella-like, zigzag folds, transverse folds or folds that are interconnected in a reticulate manner /7, 18, 27, 29/. Due to dearth of information on chiropteran gut morphology and the very diverse variation observed in the species studied, it is difficult to predict the patterns or shapes of villi that are likely to be encountered in any one unstudied species. Although the pattern in Miniopterus to some extent resembles that of Rhinolophus described elsewhere /18, 27/, these two species are not closely related phylogenetically. The pattern reported for Rhinolophus ferrumequinum /27/ and that reported for Rhinolophus hildebrandti /18/ show a great deal of resemblance. Whether these patterns are characteristic of a family or genus, however, remains to be elucidated but definite taxonomic relationships are obvious.

A knowledge of the specific landmarks of the intestinal parts may be essential when conductiong physiological and/or morphometric studies involving a certain part of the gut. However, without the aid of any specific external landmarks, the rectum of the entomophagous bat may be picked out only on the account of its greater diameter in relation to the part of the intestine immediately precedent.

The specific role of the pits found in the proximal part of the intestine is unclear but they are probably important in trapping the semisolid part of the ingesta and exposing it to a larger surface area for digestion and absorption as predicted earlier /18/, or may be involved in enzyme production. The presence of non-mucus debris in these pits (Fig. 3) supports the first conjecture and these pits do not appear to be important in mucus secretion.

The absence of the colon in the intestines of the entomophagous bat has been justified elsewhere /18, 23/. The colon is important in absorption of water, electrolytes and short chain fatty acids and in some cases may be involved in microbial fermentation /10/. It is noted that in carnivores and insectivores, the intestine is unimportant as a fermentation chamber and the caecum is very simple or absent /34/. The insects on which most microchiroptera thrive have very little structural carbohydrates and contain low water levels /23/ estimated at 60% /1, 3, 6, 33/ and as such, a colon or fermentation chambers such as caeca may be unnecessary. However, high amounts of mucus are necessary to assist the voiding of faeces which may include hard indigestible parts from arthropod exoskeletons /26/ and hence the presence of the short rectum with numerous goblet cells.

Flight as a form of locomotion is energetically very expensive /4, 30--32/ and its evolution has involved a delicate interplay between morphology and physiology in meeting the very severe constraints encountered in the aerial locomotion. In bats, the evolutionary pressures appear to have been geared towards attaining a small body weight with the result that the smaller species are more maneuverable than the larger ones /22/. In this respect, the organs less related to flight appear to have been reduced or sacrificed and probably this is how parts of the large intestine such as the colon and caecum have been lost.

This study highlights interesting structural adaptations in the intestine of the entomophagous bat that may be related to phylogeny, diet and energetics in this unique group of mammals. It would be interesting to study more species belonging to different families and genera and with different feeding characteristics and foraging strategies in order to understand the various mucosal topographical categories present in the extant species. Physiological studies to determine the functions of the pits in the proximal part of the intestine are strongly advised and functional parameters such as surface area available for absorption in different groups of bats can easily be determined using the recently modified stereological techniques /19/. Generally, a study of the ecology of the tropical bats would largely assist in the interpretation of some of the unique anatomical and physiological findings associated with these animals.

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MORPHOMETRY OF THE KIDNEYS OF FRESH WATER PERCOID AND OSTARIOPHYSIAN TELEOSTS

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Kidneys of four fresh water teleost species representing percomorpha (Oreochromis niloticus and Micropterus salmoides) and Ostariophysi (Cyprinus carpio and Clarias mossambicus) were studied for quantitative structural characteristics using tissues fixed by perfusion. The renosomatic index (weight of kidney/weight of fish) was higher in the ostariophysian than percoid fish ($p < 0.05$) but values of volume of nephronic tissue/body weight were not significantly different. Results obtained by point counting morphometry showed that the kidneys of the percoid species were made up of 56.85-64.4% nephronic tissue, 30.45-32.47% blood vessels, 2.84-8.20% ureter and ureteral ducts and 2.07-2.48% connective tissue. The kidneys of the ostariophysian species comprised of 35.27-36.71% nephronic tissue, 27.86-29.29% blood vessels, 1.59-4.20% ureter and its ducts, 29.07-29.51% interstitial cells and 2.70-3.60% connective tissue. The mean values for the volume proportions of the components of nephronic tissue were not significantly different in the percoids and ostariophysians and they were; renal corpuscles 3.84-6.54%, neck segments 0.50-1.11%, proximal segments 67.17-69.72%, distal segments 18.16-20.55%, and collecting tubule—collecting duct (CT—CD) system 5.66-7.41%. These results show that the quantitative structural characteristics of the kidneys of fresh water species from different orders are quite similar and emphasise the all persuasive influence of the environment on renal structure and function in teleosts.

Keywords: Teleosts — kidney — light microscopic morphometry — fresh water — percoid — ostariophysian

Introduction

Percoid fish are a recent group of teleosts that has successfully invaded fresh waters of all continents and ostariophysian fish (cyprinids and silurids) are mainly stenohaline fresh water fish. In correlation with the differing requirements in the diverse aquatic habitat, the organ systems

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used in osmoregulation exhibit great variations among the different species /11/. Consequently, kidney structure, in particular the segmentation of the nephron, differs in fish occurring in different aquatic habitats. A great variety of nephron types are discernible in kidneys of fish and typical nephrons (multisegmental) and atypical nephrons (paucisegmental and aglomerular) occur in fresh water fish /11, 13/. Kidneys of fresh water percoid and ostariophysian fish so far studied have multisegmental nephrons. Furthermore, ostariophysian fish kidneys contain abundant peritubular cells which are scarce or absent in some members of the percomorpha /13, 15, 16, 18/.

In addition, comprehensive data on quantitative structural characteristics of the fish kidneys are generally scarce. The available data so far includes the measurements of kidney length and weight, the number, size and area of glomeruli, the renal tube length and proportions of various segments, height of tubular epithelium and nuclear areas and the size and volume of mitochondria for a limited number of species /3, 7, 8, 16, 21, 23, 29/.

This study examines some quantitative structural characteristics of the kidneys of fresh water percoid and ostariophysian fish to given insights into structure-function in fish kidneys. However, the number of species in study was small and more work is required to give more comprehensive insights on structure-function relationship in fish kidney. Moreover, quantitative structural studies could be of importance in view of assessing abnormal changes of the kidney caused by increasing pollution of aquatic habitats and those arising in aquaculture.

Materials and Methods

Experimental animals

Twenty-two (22) fish specimens of the orders Percomorpha (6 Nile tilapia and 6 blackbass) and Ostariophysi (5 common carp and 5 catfish) were obtained from Sagana Fisheries, Masinga dam and Lake Naivasha in Kenya. The fish were transported in aerated polythene bags to the department of Veterinary Anatomy, University of Nairobi and maintained for 3-5 days in plastic tanks containing Nairobi tap water and room temperature (20-30 ^{o}C) before use. The fish were fed daily to satiation using a diet of commercial tropical flakes for fresh water fish.

Animal preparation

Fish were killed by destruction of the brain, weighed and the body cavity opened to expose the kidneys and the heart. A polythene cannula at a height of 80-120 cm of water /7, 11/ was introduced into the heart and ligated at the bulbo-ventricular junction. Blood was removed from the circulatory system by gravitational perfusion of chilled saline /30/ for 5-10 minutes. Perfusion fixation of the kidney was performed in situ for 15-20 minutes using 2.5% glutaraldehyde in 0.15 M phosphate buffer, pH 7.2. By this method the fixative was rapidly distributed through the branches of segmental arteries to all the regions of the elongated kidney. The right and left kidneys in each fish were carefully excised from the body wall, weighed and their volume determined by fluid displacement /25/ before being immersed in 2.5% glutaraldehyde for 12 hours.

Light microscopic morphometry

For each species the right and left kidneys (either partially or completely fused) were cut into eight serial blocks and routinely processed for paraffin sectioning. From each block two 7 μm thick sections were cut using a Leitz microtome and stained with Gomori's trichrome, Harris' haematoxylin and alcian blue. The tissue shrinkage factor assessed by measuring the length of a phase of tissue block before and after processing was 0.88.

The point counting method described by /1, 28/ and adopted for the mammalian and avian kidneys /24, 26, 27/ was used to determine the volume proportions of the main components of the kidney at a magnification of x60 and the volume proportions of the components of the nephronic tissue at a magnification of x100. A Zeiss microscope fitted with a 100 point Zeiss integrating eyepiece graticule was used for all the counting. The main components of the kidney quantified were the nephronic tissue, blood vessels, interstitial cells or tissue, ureter and its ducts and connective tissue (Figs 1 and 2). Absolute volumes of the main components of the kidney were calculated by multiplying the volume proportions of the component with the kidney volume obtained by water displacement. The components of the nephronic tissue quantified were renal corpuscles, neck segments, proximal and distal segments and collecting tubules and small ducts (Fig. 3).

Glomerular diameters were determined using an eyepiece micrometer for the renal corpuscles showing both the vascular and urinary poles. Fifty glomeruli were measured.

Results

Histological observations

Light microscopic observation of paraffin sections revealed that kidneys of all the species were glomerular with multisegmental nephrons. There was no evidence of cortico-medullary zonation; distal segments were irregularly intermingled with proximal segments, collecting tubules and ducts. The interstitium of the percoid fish kidneys appeared fairy clear (Fig. 1) in contrast to that of the ostariophysian fish that had abundant interstitial

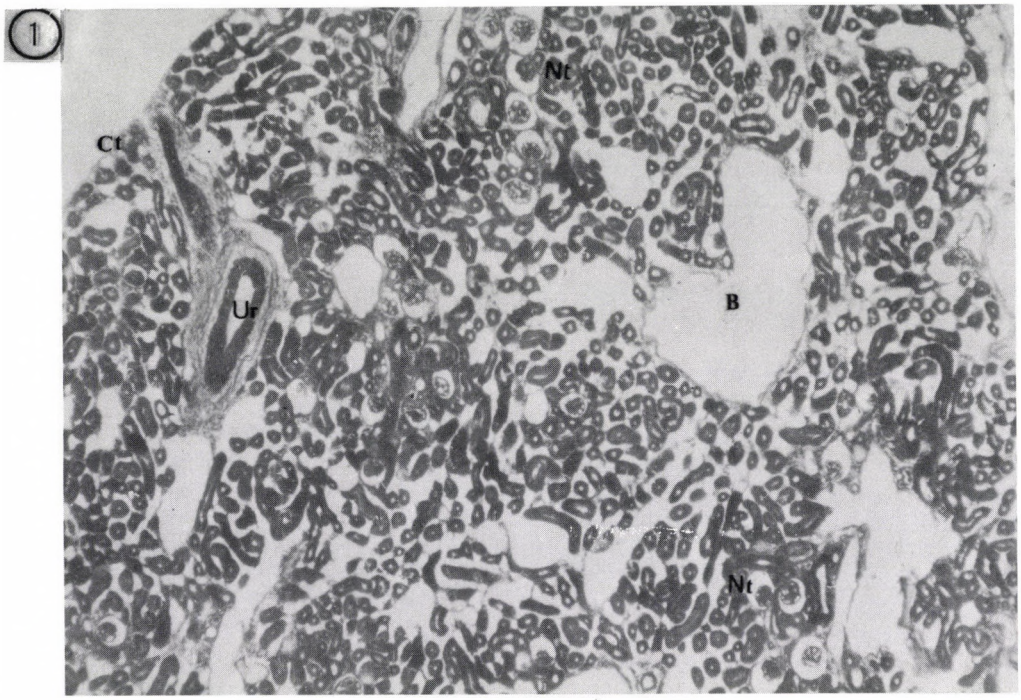


Fig. 1. Part of perfusion fixed kidney section of the blackbass, Micropterus salmoides, showing the lack of zonation and the absence interstitial cells/tissue. The kidney of Nile tilapia, Oreochromis niloticus, had a similar appearance. Nt: nephronic tissue, B: blood vessels, Ur: ureteral duct, Ct: connective tissue. x 80

cells (Fig. 2). In all the fish, the segments of the nephron distinguished were the neck and proximal segments I and II, distal segments and a collecting tubule-collecting duct system draining into the ureteral duct system and the ureter (Fig. 3). These segments were distinguished by their histological, topographical and staining characteristics as follows. The neck segments were contiguous to the renal corpuscles and were composed of low cuboidal cells with apical cilia. The proximal segments were made up of columnar cells with an apical brush border. Proximal segment I (PI) had a taller and more densely packed brush border than proximal segment II (PII). In addition, PI possessed a well-developed vacuolar apparatus. The distal segments were formed by low columnar cells with metachromatic cytoplasm but lacking apical brush border. The collecting tubule-collecting duct system was constituted by the profiles of columnar cells with metachromatic cytoplasm surrounded by thin discontinuous layers of smooth muscle and connec-

tive tissue. The ureter and ureteral duct system were surrounded by a thick layer of connective tissue.

Morphometric observations

The values (means ± S.D.) for the renosomatic index, the volume proportions and absolute volumes of the main components of the kidneys of the percoid and ostariophysian teleosts are shown in Table 1. The renosomatic index ranged from 2.22 ± 0.07 mm^3/g in the blackbass to 6.13 ± 0.71 mm^3/g in in the catfish. The kidneys of the ostariophysian teleosts were larger than those of the percoid fish ($p < 0.05$). The volume proportion of the nephronic tissue was lowest in the common carp (35.27 ± 1.30%) and highest in the tilapia (64.64 ± 1.32%). Blood vessels made up 27.86 to 32.47% of the kidney in all the species investigated. The ureter and the ureteral duct system were best developed in the blackbass in which it formed 8.20 ± 0.41% of the

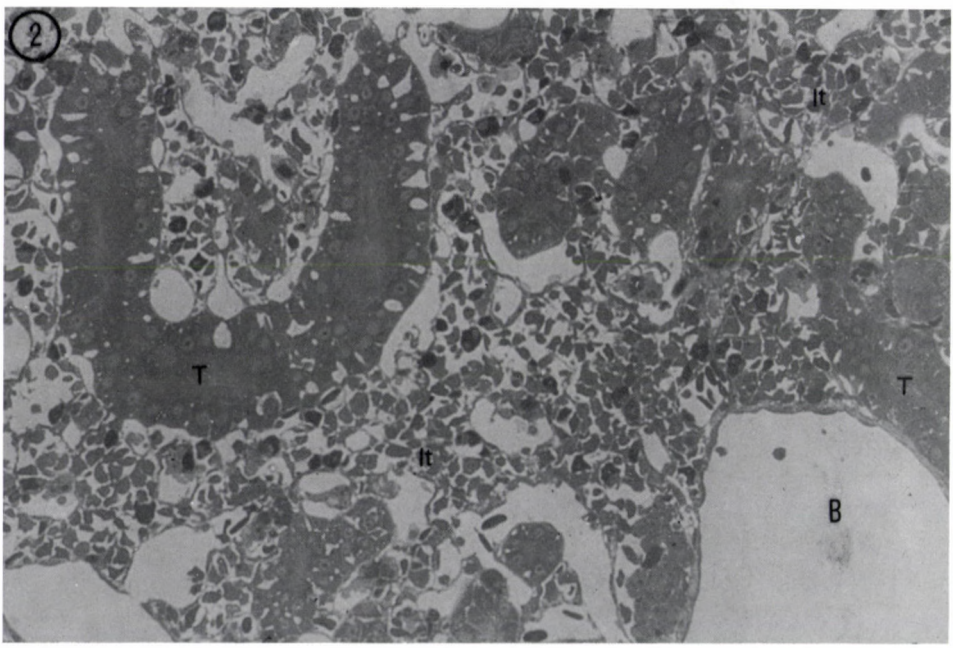


Fig. 2. Part of kidney section of the catfish, Clarias mossambicus, showing abundant interstitial tissue. The kidney of the common carp, Cyprinus carpio, has a similar appearance. It: interstitial tissue, T: renal tubules, B: blood vessels. x 625

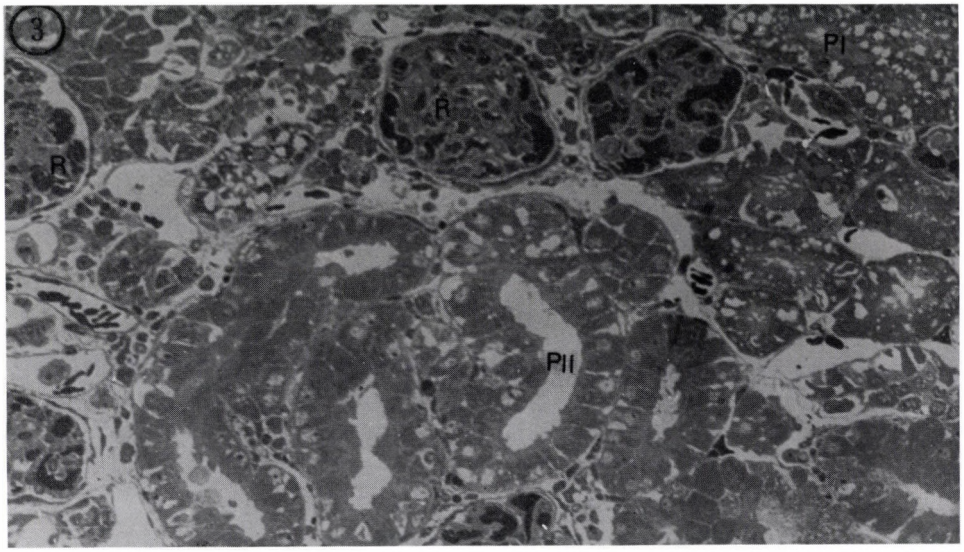


Fig. 3. Part of a section of the kidney of the Nile tilapia, Oreochromis niloticus, showing parts of the nephron. R: renal corpuscle, PI: first proximal segment, PII: second proximal segment. x 625

kidney volume and least developed in the catfish in which it formed 1.59 ± 0.18 of the kidney volume. Ostariophysian fish kidneys contained abundant interstitial cells that formed 29.07 ± 2.01% and 29.51 ± 1.64% of the kidney volume in the common carp and the catfish, respectively. Connective tissue formed 1-3% of the kidney in all the species investigated.

Table 2 shows the mean (± S.D.) value for the volume proportions of the nephronic segments expressed as part of the nephronic tissue. The volume proportion of renal corpuscles ranged from 3.84 ± 0.48% in the catfish to 6.54 ± 0.95% in the blackbass. Neck segments formed relatively low volume proportions of the kidney and the values ranged from 0.50 ± 0.15% in the tilapia to 1.11 ± 0.29% in the common carp. Proximal segments were the main component of the nephronic tissue and formed 67.17 ± 69.72% of the nephronic tissue amongst the species studied. Volume proportions of the distal tubules ranged from 18.16 ± 1.25% in the common carp to 20.55 ± 1.91% in the catfish. The CT-CD system formed relatively low volume proportions of the nephronic

tissue and the values ranged from 5.66 ± 1.76% in the tilapia to 7.41 ± 1.16% in the catfish.

Allometric equations and correlation coefficients relating body weight and the volumes of the nephronic and vascular space for all the species and groups investigated are shown in Table 3. The absolute volumes of the nephronic tissue and the vasculature were well correlated with body weight in all the species and groups studied. There was no significant difference in the volume of nephronic tissue in the percoid ostariophysian teleosts. However, the volume of the vascular space was higher in the ostariophysians than in the percoids ($p < 0.05$).

The mean (± S.D.) values (adjusted for shrinkage) for the glomerular diameters of the species studied were Nile tilapia, 59.92 ± 8.36 μm; blackbass, 53.43 ± 6.40 μm; common carp, 54.14 ± 7.85 μm and catfish 50.11 ± 6.76 μm. There was no significant difference in the glomerular diameter between the percoids and the ostariophysians.

Discussion

Perfusion fixation at physiological blood pressure results in uniformly and adequately preserved kidney tissues characterised by uniform arrangement of renal components. Good preservation of tissues is important in morphometric studies since the aim is to obtain data representative of the live animal. It has been demonstrated /7, 9, 12, 14/ that perfusion improves the preservation of fish renal components for microscopy. Similar observations /20, 27/ have also been made on mammalian and avian kidneys. According to previous observations /27/ the main difference between immersion and perfusion fixed avian kidneys are the volume proportion of the vascular compartment. It is therefore important to ensure that the perfusion pressure equals the physiological blood pressure during perfusion fixation.

The Gomori's, Harris' haematoxylin and alcian blue staining procedure used in this study facilitated identification of renal tubule segments by their colour (Fig. 3), and this coupled with the observable histological characteristics was adequate for the identification of all the renal components for quantification. Alcian blue when used with other stains such as PAS has been shown to bind to biochemical substances in fish epithelia /10, 12/ resulting in different shades of metachromasia in renal tubule segments, thus allowing identification. The criteria for identifying the different

Table

Renosomatic index (RSI) and the volume proportions (V_V%) and absolute volumes (abs. vol., mm^3) tissue of the percoid and the

Species	RSI	Nephronic tissue V_V%	Nephronic tissue Abs. vol. (mm^3)	Blood vessels V_V%	Blood vessels Abs. vol. (mm^3)
Nile tilapia (*O. niloticus*) /6/	2.83 ± 0.34	64.64 ± 1.32	160.74 ± 47.94	30.45 ± 0.99	75.56 ± 18.90
Blackbass (*M. salmoides*) /6/	2.22 ± 0.27	56.85 ± 1.34	557.31 ± 158.63	32.47 ± 1.15	317.40 ± 86.01
Common carp (*C. carpio*) /5/	4.24 ± 0.97	35.27 ± 1.30	648.86 ± 348.81	27.86 ± 1.14	521.50 ± 303.35
Catfish (*C. mossambicus*) /5/	6.13 ± 0.71	36.71 ± 0.67	172.50 ± 96.80	29.29 ± 0.99	138.43 ± 79.41

The values are means ± S.D. The number in brackets against the name of the species stands

Table 2

Volume proportions (V_V%) of the nephronal segments (renal corpuscles, neck, proximal and distal segments and collecting tubule-collecting duct system) for the percoid and ostariophysian fish studied expressed in the nephronic tissue

Species	Renal corpuscles (%)	Neck segments (%)	Proximal segments (%)	Distal segments (%)	Collecting tubules and ducts (%)
Nile tilapia (*O. niloticus*)	4.50 ± 0.51	0.50 ± 0.15	69.72 ± 2.21	19.62 ± 3.36	5.66 ± 1.76
Blackbass (*M. salmoides*)	6.54 ± 0.29	0.76 ± 0.37	67.17 ± 1.87	19.17 ± 0.86	6.36 ± 1.27
Common carp (*C. carpio*)	4.16 ± 0.95	1.11 ± 0.29	69.50 ± 1.77	18.16 ± 1.25	7.07 ± 0.74
Catfish (*C. mossambicus*)	3.84 ± 0.48	0.94 ± 0.24	67.26 ± 2.22	20.55 ± 1.91	7.41 ± 1.16

Values are means ± S.D.

1

of the nephronic tissue, blood vessels, interstitial cells, ureter and its ducts and connective ostariophysian fish in the present study

Ureter and its ducts		Interstitial cells		Connective tissue	
V_v%	Abs. vol. (mm^3)	V_v%	Abs. vol. (mm^3)	V_v%	Abs. vol. (mm^3)
2.84 ± 0.49	7.15 ± 2.68	—	—	2.07 ± 0.38	5.24 ± 2.17
8.20 ± 0.41	80.73 ± 26.14	—	—	2.48 ± 0.43	24.48 ± 8.27
4.20 ± 0.50	78.69 ± 45.70	29.07 ± 2.01	529.44 ± 22.18	3.60 ± 0.74	70.39 ± 53.9
1.59 ± 0.18	7.46 ± 4.43	29.51 ± 1.64	135.17 ± 69.27	2.70 ± 0.35	12.42 ± 7.15

for the sample size.

Table 3

Allometric equations and correlation coefficients (r) for the relationships between the body weight and the absolute volumes of the nephronic tissue and blood vessels in percoid and ostariophysian fish

Groups and species	Nephronic tissue		Vascular space	
	Allometric equation	r	Allometric equation	r
1 Percoid	$4.581\ W^{0.79}$	0.97	$1.39\ W^{0.89*}$	0.97
(a) *O. niloticus*	$0.445\ W^{1.318}$	0.95	$0.545\ W^{1.103}$	0.95
(b) *M. salmoides*	$1.811\ W^{0.94}$	0.94	$1.792\ W^{0.834}$	0.87
2 Ostariophysian	$5.14\ W^{0.79}$	0.98	$3.981\ W^{0.798*}$	0.98
(a) *C. carpio*	$1.25\ W^{1.018}$	0.98	$0.574\ W^{1.108}$	0.98
(b) *C. mossambicus*	$2.133\ W^{1.013}$	0.97	$1.589\ W^{1.03}$	0.96

*Significant difference, $p < 0.05$.

nephronic segments and collecting tubules and ducts were based on histological differences /11, 13/ that generally agree with observations in the present study.

The teleostean kidney is not characterised by cortico-medullary zonation as seen in mammals nor the spatial separation of the early distal tubule from the proximal tubule and collecting tubule as seen in other classes of fish such as the dipnoans, elasmobranchs and lampreys. Thus the sampling technique usually employed for zonated kidneys to ensure adequate representation of both the cortex and the medulla was not necessary. In the current investigation, the adequate sample of tissue sections was based on a pilot study in which cumulative mean plots showed that analysis of fifteen or more section was required to determine proportions of lysosomes and mitochondria and the surface area of basal membrane infoldings in the cells of the various segments of the renal tubule by linear integration at electron microscopic level for the kidney of the three-spined stickleback /29/. Quantitative structural data could be useful in explaining the notable differences in kidney function in teleosts species occurring in different habitats. It has been shown that the transfer of fresh water fish or sea water fish to the opposite environment results in changes in glomerular number and size and in the height of the tubular epithelium /5—7, 15, 16, 22, 29/. These changes suggest that besides physiological parameters the quantitative features of the nephron could be a reliable indicator of kidney function in the diverse aquatic ecosystem. The observations in the present investigation showed that quantitative structural characteristics of two diverse groups of fresh water teleosts were comparable. This indicates that the environment, which dictates the function, influences renal structure in a similar manner, the order notwithstanding.

The glomerular parameters (diameters and volume proportions) obtained in this investigation point to a large filtration surface. The glomerular diameter obtained for O. niloticus in the present study is comparable to that reported for a closely related tilapid, Oreochromis mossambicus /16/. However the glomerular diameters obtained for the catfish and the common carp in this investigation were smaller than those reported previously /21/. Glomerular diameter is an important factor of the renal filtration surface although the extents of glomerular capillary branching, the number of the glomeruli and the ultrastructural characteristics are important characteristics of glomerular filtration surface.

Previous studies on the fish nephron have reported the proportions of the various segments of the nephron in Pleuronectes lethostigma, Anguilla rostrata and Pleuronectes platessa /3, 4, 8, 23/. In these species the proportion's of the neck, proximal and distal segments in the nephron were 0.07-8%, 79.5-100% and 0-11%, respectively. In the present study the neck segments comprised less than 1.1% of the nephronic tissue. This low volume proportion indicates the relatively low contribution of the neck segment to renal function in fish. It appears that the neck segment has become progressively smaller in evolution and is absent in most mammals and birds. Our results about the proximal tubule in fishes conform with the observation that the proximal tubule is the main component of the nephron in all vertebrates /2/. The volume proportions of the distal nephron (distal tubule and collecting tubule-collecting duct system) were fairy high in all the fishes studied. The well-developed distal nephrons make fresh water fish kidneys efficient organs for diluting urine since distal nephrons are impermeable to water and preferentially reabsorb solutes. Moreover, previous reports /3, 4, 8, 17, 23/ show that the proportion of the distal nephron to the whole nephron is higher in fresh water fish than in marine fish. The volume proportion of all the blood vessels was generally more than 25% of the kidney. There was a significant ($p < 0.05$) difference in the volume of the blood vessels in the percoids and ostariophysians. This difference was accounted for by the absence of a renal portal system /17/ in O. niloticus which is a percoid.

In conclusion the morphometric characteristics of the fresh water fish kidney elucidated in the present study appear to indicate adaptational osmoregulatory characteristics that favour survival in fresh water. The numerous glomeruli and the fairly high volume density of the renal corpuscles may indicate high glomerular filtration rates required in a fresh water habitat. In addition the well-developed distal nephron points to high urine diluting efficiencies needed in the fresh water environment in which fish face heavy osmotic water influxes.

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GENETICS IN HUNGARY BACK INTO THE NORMAL TRACK

After several years of struggling the Hungarian Genetical Society was founded in 1985 and the first Conference of the Hungarian Geneticists was held in 1987 in Budapest. At our second conference (Szeged, 1991) we decided to have our meetings triennially. The latest, third Conference of the Society was held in Debrecen on December 8—9, 1994.

The number of participants exceeded 300 and the most important fields of research in genetics were represented. Lectures and poster presentations were organized in the following 8 sections: Animal Genetics, Developmental Genetics, Genetic Regulation, Human Genetics, Microbial Genetics, Molecular Genetics, Plant Genetics and Population Genetics. The number of papers presented at the Conference exceeded 150.

The editors of the Acta Biologica Academiae Scientiarum Hungaricae offered the possibility to publish papers of different fields which were presented at the Conference.

Although the submitted papers were not selected by the organizers we have found it a good opportunity to collect them in one volume in order to give signs to the scientific community about the normalization of the state of affairs in genetics in Hungary.

Secretary of the Conference
Sándor Bíró

President of the Conference
Gábor Szabó

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PHENOTYPIC HETEROGENEITY OF THE PROGENY OF STREPTOMYCES GRISEUS CONIDIA*

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In order to understand the complex ontogenetical processes, the development of Streptomyces (S.) griseus was applied as a model. The developmental cycle of S. griseus starts and ends as a conidium. In between, coenocytic mycelium develops which, if studied by cytomorphological or biochemical methods, exhibits conspicious heterogeneity. The hyphae develop into young, transient and old vegetative hyphae and different stages of reproductive forms. In developmentally blocked mutants these sequences of events appear mixed in all possible associations. It seems as if the program of development could be divided into several subprograms. The quantitative evaluation of the results show that the individual morphological markers exhibit certain independence from each other realized with a given probability. The conidia of S. griseus are also heterogeneous concerning all morphological and physiological traits examined so far (shape, size, light refraction, staining and shape of nucleoids with Feulgen, methyl green — pyronine, intensity and form of polysaccharide distribution, heat resistance, etc.).

Kinetics of the survival curves of two S. griseus strains — a well-sporulating and its developmentally blocked mutant /24/ — are different from each other, one has many more heat resistant conidia than the other but the kinetics of the survival curves of the two S. griseus strains indicate that spore populations of both react differently to heat treatment and heat resistance can be modeled by assuming the presence of two independent subpopulations of spores with different heat sensitivity. The emergence of two distinct subpopulations with (possibly) the same genetic make-up is designated: phenotypic segregation. Heat resistance is first of all species specific (genetically determined) but the epigenetic segregation seems to be characteristic of the developmental process. This process can in certain mutants be affected by environmental conditions and more importantly by the so-called autoregulators (A-factor and factor C). Factor C and A-factor are needed to normal development, if their quantity or the time of addition to the culture was not optimal, the quantity of spores decreased.

Keywords: Development — Streptomyces — heat-treatment — Factor C — A-factor

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Introduction

In order to understand the complex developmental processes — how the individual proteins and their products are concerted into a developmental process; how the genetic, e.g. operonal regulatory mechanisms are related to the regulation on the epigenetic level — the development of S. griseus was applied as a model.

Streptomyces are Gram-positive prokaryotes exhibiting a life cycle which starts and ends as a conidium (so-called spore). The spore develops into a filamentous hypha that very often forms a mycelial network. The hyphae are divided by cross-walls into units with different numbers of nucleoids. After a period of vegetative growth, morphologically different reproductive hyphae appear in which the conidia or spores are formed /5, 17, 24/.

The above process: A model to study differentiation, is intensively investigated, both from the cytomorphological point of view as well as the genetic and environmental regulatory mechanisms and compounds influencing it /3, 16, 21, 22, 27/.

The mycelium may be looked upon as a multicellular organism. Thus the possibility to study differentiation of Streptomyces (S.) as a relatively primitive multicellular model bears importance not only for basic biology but it may have practical importance, because the production of secondary metabolites (e.g. antibiotics) and some stages of differentiation often coincide in time /5/.

The genetic regulation of differentiation in Streptomyces was recently reviewed by Chater /2/. The factors which influence differentiation in Streptomyces belong to two categories. They may be exogenous ingredients of the medium in which the microorganism grows or endogenous compounds produced by the cells themselves. These latter were named autoregulators /10/. In certain strains the composition of the medium may determine differentiation specifically e.g. certain compound may induce aerial hyphae production with spores, or only aerial mycelium without spores /21, 22/.

Autoregulators /10/ are endogenous molecules produced by the Streptomyces itself, a kind of microbial hormone. Their mode of action was studied mainly in the case of A-factor by Khokhlov and coworkers and Horinouchi and Beppu /6, 7, 11/ and factor C by Szabó and coworkers /16, 19, 23/. We believe that the autoregulators are endogenous molecules that have appeared at

the multicellular level of organization in the course of evolution. Their biological role is not fully understood.

In order to describe the differentiation of S. griseus, a wild type strain No. 45H and its mutant strain No. 52-1 were studied. Strain No. 45H has a complete life cycle, produces reproductive hyphae and spores both on solid medium and in submerged culture, while strain No. 52-1 does not produce spores in submerged culture.

In the following we summarize our earlier results (cited in this paper) about the development of S. griseus by describing the changes of the complex cytomorphological markers and shall try to find regularities in the process of differentiation at the epigenetic level.

The effect of two autoregulators, A-factor and factor C, upon differentiation at the same epigenetic level will be summarized, too.

Materials and Methods

All the methods of cultivation and techniques to study cytomorphology applied in the experiments presented here were described in our previous papers /1, 15, 17, 23—25/.

Results

The cytomorphology of the life cycle of S. griseus in submerged culture

A cytomorphological marker is a complex trait, it is the result of coordinated activities of enzymes, other proteins and structural elements. On solid medium the Streptomyces colony itself shows development to the naked eyes, first having a smooth leathery surface which is later covered by a powdery layer of aerial mycelia that also may change its color with time. The hyphae in the colony or in submerged culture differentiate into vegetative and reproductive forms. These hyphae regularly develop into young, transient and old vegetative and different stages of the reproductive branches ending up with the release of spores. It is known that because of the asynchrony of growth, different vegetative and reproductive hyphal parts are seen in the same visual field. This normal heterogeneity should be distinguished from the type of heterogeneity which was described by us in the developmentally blocked mutant. In such strains the characteristic markers

of the vegetative and reproductive development may appear mixed, in all possible associations within the same hyphal segment. This statement was true for all morphological traits studied so far, like the thickness of the cell wall, the staining of the cytoplasm, the distribution of the polysaccharides — examined with the P.A.S. reaction — the morphology of the nucleoids stained with Feulgen reaciton or methyl green-pyronin. Investigation by cytomorphological methods reveals differences which are not distinguished, when examined with biochemical methods. If one compares e.g. the polysaccharide content in the mycelia of the two above-mentioned strains in submerged culture, finds quantitative differences at the first 48 hours of cultivation which later practically disappears /14/. Cytomorphological staining for carbohydrates, P.A.S. positivity, however, shows a regular sequence of changes characteristic to the vegetative and reproductive hyphae and spores, respectively. The same holds for all the cytomorphological traits studied so far. We have shown previously /16, 18, 20, 23/ that the life cycle, the development of S. griseus, the various types of hyphae (young, transient, old vegetative or reproductive branches) are characterized by a given form of nucleoid, distribution of P.A.S. positive substance, staining of the cytoplasm and cell wall thickness.

The question arose, how much the correlation between the different markers observed in the wild type strain (No. 45H) would change in the hyphae of the developmentally blocked mutant (No. 52-1). It was established that in No. 52-1 the course of development has changed: the cytological markers did not appear in such an order or in such a clear-cut form characteristic to a given stage of development as was observed in the wild type. Young hypha may contain even "old" nucleoids, or old vegetative hypha may contain reproductive nucleoid /18/. A quantitative evaluation was attempted. Four nucleoid and five P.A.S. positive types were distinguished. Each hyphal section was first examined with methyl green-pyronin and the type of nucleoid was determined, then — after the methyl green-pyronin having been washed out — the preparation was stained with the P.A.S. reaction and the same hypha was assigned to one of the five P.A.S. positive types.

Considering nucleoid and P.A.S. types — in theory — twenty forms of hyphal segments may exist. Almost all the possible combinations were in fact found. In each visual field every hypha was examined and each segment of the hyphae was classified to one of the twenty forms. After measuring the length of a given form of hypha in arbitrary units, the length of the same forms were added up. 762 units of hyphae were evaluated (Table 1).

Table 1

Combination of cytomorphological types in hyphal segments of 96 h old S. griseus No. 52-1 mycelium (lengths in arbitrary units)

Nucleoid types	Intensity:	P.A.S positive types: -	homogeneous ±	wall contours and granules +	rods and granules ++	"brackets", regular cross walls +	Sum.:
	Young vegetative	23 + (5.6)	0 (2.0)	3 (2.3)	2 − (15.8)	0 (2.3)	28
	Transient	33 (33.0)	26 + (11.8)	8 (12.7)	91 (94.0)	8 (14.5)	166
	Old vegetative	12 − (20.3)	12 (7.3)	0 (7.8)	77 + (57.8)	3 (8.9)	102
	(Aborted) "reproductive"	83 (92.2)	16 − (33.0)	47 (35.5)	263 (263)	55 + (40.3)	464
	Sum.:	151	54	58	431	66	762

$\chi^2 = 137.02$

$p \ll 0.001$

Correlation factor $= \frac{\phi^2}{q - 1} = 0.06$

○ Fit to random distribution

□ Deviate from random distribution (ln + or − direction)

(...) Expected value

-, ±, +: Intensity of P.A.S reaction

In circles those values are seen which should have been obtained if the appearance of markers happened at random, squares show numbers reflecting remarkably deviating incidence from the random distribution of jointly appearing two markers. E.g. in 23 length units of hyphae in young cytoplasm, young nucleoid could be expected which would significantly deviate from the expected value of 5.6 if their joint appearance had been a random event. Still it can be seen that old vegetative and even reproductive type of nucleoid was found — though rarely — in young cytoplasm. In the blocked mutant it still holds true that the associated appearance of the cytomorphological markers are more probable than their random occurrence, though they may develop also independently from each other. There were all kinds of forms, i.e. almost all the possible 20 combinations of nucleoid and P.A.S. positive types.

It seems as if the program of development, the germination, the vegetative and reproductive phases, could be subdivided into several subprograms — e.g. one for the nucleoid, one for the P.A.S. positive material — which may exhibit certain independence from each other. Individual subprograms may be realized with a given probability.

The above statement is true in the case of the wild type strain too. In normal development of the wild type, the probability of correlated appearance of markers is high which is the precondition of full life cycle bringing about ripe, complete conidia.

Heterogeneity of spores

The heterogeneity of hyphae did not tell us which of the association of cytomorphological markers are compatible or not compatible with viability. Does it exist because hyphae are only intermediate stages of development serving nutrients for the reproductive cells or heterogeneity is a more fundamental phenomenon which can be observed with spores also? For the answer we extended the investigations to spores. (It must not be forgotten that the term spore has also to be defined by describing the way how they were prepared. In most of our experiments reported in this publication — for standardization — the crude spore suspension was ultrasonicated, lysozyme digested and filtered. The spores harvested by shaking with glass beads and filtration through cotton are ultrasound, lysozyme and heat sensitive in high percentage. We think that the heterogeneity of spores would be true for all the physical and chemical agents applied to characterize spores.)

Table 2

Heterogeneity of spores in light refraction (anoptral contrast, i.e. negative phase contrast microscopy)

Strain	Light refraction	Number of spores	%
	very strong	77	40.5
No. 52-1	medium	50	26.3
	weak	60	33.2

We have examined several cytomorphological characteristics of the spores and determined their viability after incubating them at different temperature for different time periods.

The morphological heterogeneity concerns the shape, size and the refractivity of the spores (Table 2 and Fig. 1), the staining and shape of nucleoids with the Feulgen method (Table 3 and Fig. 2) or with methyl green-pyronin (Fig. 3), intensity and form of the P.A.S. positive material (Table 4

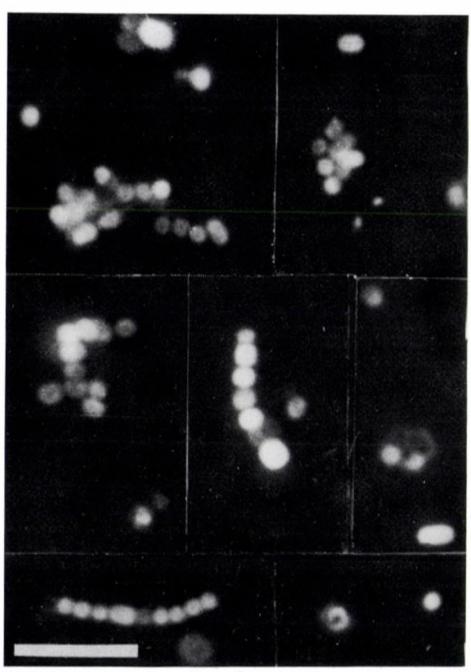


Fig. 1. S. griseus No. 52-1 spores. Anoptral contrast, oil immersion, fixed, unstained. Bar: 5 µm

Table 3

Heterogeneity of spores in DNA content (anoptral contrast, negative phase contrast microscopy)

Strain	Intensity of staining (quantity of DNA)	Number of spores	%
No. 45H	intensive	63	18.6
	medium	142	40.0
	weak	133	39.4
	Distribution of DNA in spores		
No. 45H	regular	355	77.5
	irregular	101	22.1

and Fig. 4). It can be seen, that the spores show differences in all respects we investigated. They are not homogeneous physiologically either. The spores were counted in a Buerker chamber and than the colony forming units (CFU) were determined. It is difficult to define exactly what percentage of

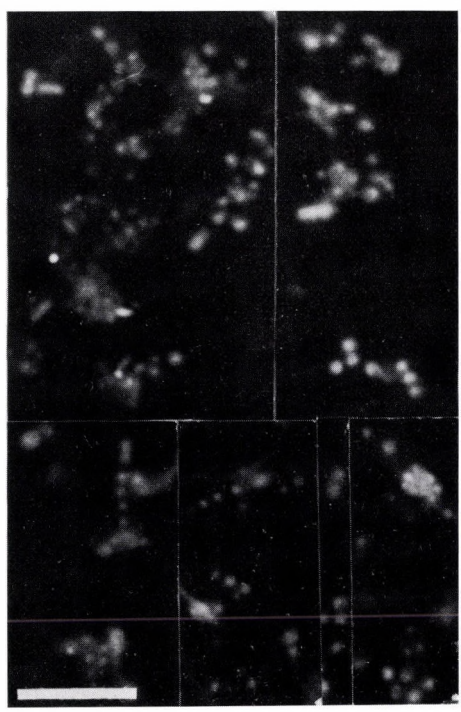


Fig. 2. S. griseus No. 45H spores and reproductive hyphae. Feulgen reaction, anoptral contrast, oil immersion. Bar: 5 µm

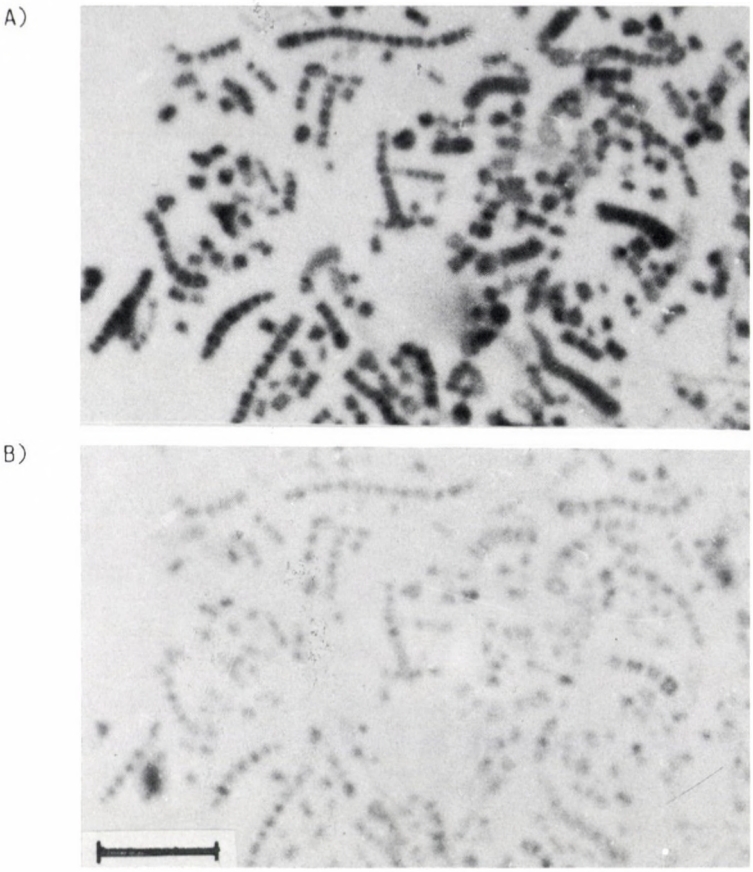


Fig. 3. S. griseus No. 45H spores and reproductive hyphae.
Methyl green-pyronin staining, oil immersion, monochromatic illumination.
A: 545 μm (RNA); B: 645 μm (DNA). Bar: 5 μm

the spores do not develop into colonies, because of the error of counting in the Buerker chamber and more importantly the methods applied to separate spores from hyphae. But the tendency that several percentage of spores are unable to germinate could be established with certainty. About 50% of spores were sensitive to ultrasonication, or lysozyme treatment. The sensitivity of the Streptomyces spores to heat treatment was determined more in detail.

Heat treatment of spores

The values in Table 5 and other experiments show that there is great variance of the survival values depending upon the strains studied.

Table 4

Heterogeneity of spores in P.A.S. reaction (anoptral contrast, negative phase contrast microscopy)

Strain	Intensity of P.A.S. reaction	Number of spores	%
No. 52-1	strong	70	64.8
	medium	38	35.2
No. 45H	strong	48	38.4
	medium	77	61.6

These static values are, however, not very meaningful. The kinetics of the process are more informative. Therefore we have followed the survival percentage with increasing incubation time at the same temperature and at the same spore density. It was shown that at 60 ^{o}C for 60 minutes the survival

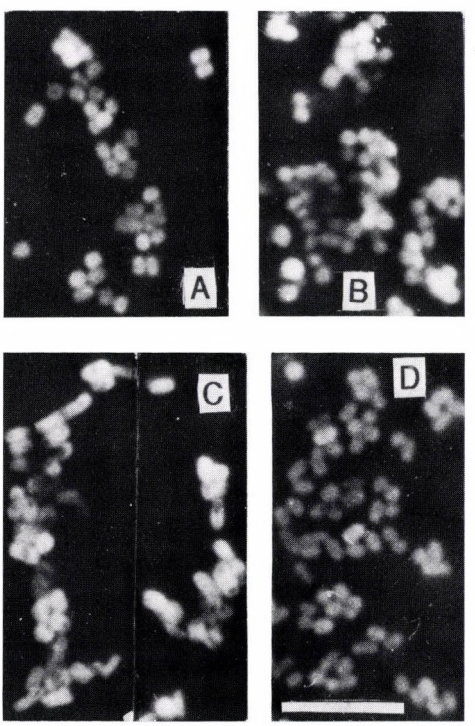


Fig. 4. S. griseus spores. P.A.S. reaction, anoptral contrast, oil immersion.
A: No.52-1; B: No. 45H; C: No. HH1;
D: IFO 13350. Bar: 5 μm

Table 5

Survival (in %) of S. griseus No. 45H, No. 52-1 and IFO 13350 spores incubated at different temperatures for 10 minutes

Strains	°C			
	25	45	60	70
No. 45H	100	55.2	44.8	0.42
No. 52-1	100	7.3	1.54	—
IFO 13350	100	71.0	0.83	—

curves of the two strains, the wild type No. 45H and of the mutant strain No. 52-1 are different (Fig. 5A, B). The values on the curve of decay fall upon two straight lines and although both strains are characterized by curves which have two straight components, an early steeper and a later slowly decreasing part, the two strains can be distinguished. One of them (No. 52-1) is characterized by the fast decreasing, beginning part of the curve. During this period a much larger percentage of spores are killed here than in the case of strain 45H.

The straight lines can be described by the exponential equation:

$$N = N_0 \times e^{-kt}.$$

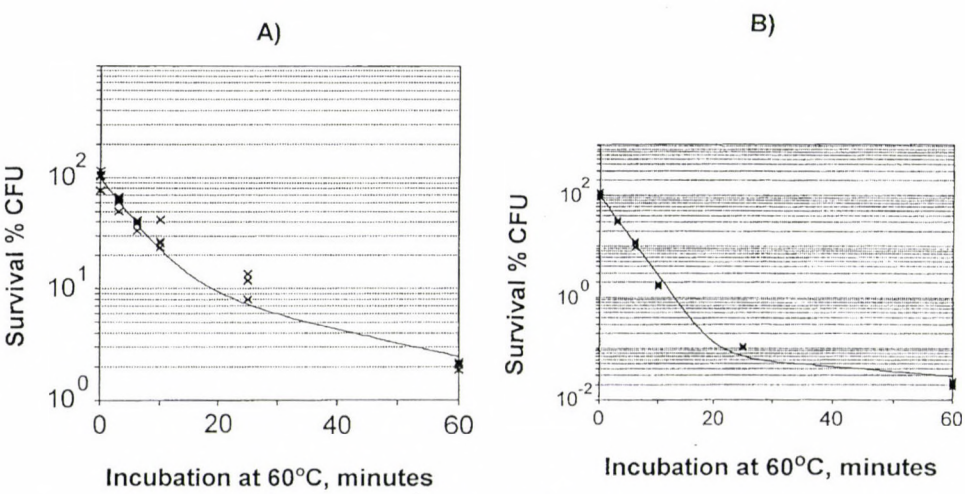


Fig. 5. Survival (in %) of spores incubated at 60 °C for different periods of time. A) S. griseus No. 45H. B) S. griseus No. 52-1

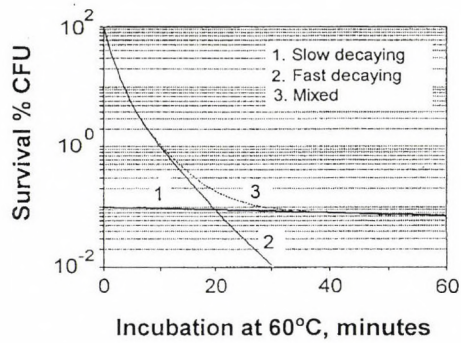


Fig. 6. Survival of two model-populations (initial number 999:1) at 60 °C assuming different decaying constants which follow normal distribution

This, when applied to the two straight lines would be:

$$N = N_{0,1} \times e^{-k_1 t} \quad \text{and} \quad N = N_{0,2} \times e^{-k_2 t}.$$

Mathematical analysis of the data (to be published later) showed that the experimentally obtained survival curves can be modeled (Fig. 6) by assuming the presence of two independent subpopulations of spores with different heat sensitivities, both exhibiting normal — modeled by binomial — distribution (Fig. 7A, B). One of the subpopulations is more sensitive to heat than the other. According to the model applied, the difference between the survival curves of different strains derives if one assumed that the proportion of the two subpopulations differed from strain to strain and also

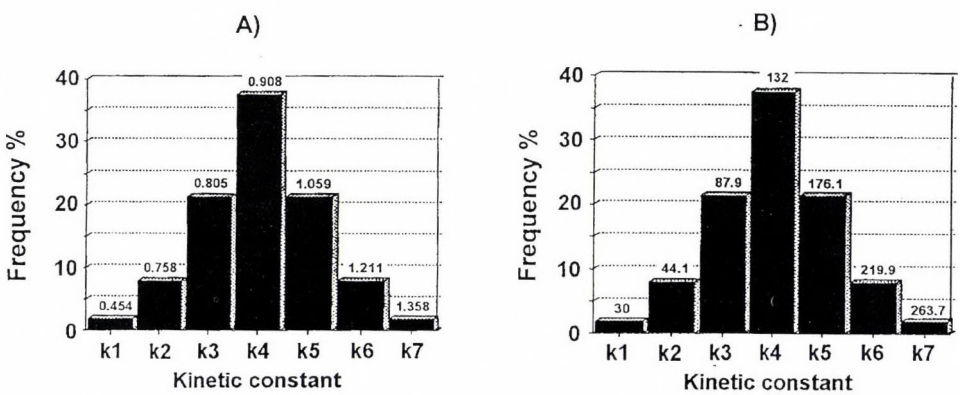


Fig. 7. Distributions of the kinetic constants in the A) fast-decaying and in the B) slow-decaying model-population (the values should be divided by 10.000)

Table 6

Proportions and kinetic constants of heat sensitive and heat resistant S. griseus spores calculated for 0 time*

Strain	Sensitivity to heat	Proportion %	Kinetic constant** k' (= 10^{-4}/sec)
No. 52-1	sensitive	99.88—99.93	61.00—128.0
	resistant	0.12— 0.07	3.30— 4.70
No. 45H	sensitive	28.36—87.00	18.20— 32.00
	resistant	71.66—13.00	1.27— 4.62

*Heat treatment at 60 °C

**In the equation: $N_t = N_0 \times e^{-k't}$

N_0 = CFU/ml before the heat treatment

t = duration of the heat treatment (seconds)

N_t = CFT/ml after heat treatment for t seconds

within the same strain depending on as yet not defined conditions. In concordance with the experiments, the calculations based on the model show that the fast decaying spores are killed in the first 25 minutes of heat treatment at 60 °C. After longer head treatment mainly the presence of the slow decaying spores has to be counted on (Figs 6, 7A, B).

With the help of the above equations the ratios in the starting subpopulations and the kinetic constants of the killing effect were determined in at least three experiments (Table 6). The starting ratios of the two subpopulations for No. 52-1 were between 99.88—99.93 and 0.12—0.07. For No. 45H the values were 28.36—87.00 and 71.66—13.00, respectively. When comparing the two strains, the constants of the exponents for the fast and slow decaying populations fell near to each other. In the case of No. 52-1 the constants (k1) for the fast decaying spores varied between 61.00—128.00, for the slowly decaying spores k2 varied between 3.3—4.7. In the case of No. 45H k1: 18.20—32.00, k2: 1.27—4.62, in units 10^{-4} s^{-1}.

The effect of autoregulators upon the heterogeneity of Streptomyces hyphae and spores

Two of the autoregulators were studied about their effect upon the cytomorphology and physiological heterogeneity of Streptomyces strains (factor C and A-factor).

Factor C

The endogenous regulator called factor C was isolated and studied by us /1, 20, 23/. It is a protein of a molecular mass of 34 000 and it is biologically active down to a concentration of 0.5 ng/ml. It was detected on the basis of its cytomorphological effect /16, 23/. If factor C was added to the mutant strain No. 52-1 in submerged culture (which did not conidiate under these conditions) not later than 24 hours of cultivation, the development of reproductive branches is restored (it can be observed after 60-72 h of cultivation). These branches are very similar to the wild type reproductive hyphae: intensively stained basophilic cytoplasm, thicker cell wall, high DNA and RNA content, accumulation of the P.A.S. positive material (especially in the prespores) are seen. The nucleoids, however, do not become separated in the prespores as it is characteristic to the wild type /3, 24/. Although the wild type cytomorphology is approached, the development is not complete, the spores are much less in number than in the submerged culture of the wild type and the reproductive forms are less resistant to ultrasonication (not shown). Thus we may say that factor C supplied at the appropriate time of development and in optimal concentrations partially restores the normal course of the life cycle, the dissociated subprograms are getting more co-ordinated. Biochemical analysis of the strains, e.g. protein patterns in PAGE also indicates that factor C partially restores wild type development of the mutants /25/.

No factor C-less mutant was found so far. Strains which do not sporulate in submerged culture produce factor C either in very low amount or not

Table 7

Effect of factor C upon the growth and sporulation of S. griseus No. 52-1 on solid medium (over cellophane)
(Chemical analysis took place in the ultrasound-sensitive fractions)

Factor C	DNA	RNA	Protein	No. of spores/ml
	(μg/ml)*			
None	49.60 (+5.3)**	70.60	107.00	4.04×10^8
125 U/ml	12.80 (+20.7)**	33.00	71.20	1.59×10^9

*Related to the volume of the culture media (20 ml per 90 mm Petri dish).

**Calculated value based on the number of spores and their known DNA content.

Table 8

Effect of factor C upon S. griseus No.52-1 on solid medium (Chemical analysis took place in the ultrasound-sensitive fractions)

Sample	RNA / DNA	Protein / DNA	DNA + RNA / Protein	Spore / µg DNA
Control	1.425	2.159	1.123	2.717×10^7
Factor C	2.578	5.563	0.643	4.143×10^8

in the proper time of development. Factor C when added to the low factor C producing strain stimulated its own synthesis /20/.

The addition of factor C to the mutant strain stimulated the number of spore production even on solid medium (Tables 7, 8). While the heterogeneity of the mycelium and spores decreased by factor C treatment (unpublished results) there was no difference in the heat resistance of the spores with or without added factor C to cultures on solid medium.

A-factor

S. griseus mutants deficient in A-factor (2-isocaproyloyl-3R-hydroxy methyl-γ-butyrolactone) do not form aerial mycelium and spores /11/. The addition of exogenous A-factor to A-factor deficient strains restores spore formation /4/. Streptomyces bikiniensis HH1 (now known to be S. griseus) is an A-factor deficient mutant neither produces streptomycin nor forms aerial mycelium owing to the lack of DNA sequences (the gene afsA) responsible for A-factor synthesis /7, 8/.

Studying strain HH1 and compared to its wild type progenitor S. griseus IFO 13350 we have found that mass sporulation occurred in the mutant A-factor minus HH1 on agar medium without A-factor although no aerial hyphae were formed /15/. The reproductive branches can be observed inside the agar medium /16/, which do not emerge from the surface of the medium. The shape of the spores and that of the reproductive hyphae is more heterogeneous than those in the reproductive branches or spores of the wild type aerial mycelia. Heat resistance of spores from strain HH1 is similar to spores of the wild type strain or to spores obtained from the mutant after A-factor treatment (Table 9). The survival curve at 60 ^{o}C also consists of two parts, a steeply and a slowly decreasing one. The heat resistant fraction of HH1 is as resistant as spores after A-factor treatment.

Table 9

Kinetic contants of heat inactivation of *Streptomyces* spores (10^{-4}/s)

Strain	k1	k2
45H	30.00	1.50
	32.00	4.62
	18.20	1.27
52-1	61.00	3.47
	128.00	3.30
	93.00	4.70
HH1(-Af)	104.67	7.66
	118.90	3.85
	89.3	6.80
HH1(+Af)	112.10	7.70
	87.28	1.60
	76.80	4.25

A-factor restores aerial mycelium formation in strain No. HH1 and also stimulates spore formation. The number of spores increases by one order of magnitude, the shape of the spores, cytomorphology becomes less heterogeneous, but does not reach the homogeneity of the wild type spores.

We have compared the spore densities of strain No. 45H and No. 52-1 by density gradient centrifugation. Both spores were found at a density of 1.3 g/ml. It could be seen that the heat sensitive spores of No. 52-1 and the heat resistant ones of No. 45H have the same densities indicating no correlation between spore density and heat resistance. Heat sensitive spores could be separated from the heat resistant ones for morphological investigations by having them germinated after heat treatment at 60 ^{o}C for 60 minutes and disintegrate the outgrown hyphae by ultrasonication. No cytomorphological difference between these two subpopulations was found.

It may be concluded that although several morphological traits of the spores as well as their heat resistance were found heterogeneous, no correlation between any of the examined characteristics and heat resistance could be found.

Discussion and Conclusions

Summarizing our studies about the cytomorphology of S. griseus strains show that the hyphae are heterogeneous for the cytomorphological markers. Normal — traditionally known — heterogeneity appears regularly in concert with the life cycle and also minor variations can be observed in the same hypha between neighbouring parts of that segment. There is, however, a third type of heterogeneity when definitely expressed markers develop in all possible associations. This third type of heterogeneity is characteristic for certain developmentally blocked mutants. This process can be reversed towards the normal pattern with environmental factors but not completely. Among the environmental ingredients some are specifically, endogenously produced by the Streptomyces itself. They are the autoregulators of which A-factor and factor C were studied.

The cytomorphological heterogeneity of hyphae can also be observed in the spores. If one considers that the replication of DNA is almost error-free with the probability of mutation of 10^{-9} per nucleotide, the heterogeneity (non-identity) of the reproduced cells is conspicuous. Heterogeneity concerns all the cytomorphological markers examined so far (size, shape, the distribution and concentration of DNA and RNA, the staining properties of the cytoplasm, the nucleoid and cell membrane, the thickness of the cell wall, refractibility (i.e. the density of the cytoplasm) and also the physiological properties (markers) of the spores: e.g. viability at different temperatures. It seems as if it were a general rule that for evolutionary adaptation to carry the almost unchanged genetic material in phenotypically variable cells has a positive selection value. The variation of one of the examined traits, heat resistance, behaves accordingly. Its variability towards heat is considerable. The proportion of the subpopulations of spores which is significantly resistant to heat may vary from below 1% sometimes over 10%. We have found that studying the kinetics of the killing effect of heat in time was more informative than to determine the CFU-s after heat treatment at different temperatures during a single incubation time.

The kinetics of the survival curves indicated two subpopulations of spores to be present. This would have not been revealed by incubating the cells at different temperatures for a given time interval. This analysis renders possible to calculate heat denaturation constants and activation energy of heat denaturation (of a hypothetical cell component responsible for non-viability). It is worth noting that the activation energy of heat

Table 10

Activation energy of heat denaturation of spores

Strain		Temp. of treatment, °C	kJ/mol
Streptomyces:	heat sensitive	60 °C	96.684 (± 2.064)
	heat resistant	60 °C	104.578 (± 1.570)
*B. subtilis (wild type)		85 °C	103.803
*B. subtilis ssp. α^- β^- (heat sensitive)		85 °C	99.444

*Calculated from the results of Setlow and Setlow /13/.

denaturation of S. griseus spores are almost the same as that of the spores of B. subtilis (Table 10, /13/).

Heat sensitivity may be determined by polygenic systems but the distribution of heat resistance obtained in the experiments, the deflection between the two straights of the curve representing the fast and slow decaying spores cannot be modeled with one gaussian distribution. Therefore at least two independent gaussian distribution of heat sensitivities have to be assumed. There are at least two subpopulations among the spores of which one is significantly more resistant than the other. These variants are the products of phenotypic variation because three consecutive selection at 60 °C for ten minutes did not increase the percentage of the survivor over 45 °C (Fig. 8).

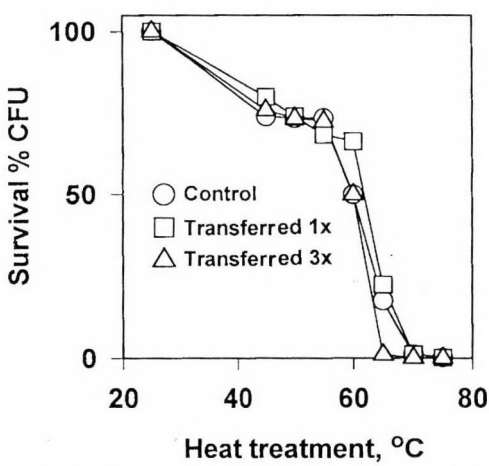


Fig. 8. Survival curves of S. griseus No. 45H spores treated for 10 min at different temperatures before or after one or three transfers at 60 °C

In order to understand the mechanism of phenotypic variation we speculated earlier — concerning the heterogeneity of mycelium — that the cytologically observed markers are complex traits determined by several genes belonging to one of the genetic subprograms. These subprograms develop relatively independently from each other because their coupling in space and time is not as strict as of the cogwheels in a clock. The regulation of the co-ordination of these subprograms would follow a course which is subjected to apparently random changes of the internal and environmental milieu.

It is not unlikely — as it was observed in complex systems — that small differences occurring at a given time may trigger events which lead to bifurcation, which in developmental systems appears as a random choice between two possible outcomes /12/. These random "decisions" would determine the end result indicating random association of the markers.

The random association of subprograms may result abortive spores. In fact several percentage of spores do not germinate even at favorable conditions. It is, however, known that during ontogenesis the random minor errors, deviations from the normal route of development are compensated by the phenomenon of canalization /9, 26/. The molecular mechanisms of canalization is not yet known.

It is peculiar that — concerning heat resistance — the spore population consisted of two subpopulations each of which displays a separate gaussian distribution. The less sensitive subpopulation seems not to differ genetically from the other, the more sensitive major part of the spore population. The existence of two subpopulations concerning heat resistance indicate that heat resistance may develop at least by two mechanisms. It would be desirable to see if the observations obtained with heat resistance would be valid with other cellular markers too.

The two autoregulators studied, A-factor and factor C partially restored the cytomorphological abnormality of the mutant strains which either do not produce the autoregulator at all (in case of A-factor) or do not produce it in quantity enough (in case of factor C). With autoregulators added, the development of the mutant strains became similar to the wild type, the process of development is more often completed, the number of spores increased. It seems as if the canalization of the development became more efficient although the proportion of spores resistant to heat over 45 ^{o}C did not increase. Thus we assume that there is a special class of excreted regulatory molecules (autoregulators) which are necessary to the development in time and space. The proportion of the highly resistant fraction

of spores is first of all strain specific (genetically determined); strain No. 45H (the wild type) definitely has a higher percentage of spores resistant to heat than No. 52-1 (the mutant), although the proportions show variation depending probably on — not yet clarified — environmental factors.

These observations emphasize the commonplace knowledge that very large differences may hide behind one given phenotype. Our results only underline that the extent of this inherent heterogeneity is larger than generally believed, to such an extent that with one genotype at least two qualitatively different subpopulations segregate. For the phenomenon described we propose the term: epigenetic segregation.

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GENETIC ANALYSIS OF THE COMPONENTS OF WINTERHARDINESS IN BARLEY (*HORDEUM VULGARE* L.)

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Winterhardiness in cereals is the consequence of a number of complex and interacting components: cold tolerance, vernalization requirement and photoperiod sensitivity. An understanding of the genetic basis of these component traits should allow for more effective selection. Genome map-based analyses hold considerable promise for dissecting complex phenotypes. A 74-point linkage map was developed from one hundred doubled haploid lines derived from a winter x spring barley cross and used as the basis for quantitative trait locus (QTL) analyses to determine the chromosome location of genes controlling components of winterhardiness. Despite the greater genome coverage provided by the current map, a previously-reported interval on chromosome 7 remains the only region where significant QTL effects for winter survival were detected in this population. QTLs for heading date under 24 h light map to the same region. A QTL for heading date under this photoperiod regime also maps to chromosome 2. A distinct set of QTLs mapping to chromosomes 1, 2, 3 and 5 determined heading date under 8 h light. Patterns of differential QTL expression underscore the complexity of winterhardiness.

Keywords: Barley — vernalization — photoperiod — winterhardiness — QTL

Introduction

Winterhardiness in cereals is the final expression of a number of interacting characters that can include vernalization requirement, photoperiod response, and low temperature tolerance. The genetic investigation of frost resistance became more intensive with the appearance of monosomic and substitution series, which confirmed beyond any doubt the complexity of this character. It was found that 15 of the 21 chromosomes of wheat influenced the phenotypic manifestation of frost resistance in some way /1, 19/. One major genre (*Fr1*) is located on chromosome 5A. Hayes et al. /9/ reported the

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presence of a putative multilocus cluster on barley chromosome 7, which is homologous with group 5 chromosomes of wheat, defined by Quantitative Trait Locus (QTL) effects for a number of winterhardiness component traits. The genetic basis of frost resistance is affected by numerous environmental factors. Veisz and Sutka /23/ found that chromosomes 5A, 5B, 5D, 4B and 7A of the forst resistant winter wheat Cheyenne improved frost resistance to various extent during different phases of the hardening process. Thomashow /21/, reviewed the wealth of physiology data regarding mechanisms of cold injury and the paucity of information regarding the genetic basis of resistance to such stresses.

Vernalization plays an important role in the expression of winter hardiness. Five major genes are responsible for this trait in hexaploid wheat (Triticum aestivum). Vrn1, Vrn4 and Vrn3 are located on chromosomes 5A, 5B and 5D /25/ Vrn2 on 2B and Vrn5 on 7B /15/. Takahashi and Yasuda /20/ proposed that three major genes — sh, Sh2 and Sh3 — located on chromosomes 4, 7 and 5, respectively, were responsible for winter vs. spring growth habit in barley (Hordeum vulgare). A multiple allelic series at the Sh2 locus and complex epistatic interactions among loci were postulated to explain the range of growth habit seen in barley germplasm /16, 20/. The homologous relationships of the Sh and the Vrn loci have not been rigorously established.

The most frequently employed measure of winter vs. spring growth habit in cereals is heading date. Takahashi and Yasuda /20/ based their genetic analysis of growth habit on heading date under continuous light without vernalization in order to maximise differences in trait expression, and concluded that at least three internal physiological factors — vernalization response, photoperiod response and earliness — were responsible for heading date in barley. Vernalization responses are strongly influenced by photoperiod /18/. Likewise, photoperiod responses can be masked or canceled by vernalization /20/.

Hardening and maximum expression of cold tolerance are often confounded as the result of the association between the loci carrying frost tolerance and vernalization response, found both for wheat on chromosome 5A, and for barley, on chromosome 7 /6, 9, 20/. Recent evidence points to linkage rather than pleiotropy. In barley, Doll et al. /4/ reported that double haploid lines with good winterhardiness but without a vernalization requirement were recovered from winter x spring crosses. Hayes et al. /9/ reported segregation for low temperature tolerance in the progeny of a winter x

spring cross, where the winter parent was photoperiod sensitive but had no vernalization requirement.

Progress in understanding the genetic mechanisms of winterhardiness, vernalization and photoperiod responses is hindered by the often quantitative nature of trait expression and complex interactions among traits. The development of molecular marker technology and Quantitative Trait Locus (QTL) mapping offers a new approach to resolving this complexity /13, 17/. Several molecular marker maps of barley have been developed /8, 11, 12/. These maps have been used to locate QTLs affecting yield, quality and disease resistance traits /3, 10/. Based on analyses of a winter x spring cross, Hayet et al. /9/ reported the presence of a QTL on the long arm of chromosome 7 that was associated with low temperature tolerance in both field and controlled environment tests. QTL effects for heading date under 24 h light and other winterhardiness-related traits mapped to the same region. Additional markers have since been mapped in this same population, providing a greater degree of map resolution and genome coverage. Therefore, the objectives of this work were to (i) reassess cold tolerance QTL expression in view of greater map density and coverage, and (ii) locate QTLs for vernalization and photoperiod response.

Materials and Methods

Two barley cultivars, "Morex" and "Dicktoo", and one hundred F_1-derived double haploid (DH) progeny were used in these experiments. Morex, released in 1978 by the Minnesota Agricultural Experimental Station, is the U.S. six-row spring barley malting standard. Dicktoo is a six-row winter feed barley released by the Nebraska Agricultural Experimental Station in 1952. The DH lines were developed by the Hordeum bulbosum method, as described by Chen and Hayes /2/.

The 100 DH lines and the parents were phenotyped for winterhardiness component traits in field and controlled environment tests. The cold tolerance testing procedures were described by Hayes et al. /9/ and involved field plots at Bozeman, Montana, USA. The heading dates of the 100 DH lines and the two parents were also measured in a series of controlled environment tests involving four combinations of vernalization and photoperiod. In all cases, the vernalization treatment consisted of six weeks of hardening seedlings at 6 ^{o}C with an 8 h light/16 h dark photoperiod regime. Heading date with and without vernalization with an 8 h light/16 h dark photoperiod regime was assessed in the phytotron facilities of the Agricultural Research Institute (Martonvásár, Hungary) with the light intensity and temperature program described by Tischner /22/. Heading date with and without vernalization under a continuous light regime was determined under greenhouse conditions at Corvallis, Oregon. Ambient temperatures were 20 ± 2 ^{o}C, and supplemental illumination was provided by high pressure sodium lights suspended

1.5 m above the bench surface. Each genotype was replicated twice. These four data sets will subsequently be referred to by the hours of light duration (8 h or 24 h) and a suffix indicating the vernalization treatment, where v = vernalized and uv = unvernalized.

A total of seventy-eight markers were scored on the 100 DH progeny in a co-operative effort involving Linkage Genetics, Inc. (Salt Lake City, Utah), Montana State University (Bozeman, Montana), and Oregon State University (Corvallis, Oregon). The marker nomenclature of Kleinhofs et al. /12/ is employed in this report. A linkage map was constructed using Mapmaker/EXP 3.0 /14/. Seventy-four markers were resolved into eleven linkage groups. Assignments of these linkage groups to the seven chromosomes of barley were made based on previously mapped markers. QTL analyses were conducted using the interval mapping procedures of Mapmaker/QTL 1.1. The minimum LOD threshold was usually specified at 3.0.

Results

Phenotypic trait expression

The winter survival data of Hayes et al. /9/ are presented for the sake of reference and because of the greater genome coverage that has been achieved since the publication of that report. Morex and approximately half of the DH lines experienced complete mortality in Montana. The mean survival of Dictoo was 43% and the percent survival of the remaining DH lines ranged from 1 to 85 (Fig. 1e). The standard error was $\pm$ 11.1%.

Heading date frequency distributions for the DH population in controlled environment were continuous (Fig. 1a—d), indicating quantitative inheritance. Population means and ranges, and the numbers of phenotypic transgressive segregants were quite different in the various vernalization and photoperiod regimes, indicating environmental mediation of gene expression. In the controlled environment tests population means and ranges for heading date were strongly affected by photoperiod and to a lesser extent by vernalization. In terms of mean performance, vernalization had little effect in the 8 h experiments (Fig. 1a—b): the average heading date was 108 $\pm$ 1.4 days with vernalization and 111 $\pm$ 1.6 days without vernalization. Genotypes varied in their response to vernalization. For example, vernalization reduced the time to heading for Morex by 19 days and by 8 days for Dicktoo. Under 24 h light, the population means were dramatically reduced, as compared to the 8 h experiments, and there were significant number of transgressive segregants (Fig. 1c—d). Standard errors for the unvernalized and vernalized experiments were $\pm$ 1.5 and 1.1 days, respectively. The heading dates of the two parents were similar to each other. Differences among the

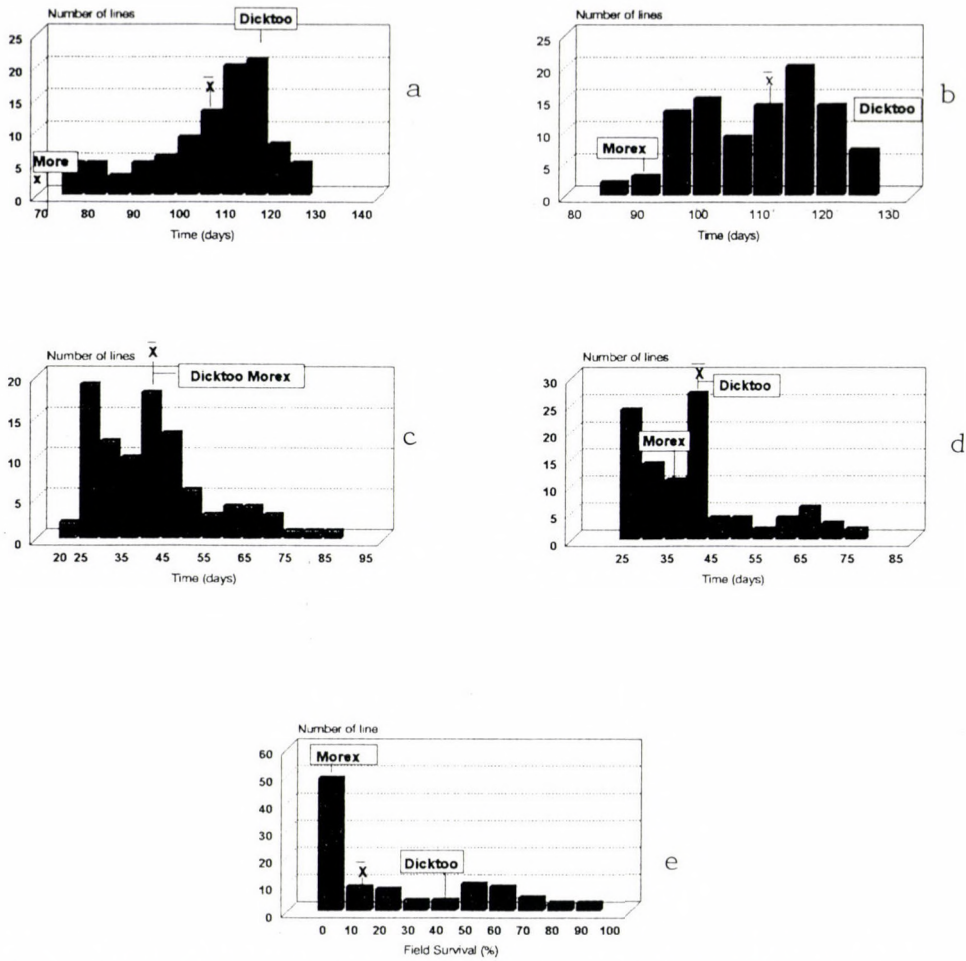


Fig. 1. Frequency distributions for heading date and field survival in a population of DH lines derived from the cross of Dicktoo x Morex, (a) heading (8 h vernalized), (b) heading (8 h unvernalized), (c) heading (24 h vernalized), (d) heading (24 h unvernalized), (e) winter survival

progeny were accentuated, particularly without vernalization. Vernalization had little effect and in some cases even delayed heading. For example, vernalized Morex was 5 days later but vernalized Dicktoo was 3 days earlier.

Quantitative Trait Loci

The large QTL effect for cold tolerance on chromosome 7 reported by Hayes et al. /9/ was substantiated with the greater density and resolution of the current map. Despite the presence of phenotypic transgressive segregants (Fig. 1e), this remains the only region of the genome in which a QTL effect for winter survival was detected. As shown in Table 1, the winter survival LOD score for the QTL peak defined by Dhn1—BCD265b was 27.1 and this interval accounted for more than 79% of the variation for winter survival in this population. QTL LOD scores declined at the upstream marker WG364b and the downstream marker BCD265b, while three peaks of approximately equal magnitude were seen in the intervening intervals. There were only modest differences between peaks in terms of maximum LOD and r^2, so selection of any particular peak as the location of the underlying gene is arbitrary. Based on the available data, determination of the exact position of the gene or genes detected by these QTL effects will require analysis in alternative genetic stocks, that will allow for higher resolution mapping.

QTLs for heading date under 24 h light, both with and without vernalization, were detected on chromosomes 2 and 7 (Table 1). Morex contributed the later maturity QTL on chromosome 2, while Dicktoo contributed the later maturity allele on chromosome 7. The LOD peaks for the chromosome 2 and chromosome 7 intervals were defined in all treatments by the ABC170—CDO064 and Dhn1—BCD265b intervals, respectively. The interval on chromosome 7 is the same as that defining winter survival. There was variation in the magnitude of the QTL effects at these two intervals in the two data sets. With vernalization, the effects of the QTLs at the two intervals were similar to each other, the chromosome 2 and 7 interval LOD scores for the 24 hv were 7.4 and 7.4. Without vernalization, the chromosome 7 effect was larger than the chromosome 2 effect. The chromosome 2 and 7 interval allele weights were 16.4 and 18.5 days, respectively.

Under 8 h light, heading date was determined by a different set of QTLs than those controlling responses at 24 h light. As shown in Table 1, QTL effects were found on chromosomes 1, 3, 5 and 7. In all cases, Dicktoo contributed the later allele. Since Dicktoo was among the latest heading genotypes under 8 h light, these loci may be those governing sensitivity to short daylength. QTL expression was essentially the same with and without vernalization. The QTL with the single largest effect was defined by the iPgd—BCD265c interval on chromosome 5.

Table 1

QTL peak effects for heading date and winter survival of 100 doubled haploid (DH) lines derived from the cross of Dicktoo x Morex

Trait	Chromosome	Marker interval	% re-combination	LOD	r^2	Weight
SUR	7	Dhn1-BCD265b	13.0	27.1	.79	47.2D#
HD8hv	1	ABC158-Brz	25.0	3.0	.16	10.9D
	3	ABG4-ABC174	20.0	2.4	.13	9.7D
	5	iPgd-BCD265c	7.7	10.1	.40	17.2D
	5	Hord-ABG452	19.0	3.8	.19	11.8D
HD8huv	1	ABC158-Brz	25.0	2.8	.17	8.8D
	3	ABG4-ABC174	20.4	3.6	.22	9.7D
	5	iPgd-BCD265c	7.7	6.8	.30	11.5D
	5	Hord-ABG452	19.0	3.8	.18	8.8D
HD24hv	2	ABC170-CD064	12.0	7.4	.34	16.1M
	7	Dhn1-BCD265b	13.0	7.4	.34	16.3D
HD24huv	2	ABC170-CD064	12.0	8.5	.36	16.4M
	7	Dhn-1-BCD265b	13.0	10.9	.44	18.5D

HD = heading date
SUR = winter survival
v = vernalized
uv = unvernalized
# = Letter suffix indicates parent contributing higher value allele, where D = Dicktoo and M = Morex

Discussion

Using a 39-point linkage map, Hayes et al. /9/ found a QTL for winter survival in the BCD265b-mR interval on chromosome 7 in this population. With the current 74-point map, this chromosome region is still the only site of significant QTL effects for this trait. The presence of large numbers of positive phenotypic transgressive segregants may simply be a consequence of the variability associated with measurement of low temperature tolerance /5/. If, however, these represent genotypic transgressive segregants, then there must be other QTLs in regions of the genome that have not been completely mapped, or there may be epistatic interactions between the chromosome 7 QTLs and other genome regions that did not have a significant single locus effect. More definitive mapping of this low temperature tolerance effect

will require alternative genetic stocks, such as lines that are isogenic for critical chromosome intervals.

The data also confirm the earlier findings of Hayes et al. /9/ regarding coincident map location of QTL effects for low temperature tolerance and heading date under 24 h light and provide additional information on the location of genes governing growth habit and response to photoperiod and vernalization. This QTL effect may correspond to the Sh2 gene described by Takahashi and Yasuda /20/. The accession of Dicktoo used in these studies does not have a vernalization requirement, yet it carries an allele on chromosome 7 that is vernalization responsive. Takahashi and Yasuda /20/ reported that there was a multiple allelic series at the Sh2 locus that accounted for gradations in winter growth habit. Thus, this accession of Dicktoo may carry an allele conferring an intermediate level of vernalization response. Two heading date QTLs were mapped to the long arm of chromosome 5 with the 8 h light treatments. However, the relationship of these QTLs with Sh3 cannot be established. Takahashi and Yasuda /20/ reported only a single point linkage of Sh3 with B (black caryopsis) with a recombination percentage of 35.8. B is on the long arm of chromosome 5. No QTL effects were detected on chromosome 4, site of the sh locus. Takahashi and Yasuda /20/ reported 6.3% recombination between sh and mHs, a common marker in the test populations. These authors reported that certain accessions of U.S. spring barley carried the winter habit allele (Sh) at this locus. Thus, it may be that Dicktoo and Morex have the same allele at the Sh locus.

The heading date QTL on chromosome 2 was detected under 24 h photoperiod. The heading date QTLs on chromosomes, 1, 3 and 5 were detected under 8 h light. Genes affecting heading date under short daylength were also previously reported on chromosomes 1, 2, 3 and 5 in other barley germplasm /7, 24/. Again, definitive conclusions regarding the allelism of QTL effects and these previously reported Mendelian loci are not possible due to the lack of common markers.

These data confirm the overriding importance of photoperiod and vernalization in determining phenotypic expression of heading date. Under long day condition variation in heading date was determined by QTLs on chromosomes 2 and 7. Dicktoo contributed the late allele on chromosome 7 and Morex the late allele on chromosome 2. Independent assortment at these unlinked loci gave rise to the transgressive segregation observed under this photoperiod condition. Under short day condition (8 h light), variation for heading date was determined by QTLs on chromosomes 1, 3, 5 and 7. Dicktoo con-

tributed the late alleles at all loci. As a consequence, the parents were at the extremes of the frequency distribution.

The results of this study indicate future course of investigation, the assessment of orthologous QTL expression in other germplams. We are, therefore, conducting map-based analyses in other winter barley populations.

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COMPARATIVE STUDIES ON POTATO TUBER DEVELOPMENT USING AN IN VITRO TUBER INDUCTION SYSTEM

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A method for synchronized in vitro tuber induction in a Hungarian cultivar of Solanum tuberosum designated "Keszthelyi 855" has been developed. It was shown that in this system tuberization and stolon elongation primarily depend on the level of sucrose in the medium. The cytokinin, 6-bensylaminopurine (BAP), also enhances the efficiency of tuber formation, however, only at sucrose concentration above 4% (w/v).

The synchronized plant culture provided starting material for isolation of genes specifically expressed in tuberizing Solanum species during the early stage of tuber development. In comparison with the non-tuberizing Solanum brevidens, three types of specific transcripts have been obtained by differential screening. Based on DNA sequence analysis the genes isolated code for the major tuber proteins, patatin and proteinase inhibitors.

Keywords: Patatin — proteinase inhibitors — Solanum brevidens — Solanum tuberosum — tuberization

Introduction

Tuberization is a complex process leading from stem differentiation to a storage organ filled with starch. The onset of tuber morphogenesis is under the control of various environmental, hormonal and genetic factors. Short daylength, low temperature or low nitrogen supply favour tuber formation. Plant hormones like cytokinins enhance tuber formation while gibberellic acid inhibits this developmental process (reviewed by /3/).

Recently, some of the genes encoding proteins highly expressed in tubers (p. ex. patatin, proteinase inhibitors, sucrose synthase, starch synthase) have been described (reviewed by /11/). Little is known, however, about the molecular mechanism involved in initiation of tuber morphogenesis.

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For molecular studies on the dissection of the process of tuber development the in vitro tuberization system /9/ seems to be the obvious choice. We have intended to use this system (Fig. 1) in order to understand the evolutionary basis of potato tuber development. In the present investigation we have examined effects of sucrose and a cytokinin on tuberization by means of stem segment culture of a new Hungarian potato cv. Keszthelyi 855 and by looking for transcriptional differences between a tuberizing and a non-tuberizing Solanum species, S. brevidens.

Materials and Methods

Plant materials

Plants (Solanum tuberosum cv. Keszthelyi 855 and Solanum brevidens) were vegetatively propagated from cuttings on RM salts /7/ with 2% sucrose and 1.2% Bacto Agar (RM medium) at 24 °C with a 16 h photoperiod under 500 lx intensity.

A synchonized in vitro tuberization system, similar to those described by Perl et al. /10/ and Hendriks et al. /5/, has been developed for the S. tuberosum cv. Keszthelyi 855. Single-node stem segments of two month old in vitro cultures were explanted in MS medium /7/ supplemented with 2%, 4%, 6% or 8% (w/v) sucrose in the absence or presence of 0.25 mg/ml and 2.5 mg/ml of 6-bensylaminopurine (BAP) at 22 °C in dark. Simultaneous presence of 8% sucrose and 2.5 mg/ml BAP in the medium resulted in synchronized tuberization.

DNA, RNA isolation and analysis

Basic methods (plasmid DNA isolation and cloning, colony hybridization etc.) were according to Sambrook et al. /14/. Total RNA was extracted as described by Stiekema et al. /17/.

Differential screening was carried out with 1000 colonies replicated in order to Hybond-N filters. Single stranded cDNA probes were prepared as in Sambrook et al. /14/ except that instead of 1 µg poly(A^+) RNA 50 µg of total RNAs were used in labelling reactions.

DNA sequence analysis

Double stranded cDNAs cloned in the vector pBluescript II SK- were sequenced by the dideoxy chain termination method /16/ using Sequenase version 2.0 kit (USB) and forward and reverse M13 primers, alternatively. Sequencing of the clone pAM66 was completed by subcloning an AvaII-XhoI fragment into the SmaI site of a dephosphorylated pUC18 plasmid DNA.

Comparative sequence analysis was performed with programs from the Genetics Computer Group /4/.

Results

Effect of sucrose and BAP on tuberization, in vitro

This experiment was designed to synchronize the tuberization of the S. tuberosum cv. Keszthelyi 855 from axillary buds of stem segments grown in vitro. The effect of sucrose level and the cytokinin BAP were tested in MS medium. When the segments were cultured in combination with 2% sucrose, BAP exerted no stimulating effect at any of the concentration tested. Although, BAP exhibited a promoting effect on the tuber formation at sucrose concentration above 4%. This effect was, however, only cumulative to that of the sucrose. Simultaneous presence of 8% sucrose and 2.5 mg/ml BAP increased the tuberization to 100% by the day 10 (Fig. 2).

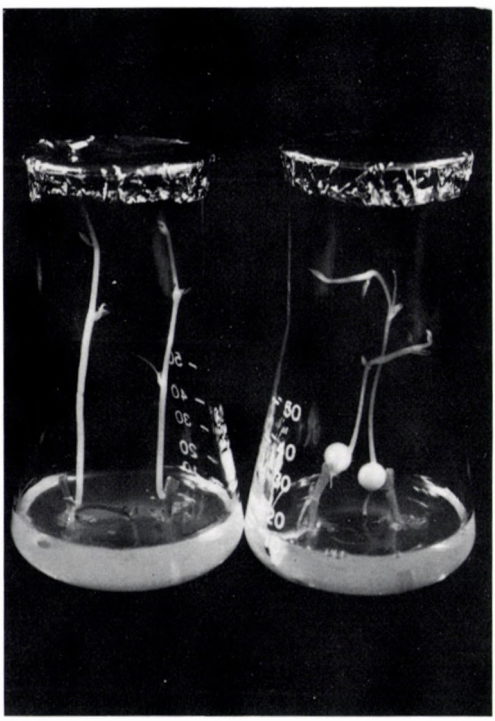


Fig. 1. Single-node potato stem segments on MS medium supplemented with 2.5 mg/ml BAP and 2% sucrose (left) or 8% sucrose (right) grown in dark at room temperature for three weeks

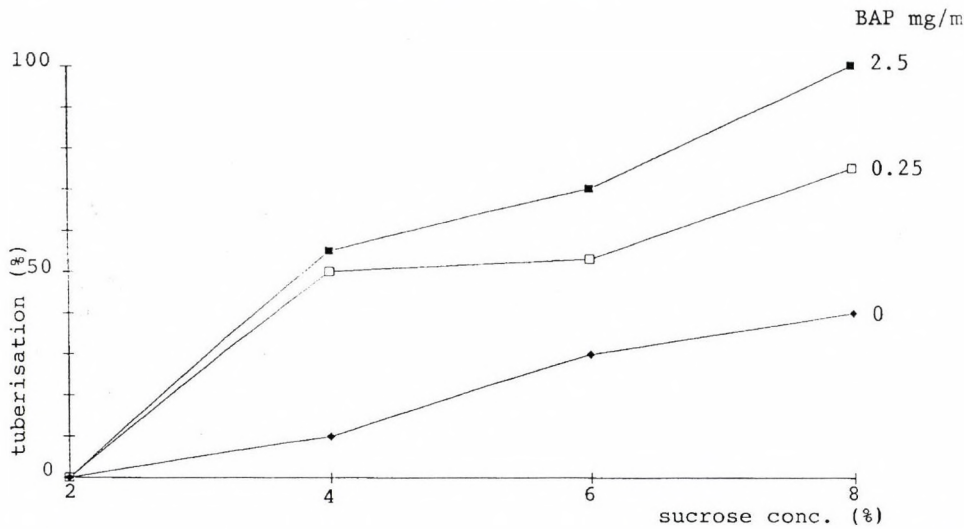


Fig. 2. Effect of BAP on the in vitro tuberization at different sucrose concentrations. Each treatment was carried out with 20 stem segments and evaluated 10 days after plantation

Effect of sucrose on shoot (stolon) formation

When a single-node segment of S. tuberosum is planted in MS medium with 2% sucrose in dark, a stolon (a shoot with reduced leaf growth) will be

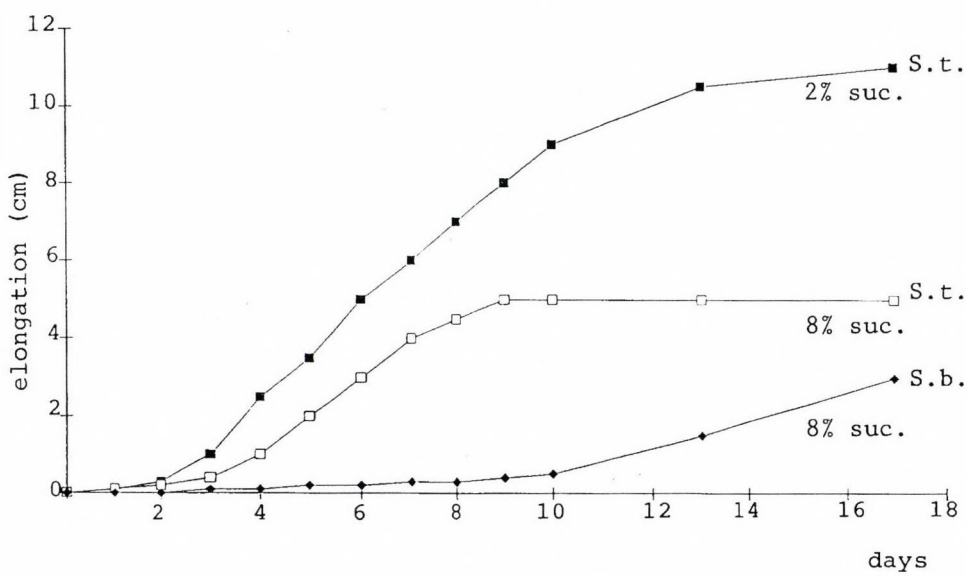


Fig. 3. In vitro shoot (stolon) elongation of S. tuberosum (S.t.) and S. brevidens (S.b.) in MS medium supplemented with 2.5 mg/ml BAP and 2% or 8% sucrose in dark at room temperature. Elongation (cm) is the average of 50 parallels

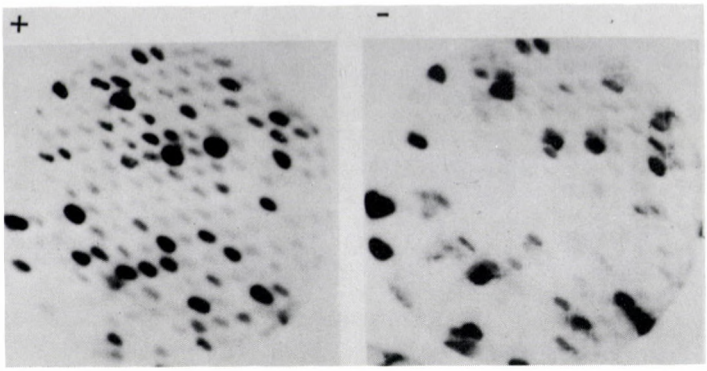


Fig. 4. Differential screening by colony hybridization. +: hybridization with first strand labelled cDNA probe prepared from S. tuberosum shoots grown from axillary buds, in vitro, 5 days after planting to tuber inducing medium; -: the same from S. brevidens

developed from the axillary bud (Fig. 1). When it is cultured with 8% sucrose, stolon formation is slower and it is stopped at day 9 when the tuber morphogenesis and growth become visible (Fig. 3).

Axillary buds of the non-tuberizing species, S. brevidens are also able for shoot formation. Although, the elongation of the shoot is much slower than in the case of S. tuberosum (Fig. 3) and no tubers or tuber-like structures are developed on this species.

Isolation and identification of cDNA clones differentially expressed in S. tuberosum and in S. brevidens

In order to isolate and study potato genes specifically expressed during tuber development, a cDNA library was constructed from 4-day-old S. tuberosum stolon tips and axillary buds induced for tuberization /1/.

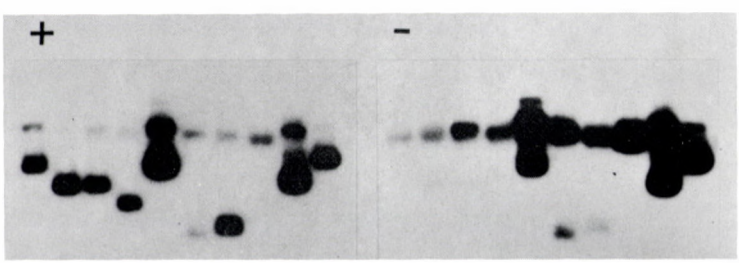


Fig. 5. South-Northern hybridization. Marks (+, -) as in Fig. 4. The differences correspond to the patatin gene of S. tuberosum

```
  1  AAAATTAGTA GTTTAAACAA GATGAAGTGT TTATTTTTGT TATGTTTGTG
                            M  K  C   L  F  L  L   C  L  C

 51  TTTGTTTCCC ATTGTGGTGT TTTCATCAAC TTTCACTTCC CAAAATCCCA
      L  F  P   I  V  V  F   S  S  T   F  T  S   Q  N  P  I

101  TTAACCTACC TAGTGATGCT ACTCCAGTAC TTGACGTAAC TGGTAAAGAA
       N  L  P   S  D  A   T  P  V  L   D  V  T   G  K  E

151  CTTGATCCTC GTTTGAGTTA TCATATTATT TCCACTTTTT GGGGTGCGTT
      L  D  P  R   L  S  Y   H  I  I   S  T  F  W   G  A  L

201  AGGTGGTGAT GTGTACCTAG GTAAGTCCCC AAATTCAGAT GCCCCTTGTG
       G  G  D   V  Y  L  G   K  S  P   N  S  D   A  P  C  A

251  CAAATGGCAT ATTCCGTTAC AATTCGGATG TTGGACCTAG CGGTACACCC
       N  G  I   F  R  Y   N  S  D  V   G  P  S   G  T  P

301  GTTAGATTCA TTGGTTCATC TAGTCATTTT GGACAAGGTA TCTTTGAAAA
      V  R  F  I   G  S  S   S  H  F   G  Q  G  I   F  E  N

351  TGAACTACTC AACATCCAAT TCGCTATTTC AACATCGAAA TTGTGTGTTA
      E  L  L  N   I  Q  F   A  I  S   T  S  K  L   C  V  S

401  GTTATACAAT TTGGAAAGTG GGAGATTACG ATGCATCTCT AGGGACGATG
       Y  T  I   W  K  V   G  D  Y  D   A  S  L   G  T  M

451  TTGTTGGAGA CTGGAGGAAC CATAGGTCAA GCAGATAGCA GTTGGTTCAA
      L  L  E  T   G  G  T   I  G  Q   A  D  S  S   W  F  K

501  GATTGTTCAA TCATCACAAT TTGGTTACAA CTTATTGTAT TGCCCTGTTA
      I  V  Q   S  S  Q  F   G  Y  N   L  L  Y   C  P  V  T

551  CTAGTACAAT GAGTTGTCCA TTTTCCTCTG ATGATCAATT CTGTTTAAAA
       S  T  M   S  C  P   F  S  S  D   D  Q  F   C  L  K

601  GTTGGTGTAG TTCACCAAAA TGGAAAAAGA CGTTTGGCTC TTGTCAAGGA
      V  G  V  V   H  Q  N   G  K  R   R  L  A  L   V  K  D

651  CAATCCTCTT GATGTCTCCT TCAAGCAAGT CCAGTAATAA CAAATGTCTG
       N  P  L   D  V  S  F   K  Q  V   Q  *

701  CCTGCTAGAC TATATGTTTT AGCAGCTACT ATATATGTTA TGTTGTAAAT

751  TAAAATAAAC ACCTGCTAAG CTATATCTAT ATTTTAGCAT GGATTTCTAA

801  ATAAATTGTC TTTCCTT
```

Fig. 6. Nucleotide- and deduced amino acid sequence of the cDNA pAM66. Best homology (98.5%) was detected to the Kunitz-type proteinase inhibitor described by Ishikawa et al. /6/ (accession No. M17329). The non-identical bases and amino acids are indicated by stippling. The soybean trypsin inhibitor (Kunitz) protease inhibitors family signature is underlined

```
1   CTCACATCAT TGTTTTCTTT CTTCTTGCAA CTTCCTTTGA AACTCTCTTG
      H  I  I   V  F  F   L  L  A  T   S  F  E   T  L  L

51  GCACGAAAAG AAAGTGATGG ACCAGAAGTC ATAGAACTTC AAAAGGAATT
    A  R  K  E   S  D  G   P  E  V   I  E  L  Q   K  E  F

101 TGAATGCAAT GGAAAACTAA GCTGGCCAGA ACTTATTGGT GTACCAGCAC
     E  C  N   G  K  L  S   W  P  E   L  I  G   V  P  A  H

151 ATTATGCTAA GGGAATAATT GAGAAGGAAA ATTCACTCAT AACTAATGTT
      Y  A  K   G  I  I   E  K  E  N   S  L  I   T  N  V

201 CAGATACTAC TGAATGGTTC TCCAGTCACA ATGGATTATC GTTGTAATCG
    Q  I  L  L   N  G  S   P  V  T   M  D  Y  R   C  N  R

251 AGTTCGTCTT TTTGATAACA TTTTGGGTGA TGTTGTACAA ATTCCTAGGG
     V  R  L   F  D  N  I   L  G  D   V  V  Q   I  P  R  V

301 TGGCTTAATT AATGGATTAT TATTGAAGTA ATTAAGCAGC CACATGTCAA
      A  *

351 AAATAATTAG GGTTCATGTA TGTTGATTAT AATATCTCCA TGTACTCTTA

401 CTATATATAA TGAAATAAAT AAGTGTGGCT TAATT
```

Fig. 7. Nucleotide- and deduced amino acid sequence of the cDNA pAM98. Best homology (97.9%) was detected to the coding region of the gene winI (2; accession No. M17108). The non-identical basis and amino acids are indicated by stippling. The proteinase inhibitor family signature is underlined. Based on homology to winI 16 bp is missing from the 5' end of the cDNA in the clone pAM98

Differential screening has been performed by colony hybridization using RNAs isolated from S. tuberosum (+ probe) and from S. brevidens (- probe) shoots and buds developed under the same conditions (Fig. 4). Plasmid DNA have been isolated from colonies showing the desired hybridization pattern and the differential screening has been repeated by the "South-Northern" method (hybridization of DNA separated on a gel and blotted to a filter with first strand labelled cDNA as a probe; Fig. 5). Finally, Northern hybridization was used to prove that cDNA clones corresponding to specific transcripts have been obtained (data not shown).

In this way, three types of tuberization specific clones have been isolated. Based on DNA sequence analysis, most of them corresponded to the well-known gene family, patatin. The other two, pAM66 and pAM98, were proteinase inhibitors. pAM66 showed homology to the Kunitz-type inhibitors (Fig. 6) while pAM98 turned out to be 98% identical to the wound-inducible gene, winI (Fig. 7).

Discussion

The role of sucrose and BAP in tuberization

Using single-node stem segments derived from in vitro cultures in the same age, synchronized tuber formation was achieved in the S. tuberosum cv. Keszthelyi 855 in MS medium containing 8% surrose and 2.5 mg/ml BAP. We have shown that tuberization is primarily depended on sucrose. At a sucrose concentration of 2% only stolons were developed from axillary buds. The rate of tuberization is in correlation with the level of sucrose in the medium. Stolon formation is also influenced by sucrose concentration. Stolon elongation is stopped when radial expansion of tubers becomes visible.

The central role of sucrose in tuber morphogenesis is well known. The development of sink organs like potato tubers is dependent on the ability of the source organ (leaf) to produce and export photoassimilates. Under in vitro conditions, the photoassimilates are substituted by the sucrose in the medium. Recent experiments with antisense RNA inhibition showed that blocking the sucrose transport or the enzymes involved in starch synthesis highly influence the rate of tuberization as well as the number, size and shape of the tubers formed /8, 12, 19/.

Although, tuberization, in vivo, is under the control of not only metabolic but also of hormonal regulation /18/ the effect of BAP, in vitro, was much less than that of the sucrose. This may be explained by the internal hormone content of the stem segments that might has been predestinated tuberization without any external hormone effect.

The role of patatin and proteinase inhibitors

Patatin is the best known "tuber-specific" gene that is encoded by a gene family comprising two classes with 10-18 members per haploid potato genome. Class I genes are highly expressed in tubers and are sucrose inducible, while class II genes are under developmental control and are expressed mainly in root tips. Patatin is regarded as a storage protein in tubers /11/. We have shown that patatin is not expressed in the non-tuberizing species, S. brevidens, although it is present in its genome /1/.

Proteinase inhibitor proteins are found throughout all life forms and comprise one of the most abundant classes of proteins in the world. Their functions appear to be twofold: prevent uncontrolled proteolysis and

protect the cells from foreign proteolytic enzymes /13/. Several proteinase inhibitors have already been isolated from potato. Some of them are transcribed during tuber development /15/. We have detected two types of proteinase inhibitors (Kunitz-type and winI) differentially expressed in S. tuberosum and in S. brevidens shoots grown from axillary buds under tuber inducing conditions. In further research we are going to study the regulation of these genes in S. brevides in order to understand the basis of transcriptional difference that might be in signal transduction pathway or in cis regulatory elements.

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STUDY OF THE ALCOHOL DEHYDROGENASE-1 (Adh1) GENE IN TETRAPLOID CORN: EXPRESSION IN THE POLLEN GRAINS AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM

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The results of two separate experiments are presented in this paper. In the first experiment pollen assays were carried out in Wf9 tetraploid maize plants in order to decide whether the lack of the ADH enzyme activity in the pollen grains was caused by a 'O' allele or an active transposon. On the basis of the results we suppose that the reduced ADH enzyme activity in the pollen grains is the result of a transposon affecting only the gametophyte.

In the second experiment a maize Adh1-S genomic clone was constructed by polymerase chain reaction (PCR) and used as a probe to detect polymoprhism around the Adh1 gene in W117 S_{18} tetraploid maize sublines. The PCR20-EcoRI clone-enzyme combination resulted in a monomorphic RFLP pattern. The PCR20-Bgl II probe-enzyme combination yielded a multiple-banded pattern.

Keywords: Alcohol dehydrogenase-1 — pollen — transposon — gDNA — PCR — RFLP — autotetraploid — Zea mays L.

Introduction

The Adh1 locus is one of the best characterized gene systems in higher plants, with electrophoretic, null-activity and controlling-element mutants available /7, 17, 19/. Adh1 encodes the major alcohol dehydrogenase enzyme (ADH; E.C. 1.1.1.1.) present in the seed, seedling, and pollen of maize, but is not expressed in mature leaves. The active enzyme is a dimer /18/ of 82 000 daltons /5/. The two allelic forms e.g. Adh1-S and Adh1-F differ in their electrophoretic mobility, organ specific expression as well as at the level of intragenic recombination /19/.

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There are many single genes in the genom with sporophytic and gametophytic expressions, e.g. Adh1, Got1, which can be observed by electrophoresis. In maize ~72% of all isozymes are expressed in both the sporophyte and male gametophyte /8/.

The fine structure of the maize Adh1 gene and the differences between the mRNSs of the Adh1-F and the Adh1-S alleles have been known since 1982. In addition, three naturally occurring alleles have been cloned and sequenced. These are the standard 1F and 1S of Schwartz and $1C^m$ alleles /14, 15/.

The objectives of our study were: (i) to assay the expression of the Adh1 gene in the pollen grains of the tetraploid corn to reveal '0' allele, (ii) to construct an Adh1-S genomic clone by polymerase chain reaction, and to use it as a probe to detect polymorphism around the Adh1 gene in W117 S_{18} tetraploid corn sublines.

Materials and Methods

Plant material

Kernels of the diploid W117 line were treated with 0.05% colchicin solution and then sowed on the gamma field to produce tetraploid plants. Plants thirty meters from the gamma source were chosen and selfed. All of the W117 sublines are the progenies of these plants.

Pollen analysis

Newly shed pollen grains from Wf9 tetraploid plants with a known ADH1 pattern in the scutellum were collected and histochemically stained for ADH activity as described by Freeling /9/.

ADH1 analysis in the scutellum

Laboratory techniques for ADH1 were performed with some modification, as detailed by Davis /2/ and Hart /8/.

Polymerase Chain Reaction

On the basis of the nucleotide sequence of the genomic clone lambda 1S.1 constructed from the Adh1-S gene by Dennis et al. /3/, two 21-nucleotide primers were sketched and produced at the Agricultural Biotechnological Research Centre:

5' GGA AGG TGA TCA AGT GCA AAG 3'

5' ATC ACA CAT GTT GCT CTC TGC 3'

Using these primers, the Adh1-S genomic clone was isolated from the Adh1-SS diploid corn inbred line produced at the Department of Genetics and Plant Breeding using PCR. Amplification reaction was performed in 50 µl. Thirty cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C were performed in a DNA thermal cycler (Perkin Elmer Cetus). The resulting ~850-bp fragment was filled with klenow enzyme and ligated into pBluescript KS (Stratagene), digested with EcoRV, and transformed into Escherichia coli DH5α. After subcloning, the Adh1-S genomic clone (PCR20) was checked by sequencing 200-bp according to the procedure of Sanger et al. /16/.

RFLP analysis

Genomic DNA was isolated from the leaves of tetraploid corn plants and separately isolated with the restriction enzymes EcoRI and Bgl II. After gel electrophoresis and Southern blotting, filter hybridization was performed with a ^{32}P-labelled Adh1-S clone (PCR20) using the method of Lee et al. /13/, RFLP profiles for inbreds in autoradiograms were scored visually and then genetic similarities (GS) were calculated among all possible pairs of inbreds by the method devised by Dice /4/.

Results and Discussion

Among the selfed Wf9 plants with a different Adh1 genotype in the scutellum, one was found to shed 96% ADH$^-$ pollen grains and to be triplex (Adh1-FFFS). Surprisingly, of the pollen grains of the other Wf9 plant with the same genotype, only 2% were ADH$^-$ (Table 1).

Table 1

Expression of the Adh1 gene in the pollen grains of triplex tetraploid plants

Plant material	Adh1 genotype in the scutelleum	Presence or absence of ADH in the pollen grains	
		ADH$^+$ %	ADH$^-$
Wf9 4x 47	FFFS	98	2
Wf9 4x 51	FFFS	4	96

In order to decide whether the lack of the ADH enzyme activity in pollen grains was caused by a '0' allele or an active transposon, the Wf9 ADH$^-$ plant was selfed, and all the seeds from the cob were analysed for the ADH1 pattern in the scutellum. After the selfing of a triplex genotype (FFFS) in the case of one locus and two alleles, two types of gametes with Adh-1-FF or Adh1-SS genotypes were found to arise out of the chromosome segregation. The random combination of these gametes results in the 1FFFF: 2FFFS:1FFSS genotypic ratio (Table 2).

Table 2

Expected segregation of progeny genotypes after selfing in the case of one triplex locus, two alleles and chromosome segregation

Gametes	1 FF	1 FS
1FF	1 FFFF	1 FFFS
1FS	1 FFFS	1 FFSS

Genotypic ratio: 1FFFF:2FFFS:1FFSS

Genotypic distribution in the scutellum of the progenies of the ADH$^-$ plant (Adh1-FFFS) after selfing

Number of seeds tested	Adh1 genotypes FFFF	FFFS	FFSS	FSSS
165	51	69	42	3

Chi^2 (1:2:1) = 4.52 p = 20-5%
F = fast, S = slow

After analyzing the FFFS triplex heterozygote Wf9 plant, the frequency of FFFF, FFFS and FFSS genotypes on the gel slabs was found to be different from what was expected, and the Adh1-FSSS enzyme pattern with a low frequency (1.8%) appeared, due to the crossing over (Table 2).

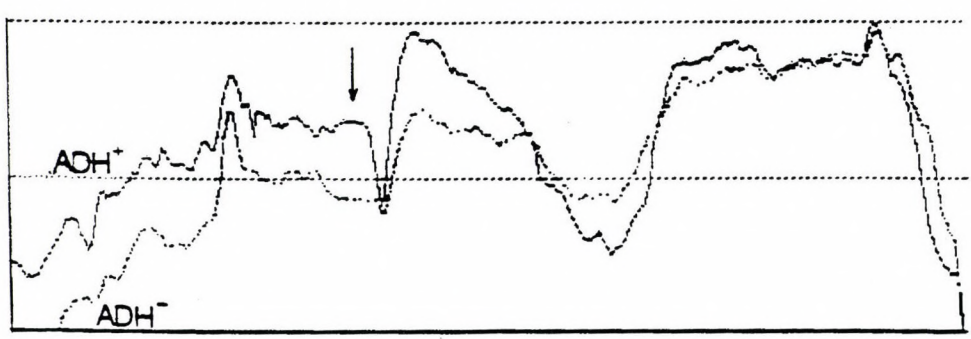


Fig. 1. Densitogram of pollen polypeptides of ADH$^+$ and ADH$^-$ plants

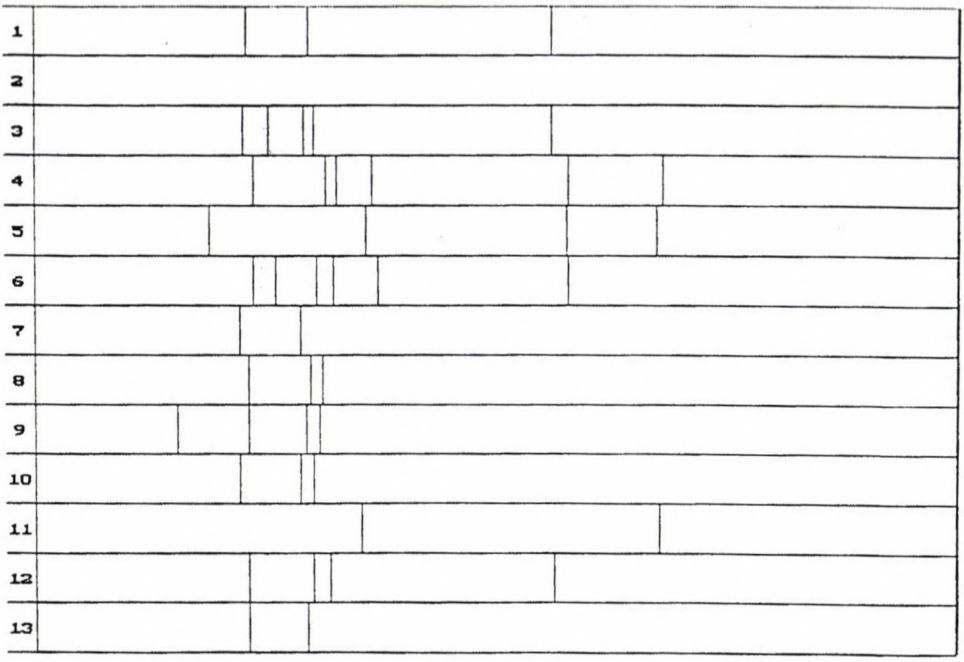


Fig. 2. RFLP patterns of PCR20-Bgl II clone-enzyme combination in W117 tetraploid S_{18} sublines

Consequently, we could not verify the existence of a '0' allele. Of the offsprings of the selfed ADH^- plant, only one did not possess ADH activity (Table 3).

The protein pattern of the ADH^+ and ADH^- plants revealed remarkable differences, not only in the ~32 Kd, but in other molecular weights (Fig. 1).

On the basis of our results we refused the '0' allele hypothesis. We suppose that the lack of the ADH enzyme activity in the pollen grains was caused by a transposon affecting only the gametophyte.

Chen et al. /1/ described the mutant allele Adh1-3F1124 which carries a transposon in the promoter region that is flanked on both sides by a TATA box. Enzyme activity and Adh1-3F1124 transcript levels are reduced in root and scutellum but not in pollen. Excision of the transposon and both TATA boxes leads to restored root expression and significantly reduced pollen expression /11, 12/. To determine the exact place of the transposon activity in the tetraploid Wf9 line, sequence analyses are to be done.

The RFLP analyses of W117 sublines showed that the PCR20-EcorI clone-enzyme combination resulted in a monomorphic pattern. The PCR20-Bgl II

Table 3

Types of Adh1 pattern in the mature scutellum, and presence or absence of ADH in the pollen grains of the offspring of ADH⁻ plant (Adh1-FFFS)

Plant material	Adh1 genotype in the scutellum	Presence or absence of ADH in the pollen grains
Wf9 4x 4	FFFS	+
Wf9 4x 22	FFSS	+
Wf9 4x 24	FFFF	+
Wf9 4x 31	FSSS	+
Wf9 4x 76	FFSS	+
Wf9 4x 83	FSSS	+
Wf9 4x 96	FFSS	-
Wf9 4x 102	FSSS	+
Wf9 4x 112	FSSS	+

Table 4

GS estimates calculated from RFLP data of PCR20-Bgl II clone-enzyme combination

W117 4x sublines	13	12	11	10	6	5	4
13		0.33	0.00	0.00	0.00	0.00	0.00
12	0.33		0.00	0.29	0.20	0.00	0.00
11	0.00	0.00		0.00	0.00	0.33	0.25
10	0.00	0.29	0.00		0.00	0.00	0.00
6	0.00	0.20	0.00	0.00		0.00	0.33
5	0.00	0.00	0.33	0.00	0.00		0.00
4	0.00	0.00	0.25	0.00	0.33	0.00	

probe-enzyme combination yielded a multiple-banded pattern. The average number of the pattern was 3.9 and the GS values ranged from 0.00-0.33 (Fig. 2 and Table 4).

Now RFLP analysis is being continued with mapped maize clones provided by Coe et al. (University of Missouri, Columbia/MO). After having a minimum of 70 polymorphic clone-enzyme combinations, the associations among tetraploid W117 sublines can be revealed by principal coordinate analysis (PCOA) based on GSs.

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PRODUCTION OF FISH CHIMERAS FROM EMBRYONIC CELLS

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The efficiency of two cell-transplantation methods were compared for the production of embryonic cell derived fish chimeras. The classic microinjection technique (blastula stage donor cells are microinjected into blastula embryos) was compared to a novel aggregation method for fish developed by our group. This method utilises the ability of dechorionated fish embryos to aggregate. Morula cells dissociated in Ca^{2+}, Mg^{2+} free medium were aggregated to recipient embryos of different developmental stage.

Donor cells and recipient embryos of different developmental stages were used for most efficient incorporation in the chimera. The fate of donor cells derived from blastulae, was followed by labelling them with FITC-dextrane (FD). The most efficient transplantations were gained by using 16-32 cell stage recipients for aggregation (18% survival of chimeras at swim up stage). Labelled donor cells were contributing to the embryos in varying ratio.

A comparison of the efficiency of aggregation was made between diploid-diploid, diploid-haploid and diploid-interspecific (diploid) hybrid chimeras. In all three cases chimeras containing different proportion of donor cells were gained. After one day incubation the embryos were dissociated by trypsin digestion and number of labelled and non-labelled cells were counted under fluorescent microscope.

Experiments were performed on Rosy barb (Barbus conchonius), Carp (Cyprinus carpio) and African catfish (Clarias gariepinus).

Keywords: Fish chimeras — embryonic cells — microinjection — aggregation-method — Cyprinus, Barbus, Blarias

Introduction

The use of fish as model system for studying vertebrate development is ever increasing /3, 10, 12/. Simple procedures for genome manipulations, short generation times, large number of gametes and the transparency of embryos are some of the advantages of using fish for these investigations /2, 11, 12, 13/.

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Pluripotent embryonic cell lines and ES chimeras, derived from these cells have been produced in mice /4, 6/, however, interest with this kind work have only recently emerged in fish. Recent publications have reported the foundation of ES-like cell lines /3/ and achievements of cell transplantation where donor cells from an embryo contributed to the recipient with varying success /7, 11/. Lin et al. /5/ used FITC-dextrane, recessive albino (alb-1) mutation and the LacZ reporter gene as marker for monitoring the cell transplantation efficiency. Five albino chimeras were used in crossing and 1-43% of the offspring showed pigmentation proving the success of germ line chimera production. However, this technology cannot yet be exploited became the ES like line produced so far is not yet fully pluripotent and its contribution to the chimera development has not been clearly established.

Weakened, non-viable e.g. X-ray irradiated /9/ or tetraploid /6/ embryos have been used as recipients in mouse chimeras for the maximalization of the rate of donor cells in the developing embryo. In Teleosts the routine production of haploid and triploid embryos /2, 8/ allows the application of a similar strategy.

Using the efficient method of Nagy et al. /6/, developed for mouse, we attempted to produce a large number of chimeras by aggregation. We found that dechorionated early embryos of fish have the property which allows the sticking of donor cells on the surface of recipient embryos and results in the spontaneous aggregation of the cells. The efficiency of incorporation of donor cells was investigated using FITC-dextrane labelling and phenotypic and biochemical markers, in diploid-diploid, diploid-haploid and diploid-interspecific hybrid chimeras.

Materials and Methods

Experimental species

Experiments were performed using embryos of Rosy barb (Barbus conchonius), Carp (Cyprinus carpio) and African catfish (Clarias gariepinus). Rosy barb broodstock were kept in 40 l tanks (10-12 fish per tank), at 26 ^{o}C, on a 14-10 hours light-dark periods. Induction of ovulation was induced by a 50% water change and by increasing the temperature 2 hours prior to dark period preceeding the planned ovulation. Eggs were stripped from females whereas sperm was obtained using a pipette. Carp broodstock were spawned at commercial fingerling farm (Dinnyés, Hungary). By injecting of dried hypophysis suspension (5 mg/kg body weight) in the abdominal cavity of the females and males (2-3 mg/kg). Sexually mature African catfish females

were injected with dried carp pituitary (4-5 mg/kg body weight dissolved in 0.65% salt solution) at approximately 10 hours prior to stripping. Stripped eggs were fertilized artificially by sperm obtained from surgically removed testis. Treated and control embryos were kept in plastic Petri dishes until hatching in Holtfreter's solution (3.5 g NaCl, 50 mg KCl, 100 mg $CaCl_2$, 200 mg $NaHCO_3 \times H_2O$ per 1 l distilled water). Hatched larvae were reared in water until the feeding stage (5-6 days of age) and afterwards in a water recirculation system.

Preparation of donor cells

For every species, fertilization was done according to Woynarovich /14/. After water hardening (approx. 5-10 minutes after fertilization) eggs were dechorionated by using Pronase E type XXV (No. P-6911, Sigma) at a final concentration of 5 mg/ml dissolved in Holtfreter's solution.

FITC-dextrane (FD, mw. 17 000, in PBS, Sigma) was used for labelling of the donor cells. Approximately 10 pl of FD was injected into the animal pole of the one cell stage embryo using glass capillaries pulled from Pasteur pipettes (2-4 µm diameter). Only normally developing, strongly stained embryos were used for aggregation.

Embryonic cells were dissociated from the yolk by incubation the embryo in phosphate buffered saline (PBS) solution. Under these condition the cells immediately start decompaction and are totally released from the yolk in 20 min. They continue dividing until reaching the 10-15 µm diameter. By gentle shaking 5-15 cells groups may be gained for aggregation.

Preparation of recipient embryos

Fertilization of eggs to produce recipient embryos was carried out when the donor embryos were at the 32-64 cell stage of development. Haploid embryos were obtained by using UV irradiated sperm /2/. Interspecific hybrids of carp and rosy barb were produced by fertilizing carp eggs with rosy barb sperm. After full water hardening these embryos were dechorionated similarly to donors and were kept in Holtfreter's solution until use. Normally embryos were selected for aggregation at the 2-4 cell stage.

The process of aggregation

Recipient embryos were first placed in wells of 96 well plate with their animal pole facing upward. Then using a capillary pipette, donor cell groups (5-25 cells) were placed on top of recipient embryonic cells. One hour after aggregation, the embryos were transferred into Petri dishes (10/dish). Nonaggregated cells were removed during the transfer. Both aggregation and postincubation to swim up stage was done in Holtfreter's solution at 26 ^{o}C.

Injection of cells

Donor cells were obtained as described above. Capillary pipettes with 30-40 um diameter was used for injection of cells into early blastula recipients (200-300 cells stage).

Comparison of chimera constructions

Following incubation for 24 hours the chimeras were digested in 0.25% trypsin solution (Sigma) for 3-5 min. The process was blocked by addition of 10% Foetal Calf Serum (FCS, Sigma). Cell suspension of the individual embryos were evaluated using a fluorescent microscope by counting random batches of the cells (approx. 50-cells in 5 repeats).

Analysis of data

Developmental stage was identified as described by Bremiller et al. /1/. Appearance of FD was examined at two developmental stages. Swim up (the start of feeding) was considered to be the most important stage, since larvae may be reared without serious loss. Dechorionation, control of embryonic development and aggregation were done under a Zeiss GSZ stereomicroscope at 50 x magnification. For the detection of FD fluorescence a Leitz Fluovert microscope was used. Successful aggregation was only considered if at least one cell was found in the developing embryo to show fluorescence. Embryos were photographed on Fujichrom (400 ASA).

Results

The determination of optimal developmental stage for the production of chimeras by aggregation

In preliminary experiments using more than 30 donor cells at aggregation we rarely observed normal development. Among these abnormal embryos the development of two embryos were occasionally observed on the same yolk. Experiments with 10-20 cells dissociated from donor morulae resulted in the highest number of survivors (data not shown).

This cell number was used for the optimization of recipient stage.

Fate of aggregated cells is shown in Tables 1 and 2. Only normally developing embryos are shown, however, abnormally developing fluroescence labelled embryos were observed in high frequency (10-20%).

As it can be seen form Tables 1 and 2 aggregation is most efficient (18.7% carp and 18.9% barb) at the early morula stage and was found to be completely unsuccessful at the early blastula stage.

Results of experiments on rosy barb and carp showed similar tendency.

Table 1

Aggregation experiments on Rosy barb for determination of optimal developmental stage of recipient

Stage of development of recipient	Number of treated	Normally developed embryos			
		gastrula stage		20 somites stage	
		number	FD + (%)	number	FD + (%)
2-4 cell	30	22	26.6	14	10
8-16 cell	58	41	46.5	19	18.9
Early blastula	31	28	0	19	0

Table 2

Aggregation experiments on Carp for determination of optimal developmental stage of recipient

Stage of development of recipient	Number of treated	Normally developed embryos			
		gastrula stage		20 somites stage	
		number	FD + (%)	number	FD + (%)
2-4 cell	12	9	16.6	4	8.3
8-16 cell	48	38	27	19	18.7
Early blastula	14	12	0	8	0

Production of chimeras by microinjection

Using African catfish embryos in these experiments, the number of treated embryos which hatched (at one day of development) was 73.5%. Of the treated embryos 77.4% was found to show FITC-dextrane labelled cells. Of the chimeric individuals, 20% were abnormal among those which contained no labelled cells only 2% were abnormal.

A total of 57.4% of the normally developing embryos were found to be chimeric according to FD labelling. Out of these individuals an average of 7.18% of the cells were donor derived FD positive. The distribution of these cells in the chimera body (head 58.1%, trunk 88.4% and tail 69.7%) is shown in Table 3.

Table 3

Production of African catfrish chimeras by microinjection*

Number of treated eggs	Survival at hatching	FD + embryos		FD- embryos	Normally developed		
		Normally developed	Abnormally developed		FD+/FD- ratio	Percentage of embryos containing FD + cells in	
						head	trunk (%) tail
102	75 (73.5%)	43 (57.4%)	15 (20%)	17 (22.6%)	7.18±8.1	88.40	69.70

*Donor = diploid, recipient = diploid.
FD = donor derivated cells labelled with Fitc-dextrane.

Comparison of cell transplantation protocols in using interspecific hybrids

Cell transplantation experiment were repeated twice. Donor cells were labelled by FD and the efficiency of integration was observed at the gastrula stage. No significant a difference was found between the two methods of chimer production (microinjection and aggregation) since 70-80% of individuals contained labelled donor derived proliferating cells in both cases. The low survival of recipients (30.8% and 82.1%) was the result of low fertilization rates. Only normally developing 2-cell-stage embryos were selected for cell transplantation experiments. Thus comparison with the recipient controls is only possible by using certain corrections. If fertilization of recipient was considered 100% normally developing hybrids were 4.9% and 8.6%, respectively. Chimeras derived from aggregation showed 17.2% and 18.3%, respectively (compared to recipient embryos 3.51 times and 2.12 times more). The low survival in the injection experiments in the second series were the result of the mechanical damage caused by the technique.

Comparison of the aggregation method using diploid and haploid recipients

Success of treatment at one day of development (20 somite stage) was facilitated by trypsin digestion of chimeras resulting in a cell suspension. This stage was chosen since FD labelling is still intensive and can be distinguished unambiguously from negative (non-labelled) cells. While haploid survival at one day is nearly half of diploids (35.2% and 60%, respectively), proportion of FD (containing FD labelled cells) embryos among haploids is 47.4% as opposed to the 38.8% among diploids. Mean ratio of FD positive cells in the chimeric individuals with haploid recipients was 39.4%

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Table 4

Comparison of cell transplantation methods using interspecific hybrids as recipient**

Treatments	Number of eggs	Gastrula stage total	Gastrula stage FD+	20 somites stage	Hatching embryos	Feeding larvae
Aggregation	58	54 (93.1%)*	44 (81.4%)	50 (51.7%)	14 (24.1%)	10 (17.2%)
Injection	73	59 (80.8%)*	47 (79.6%)	36 (49.3%)	17 (23.2%)	6 (8.2%)
Donor	110	96 (87.2%)	—	86 (78.1%)	74 (67.2%)	56 (50.9%)
Recipient***	350	108 (30.8%)	—	76 (21.7%)	21 (6%)	9 (2.5%)
Recipient (100%)	350	212 (60.4%)	—	149 (42.5%)	42 (12%)	18 (4.9%)

Table 5

Comparison of cell transplantation methods using interspecific hybrids as recipient**

Treatments	Number of eggs	Gastrula stage tota	Gastrula stage FD+	20 somites stage	Hatching embryos	Feeding larvae
Aggregation	60	47 (78.3%)*	36 (76.5%)	27 (45.0%)	15 (25.0%)	11 (18.3%)
Injection	79	55 (69.6%)*	47 (85.4%)	25 (31.6%)	6 (7.5%)	2 (2.5%)
Donor	220	201 (91.3%)	—	178 (80.9%)	169 (76.8)	152 (69.0%)
Recipient****	185	115 (62.1%)	—	71 (38.4%)	19 (10.2%)	13 (7.0%)
Recipient (100%)	185	138 (74.5%)	—	85 (46.0%)	23 (12.3%)	16 (8.6%)

*Only the normally developed embryos were used for aggregation.

**Donor = Carp X Carp, recipient = Carp X Rosy barb.

***Fertilization rate = 51%.

****Fertilization rate = 82%.

Recipient (100%) = correction for 100% fertilization rate.

(2.4%—74.3%) and chimeras with diploid recipients showed 22.1% (5.6%—52.8%). Wide range of variation was more obvious (SD = 26.3) among diploid-haploid chimeras.

Discussion

The development of the cell transplantation method by aggregation for three species was based on the idea of Nagy et al. /6/. We were looking for the optimal donor-recipient combinations and studied the contribution of donor cells to the developing embryo. As it is clear from the results the utilisation of the spontaneous aggregation capability of early dechorionated

Table 6

Aggregation experiments on Rosy barb using diploids and haploids as recipients

Type of recipient	No. of eggs treated	Somite stage survival	%	FD + embryos	%	Ratio of FD+/FD-cells
Diploid	60	36	60.0%	14	38.8%	22.09% ± 15.58
Haploid*	54	19	35.2%	9	47.4%	39.4% ± 26.3

*Rosy barb eggs fertilized by UV-irradiated Rosy barb sperm.

embryos makes the production of chimeras possible even with the hurdles caused by synchronisation of development of contributing embryos.

Preliminary experiments were done for the optimization of age and developmental stage of donors and recipients for a new cell transplantation method the aggregation (Tables 1 and 2).

Cells of disaggregated 64-128 cell stage embryos continue on with divisions and retain their pluripotency up to a 1000 cell stage, and their ability for aggreagation in the lack of Mg^{2+} and Ca^{2+} ions. During this time disaggregated cells are convenient subjects of any cell manipulation experiments. Utilising the intensive aggregation capability of early morula cells (16-32 cells), these can be used as recipients increasing the chance of efficient incorporation of donor cells and a higher rate of involvement of donor cells in the development of the chimera.

An interesting phenomenon is the actual integration of donor cells which can easily be observed by using the FD labelling. The anchoring of donor cells on the host embryo surface happens almost instantly in the Mg^{2+} and Ca^{2+} containing Holtfreter's solution. As a first indication of stable integration of donor cells in the embryo an involution can be seen, and the migration and spread of these donor cells by development. A few cells do not integrate but remain on the surface of the embryo. These retain their size and position until hatching, while the rest of the donor cells can be seen to differentiate in an even distribution.

Haploid embryos were used as recipient for increasing the proportion of donor cells in the chimera. In our earlier experiments we have observed a 0-60% development to prehatching stage of haploids showeing a slower rate of development than that of diploids. 10-20% may even hatch too, however, these are unable to feed and consequently die. Similar results were gained by Chourrout et al /2/.

Results of diploid/haploid chimera experiments are shown in Table 6. Mean FD+/FD- ratio is almost double as compared to diploid/diploid groups (39.4% as compared to 22.09%). High integration was the result of some individuals showing 70% incorporation into the recipient (max.: 74.4%) significantly increasing the mean FD+/FD- ratio. This result may be due to the selection against embryos with high haploid cell ratio since haploid development is similar to genetic deficiencies. This is well illustrated in Table 6, where number of one day survivors of haploid is only half of similarly treated diploids. The high FD+ chimeras ratio in the haploid-diploid system (47.4%) at 20 somite stage must be the result of the effective aggregation technique and the contribution of high number of diploid cells in these chimeras.

The other possible way of increasing the ratio of the donor cells in the chimera is the use of interspecific hybrids as recipient. Those crosses are the most suitable where the developing embryos develop normally to hatching but are unable to feed or can only reach this stage in very low numbers. According to our preliminary experiments carp eggs fertilized by rosy barb sperm will develop to hatching in 0-15% and a maximum of 10% resulted in developing hybrids. Based on these results we started using this system as a putative candidate for efficient recipient.

The classic injection technique was also used as a comparison (Tables 4 and 5) to the aggregation technique. Interspecific hybrids were used as host embryo in these experiments and at gastrula stage no significant difference was found in the number of FD labelled cells both methods showing approx. 80%. At the time of swim up the difference between the aggregated chimeras and the control recipient survival (3.51 and 2.12 times more) showed an important role of diploid donor cells in chimera embryo development.

The injection technique used on African catfish resulted 77.4% FD labelled embryo containing donor derived cells (average 7.18% FD+/FD- cell ratio) whereas aggregation experiments were completely negative here. At hatching no individuals were found to contain any donor cells by FD labelling, however, spontaneous aggregation between two embryos were found here as well.

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UTILISATION OF POPULATION GENETIC AND MULTIVARIATE METHODS IN THE EVALUATION OF RESULTS OF CATTLE TYPE TRAITS JUDGMENT

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The results of the evaluation of linear type traits evaluation of Hungarian beef cattle populations were analysed by means of both traditional population genetic and multivariate biometric methods, i.e. by means of cluster analysis based on factor analysis. By using the multivariate methods it was possible to evaluate the different types of cattle with respect to type traits, reproductivity and productivity properties.

It is proven by the results obtained that factor analysis is practicable in the judgement at the population level of the individual properties. A great number of type traits — 27 or more — making an interrelating system can be analysed well by this method, whilst the cluster analysis based on the factor analysis is suitable to classify the population on the basis of individual evaluation with the simultaneous consideration of all type traits.

Keywords: Beef cattle — limousine breed — linear type traits — heritability — multivariate methods

Introduction

The secondary (non-productive) traits of beef cattle determine basically the profitability of production, so their improvement is important for the elaboration of the domestic beef cattle breeding programmes /1—3/.

The investigations concentrated on the analysis of linear type scores, as the judgment of conformation plays an important role in beef cattle breeding programmes. In this context, we also investigated the reproductivity of the individual animals and the beef producing ability of the mother cows and their bull progeny.

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In the framework of this investigation we produced the data on linear type traits (27 or more traits) by common evaluation methods. In addition, we also intended to get an overview of the complexity of this huge number of type traits at population level, so that at the next stage we could perform the grouping of the members of the population on an individual evaluation basis, by taking into account all the important type traits simultaneously and with adequate emphases.

Materials and Methods

In the methodological investigations the secondary (non-productional) type traits of the Hungarian limousine herds were examined. Reproductivity and linear type traits were evaluated with special attention because they determine the production of beef cattle herds decisively /6/.

In order to evaluate the reproductivity, the Cow Utilisation Intensity Index /4, 7/ was applied. The proposed index is a simple relative key number which was revealed as a quite a useful one in the course of the genetic investigations. When calculating the Index, the actual age of the cows related to the given number calving is compared to the effective standard age.

The formula of the Cow Utilisation Intensity Index calculation is thus:

$$I_V = \frac{\text{Standard age of cow at calving determined by number of calving (in days)}}{\text{Actual age of cow at the calving in question (in days)}} \times 100$$

By using the index the reproductivity is evaluated from birth to the last calving.

The type traits of the cows were judged by means of the French beef cattle linear judgment system. By the application of the linear judgment system the properties important for the production can be improved. Those properties are called functional (type) traits. The Hungarian judgment system classifies the type traits in four main property groups, i.e. utility value, length and width parameters, muscularity, by the application of scores ranging between 1 and 9.

The heritability of type traits was estimated by the method of one factor variance analysis.

Factor analysis was used to analyse the population level interrelationship system for the great number of type traits which are included in the linear type traits judgment system, while cluster analysis based on the factor analysis was used to classify the individual values, taking into account all the important type traits simultaneously.

The correlation system of numerous conformation traits was specified by factor analysis. Then the role and importance of the conformation trait components were revealed at population level and the population was clustered on the basis of type traits by simultaneous evaluation of individual conformation traits /6/.

Since the factor analysis clustered 27 conformation traits into independent background variables (factors), the Euclidean distance matrix of the individuals was determined by the multidimensional generalisation of the formula of Pythagoras (chosen from the similarity functions of the cluster

analysis) setting out from the factor — value matrix (C). From the distance matrix the clustering of the animals was carried out using the centroid fusional algorithm.

Results

The linear type traits judgment results available for productive cows (n = 286) in the Hungarian limousine populations including 27 properties of the four type trait groups and the cow utilisation indices characterising the reproductivity of cows, the live weight of the bull calves at different ages and the fenotypic correlations concerning them were analysed as a first step.

In the case of the investigated cow populations, a relatively low but negative correlation was found (r = 0.193) between the final trait score and reproductivity of the cows (Index of Cow Utilisation Intensity).

Based on the analysis of relationships of the type trait groups it can be stated that positive correlation can be experienced almost in every case between the reproductivity and the utility value properties and between the reproductivity and the length dimensions (r = 0.067—0.378). The results demonstrate that in the utilisation of the modern type beef cattle (longer rump and limbs, flatter chest, bigger frame) the reproductivity of the cows improves slightly. It is important to emphasize that between the reproductivity and the width measurements, as well as the properties relating to muscularity, negative correlations can be revealed /r = (-0.174) - (-0.0207), and r = (-0.151) - (-0.227)/.

A negative correlation /r = (-0.103) - (-0.124)/ was experienced between the intensity of cow utilisation and the live weight at different ages (at calving, weaning live weight corrected to 205 days and at one year of age). This result puts a stress on the importance of the progeny testing of beef sires based on the reproductive performance of their daughters.

Most properties included in the judgment have good heritability. In the high number of the Hungarian limousine herds, the heritability was determined by means of the variance analysis of the half-sibs and the results are as follows (see also Tables 1 and 2):

utility value: h^2 = 0.36—0.78
length measurements: h^2 = 0.09—0.70
width measurements: h^2 = 0.46—0.78
muscularity: h^2 = 0.36—0.76

Table 1

Heritability of linear type traits

Utility value	h^2	Length measurements	h^2
Withers height	0.78	Body	0.70
Chest floor depth	0.63	Back	0.39
Shoulder attachment	0.39	Loin	0.20
Chine and loin attachment	0.54	Rump	0.087
Bone fineness	0.34		
Total score of utility value	0.73	Total score of length measurement	0.65

Table 2

Heritability of linear type traits

Width measurements	h^2	Muscularity	h^2
Withers and back	0.74	Brisket	0.74
Chest	0.78	Bladebone, shoulder	0.71
Loin	0.54	Chine, loin	0.44
Rump I	0.46	Rump	0.76
Rump II	0.59	Thigh length	0.36
Rump III	0.59	Thigh roundness	0.56
Total score of width measurements	0.73	Total score of muscularity	0.77

In beef cattle production, factor analysis was used to analyse the type traits interrelationships in the judgment scheme of linear type traits, as well as to judge the population level role of the individual properties.

After factor analysis i.e. the analysis of correlation relationships and the structures of the factors, the factor weights and the main background variables were determined. One can classify the examined variables, i.e. type traits according to two factors as I and II (Fig. 1).

The type traits corresponding to factor groups I and II are obtained on the basis of the C(A) matrix and are found in the first and the fourth quarter of the system formed by the factors.

There are two large groups of the linear type traits, an even property and a separate variable in the first quarter of the system of co-ordinates. In the large group of traits, the length measurements (traits 2-12) and

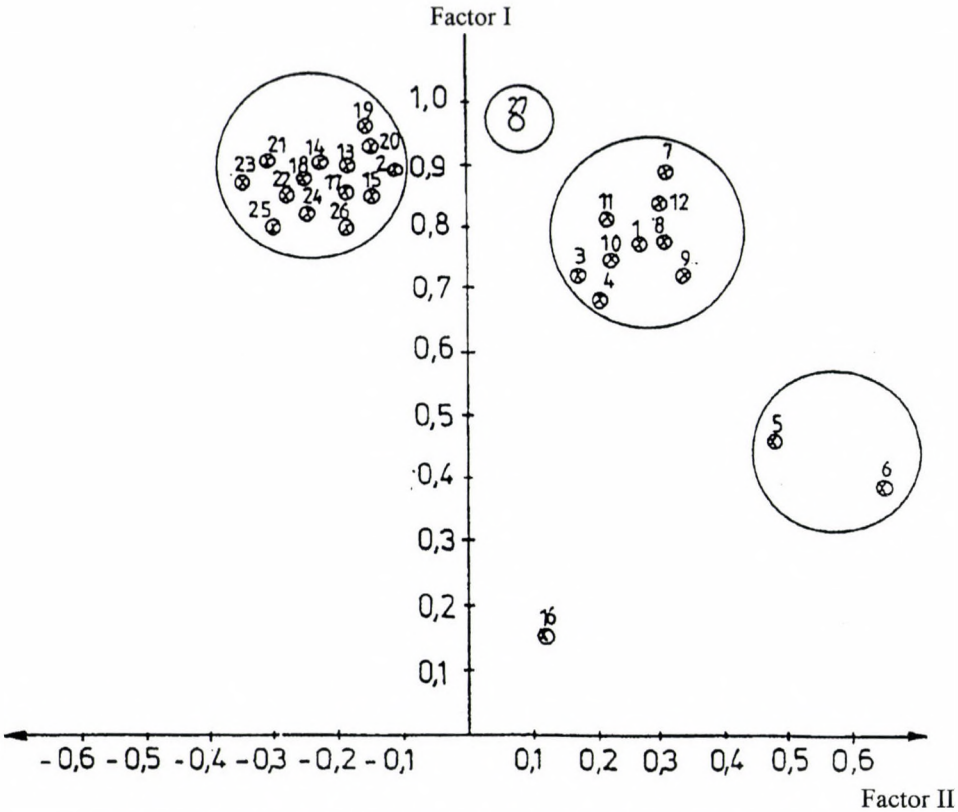


Fig. 1. Position of the conformation traits in the field of factors I and II on the basis of matrix C(A) of the factor analysis
(Analysis of the relations among the examined traits and trait groups by factor analysis)

Traits considered (see Fig. 1)

Utility value:

1. Withers height
2. Chest floor depth
3. Shoulder attachment
4. Chine and loin attachment
5. Leg and foot
6. Bone fineness
7. Total score of utility value

Length measurements:

8. Body
9. Back
10. Loin
11. Rump
12. Total score of length measurement

Width measurements:

13. Withers and back
14. Chest
15. Loin
16. Rump I
17. Rump II
18. Rump III
19. Total score of width measurements

Muscularity:

20. Brisket
21. Bladebone, shoulder
22. Chine, loin
23. Rump
24. Thigh length
25. Thigh roundness
26. Total score of muscularity
27. FINAL SCORE

Table 3

Body weights of cows and their bull progeny according to cluster groups

Cluster group	Number of animals	Live weight of cows (kg)	Body weight (kg) of growing bulls at birth	at yearling age	Cow Utilisation Intensity Index (%)
I.	36	577.1 ± 9.0	35.2 ± 0.6	414 ± 13	89.0 ± 2.1
II.	74	620.8 ± 5.8	36.3 ± 0.5	457 ± 9	88.0 ± 0.9
III.	35	643.8 ± 8.6	36.7 ± 0.8	501 ± 13	89.8 ± 1.8
IV.	50	678.7 ± 13.4	37.8 ± 1.0	500 ± 12	88.2 ± 1.8
F-value		12.379***	1.075	4.161**	0.634

the majority of the utility value properties (traits 1, 3, 4 and 7) are included. Separate even properties are the leg and foot structure and the bone fineness (properties 5 and 6) as well as the final type trait score (trait 27) variable. The latter has the greatest factor weight on the basis of factor I (Fig. 1).

In the fourth quarter of the system of co-ordinates, one can find the width (properties 13-15 and 17-19) and muscularity measurements (properties 20-26) as groups, except the rump I measurement (property 16), whilst the chest width is found here, too (property 2).

The cluster analysis started from the C matrix of the factor weights with the simultaneous consideration of all factorised type traits.

Four cluster groups were separable by means of the cluster analysis. In short, it can be stated that most linear type traits with the lowest scores belong to the first cluster group, while the highest score individuals are in the group IV. In order to check the clustering, a biometric examination of same cluster group individuals was carried out. Table 3 gives clear evidence of how successful the separation was.

In the cluster analysis made merely on the basis of the type traits, four cow groups were formed which were considerably different in other evaluation properties. The average live weight of the cows was between 5.77—678.7 kg by cluster groups (variance: 101.6 kg), the weight of their bull calves was 35.2—37.8 kg at birth and 413—501 kg at one year of age.

Discussion and Conclusions

The selection of beef cattle on the basis of total type score contributes to the cows' longer productive life. This has been shown by the moderately positive correlation of $r = 0.193$ between total type score and reproductive performance (Cow Utilisation Intensity Index). When selecting on the basis of conformation, it has to be taken into account that the relationships of the reproductive performance traits correlate positively with some type traits or trait complexes (e.g. utility traits, length measurements) while they correlate negatively with others (e.g. width measurements and some muscularity traits).

The loose but negative correlations found between the cows' body weight and reproductive performance on one hand, and the body weights of these cows' bull progeny at different ages on the other hand, suggest that the cows' reproduction has to be considered during selection for higher meat yields in order to prevent contraselection. When improving the type of the limousine breed, it is necessary to increase the average body length and withers' height of the breeding animals. In experiments, the average growth rate and yearling and finishing body weight (right before slaughter) of the breeding animals of a modern type i.e. long body and elongated extremities, were more favourable than the parameters of those breeding animals representing the old type of the limousine breed.

Effective type forming is well supported by the good heritability of type traits in beef cattle.

The evaluation of the linear type scores by multivariable methods proves that, similarly to the situation in France, the domestic purebred limousine cattle can be classified into 3 productive groups (terminal type, breeder type and mixed type) in addition to the group of cows only used for crossbreeding purposes. The distribution of the populations according to productive types may serve pure breeding efficiently in the multilevel system of breeding value estimation.

The biometrical investigations have given evidence that the method of factor analysis is well applicable for the complex analysis of the recently used type judgment systems and for the evaluation of the importance of the individual traits at population level.

Moreover, the method of cluster analysis based on factor analysis is a very efficient tool for grouping the animals on the basis of their individual characteristics, paying attention to every important type trait simultaneously.

Acknowledgement

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CHANGES IN ALKYLATION DAMAGE REMOVAL DURING IN VITRO NEURONAL DIFFERENTIATION

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Mouse teratocarcinoma cell lines allow the analysis of very early commitment and differentiation events, that are likely to be similar to those operating in the early mouse embryo. We have previously characterised the excision repair capabilities of these cells after ultraviolet light irradiation and found that differentiation is accompanied by reduction of excision repair. In the present study we examine the operation of an other DNA repair pathway participating in the removal of alkylation damage. O^6-alkylguanine-DNA alkyltransferase (ATase) activity was determined in undifferentiated and differentiated mouse P19 teratocarcinoma cell line. To obtain more information about regulation of ATase we transfected P19 cells with constructs harbouring a human ATase cDNA driven by a housekeeping promoter.

Keywords: DNA repair — O^6-alkylguanine-DNA alkyltransferase — in vitro neuronal differentiation

Introduction

The eukaryotic DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase can modulate the carcinogenecity and/or mutagenecity of alkylating agents both in vivo and in vitro /9/. It transfers methyl group from O^6-methylguanine (O^6mG) to a cystein acceptor residue in its active center by a suicidal mechanism, leading to restoration of guanine /5/. Considerable cell and tissue specific differences exist in both the levels of DNA damage produced by the alkylating agents and in the ability to repair such lesions. Direct correlation exists in vivo between the tumour locations and the level of ATase in that particular tissue /6/. Significant reduction was observed in the ATase level in differentiating brain tissue and this low level of activity coincided with the high induced rate of tumours by alkylating agents

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/1/. Beside these well characterised phenomena, little is known about the molecular mechanisms which underlie these modifications.

Mouse teratocarcinoma cells are excellent in vitro models for early embryogenesis, since they can be induced to differentiate into a particular path and allow analysis of very early commitment and differentiation events that are likely to be analogous to processes operating in the early mammalian embryo /8/. In this study in order to learn more about the regulatory changes of ATase during embryogenesis of brain tissue P19 embryocarcinoma cells, which can be induced to differentiate to neuronal direction, its differentiated derivatives and transfectants were analysed.

Materials and Methods

Cell culture and differentiation

P19 mouse teratocarcinoma cells and the transfectants were cultured and differentiated as described /7/.

ATase activity

ATase activity was determined by measuring the transfer of ^{3}H label from $^{3}HMNU$ treated substrate to cellular protein extracts according to the standard protocol /4/.

Electroporation protocol

P19 cells were electroporated with linearised construct harbouring a truncated human ATase cDNA /2/ as described /7/.

Cell survival assays

Cells from exponentially growing cultures were counted and treated with methylnitrosourea (MNU), or mitozolomide (MTZ) (2 hours with MNU, 5 hours with MTZ). After 2-3 weeks colonies were fixed, stained and scored.

Molecular biological analyses

A 650 bp human ATase cDNA was used as a probe in the Southern, Northern blot analyses and rat GAPDH and mouse NF-M probes were additionally used for hybridizations of the Northern filters. The copy number of the transfected human ATase fragment in the transfectants was determined by applying dot blot hybridization of diluted samples with the ATase probe. Calculation was made by comparing the signal intensities of the samples to purified diluted human ATase cDNA.

Effect of O^6-methylguanine on ATase activity in cellular extracts in vitro

Extracts from undifferentiated and differentiated P19 transfectant (3/20) were incubated with different concentrations of O^6mG for 2 hours. The remaining ATase activity was then measured as described /5/.

Results

Basal level of ATase activity in P19 cells and its transfectants during in vitro differentiation

As can be seen in Fig. 1, ATase activities in undifferentiated transfectants of P19 cells, which harboured the human cDNA construct are almost 3-fold higher than that in the P19 parental line, where the activity is almost as low as in the adult brain tissue of the mouse (Fig. 2). This low endogenous ATase activity in the P19 cells was the reason why the transformation experiment was decided. By the electroporation of human ATase constructs where the ATase cDNA was under the control of a constitutive promoter, which shows no changes in expression during differentiation a situation could be created where only the translational or posttranslational regulatory changes of ATase could be studied. Interestingly enough the ATase activity decreased both in differentiated P19 cells and in the differenti-

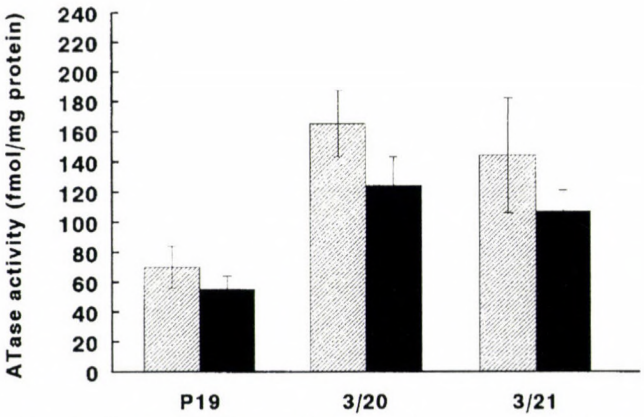


Fig. 1. Changes of ATase activities during neuronal differentiation of P19 mouse teratocarcinoma cells and two transfectant P19 cell lines. Activities of undifferentiated (hatched columns) or differentiated (filled columns) extracts are the means of four independent determinations

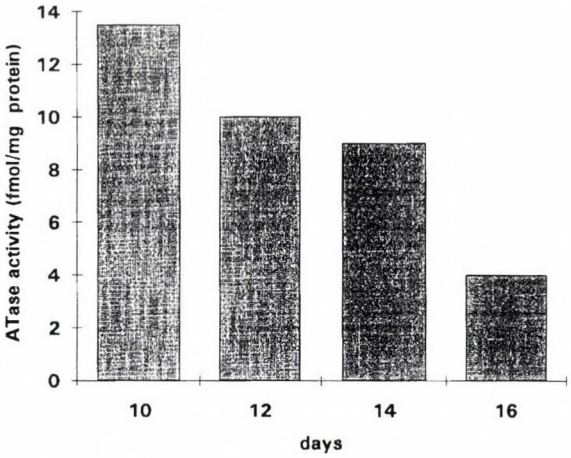


Fig. 2. ATase activities of the developing mouse brain. The columns represent an average of duplicate measurements

ated, transfected colonies similarly to the previously published results of enzyme levels in differentiating brain /1/ (Fig. 1).

Basal ATase activities in the transfectants did not correlate with the copy numbers of the transfected human ATase cDNA (Fig. 3). The highest activity was detected in the 3/20 transfectant which harbours 12 copies.

Effect of simple alkylating agents on P19 cells and transfectants

In order to see whether the difference in the basic activity of ATase in P19 and transfectant cells is also reflected in the resistance to certain simple alkylating agents, survival curve analysis had been carried out. Figure 4 presents survival data of parental P19 cells and one of the charac-

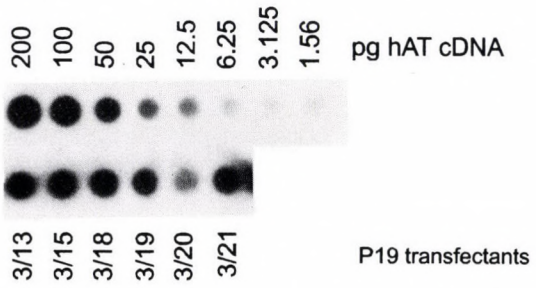


Fig. 3. Human ATase cDNA copy numbers in transfected P19 cells

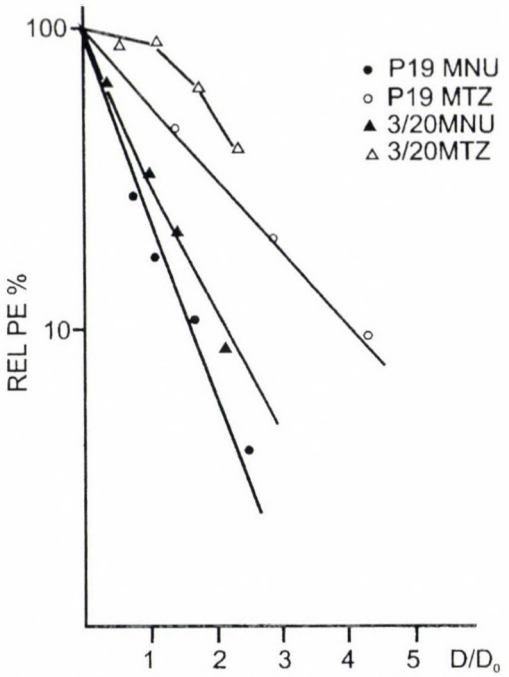


Fig. 4. Single cell survival curves of P19 (o) and 3/20 (Δ) transfectant cells to MTZ (open symbols) and MNU (closed symbols)

teristic transfectant, 3/20. Both in the case of MNU and MTZ a more resistant phenotype could be observed with 3/20 than with P19 cells.

Molecular changes of ATase expression in differentiating cultures

For the better molecular understanding of the reasons for the decrease of the ATase activities during differentiation Northern and Western analysis of the parental P19 and its transfected derivatives had been performed. As Fig. 5 shows there were no significant differences (even with densitometric quantification) in the mRNA and ATase protein levels between undifferentiated and differentiated cells.

In vitro reactions of ATase with O^6mG

Since the molecular analysis did not give any explanation for the decrease of ATase activity in differentiating cells we turned our attention to the study of the enzymatic characteristics of ATase in embryocarcinoma and

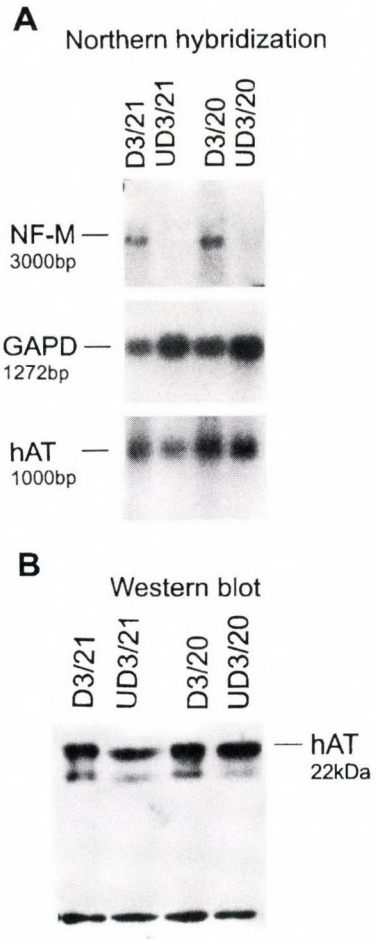


Fig. 5. ATase mRNA and protein levels of P19 transfectants

their differentiated nerve cell derivatives. Figure 6 shows that the in vitro sensitivity of ATase to O^6mG in extracts from undifferentiated transfectant 3/20 is higher than that form the differentiated cells.

Discussion

Endogenous ATase activities have been reported to undergo species, tissue and cell type-specific variations /6/. These variations could be traced in in vitro differentiating cells too, as it was shown for rat hepatoma cells /3/ and mouse neuronal cells (this publication and Czibula et al., manuscript in preparation). The changes of ATase activity in the in vitro situation are similar to those found during organogenesis. These dif-

ferentiation-dependent changes in the repair of O^6mG in the alkylation damaged DNA are not in concordance with the base excision repair pathway, the other major pathway of repair of the damage induced by monofunctional alkylating agents /3, Czibula et al., manuscript in preparation/. While in rat hepatocytes both the ATase mRNA and protein levels increased together with the increased ATase activity /3/, in the differentiating P19 transfectants these characteristics were not found. This suggested that the reason for the reduction of ATase activity in these cells could be the result of posttranslational modification of the enzyme. This speculation was indirectly supported by the in vitro data obtained with O^6mG treated cellular extracts (Fig. 6). According to our explanation the ATase protein from undifferentiated cultures is able to change conformation after treatment with O^6mG, in the differentiated extract, however though the enzyme protein is present, due to the posttranslational modification the activation is not possible and this is reflected in the virtual decrease of ATase activity in the differentiated cultures and in the mouse brain during ontogenesis.

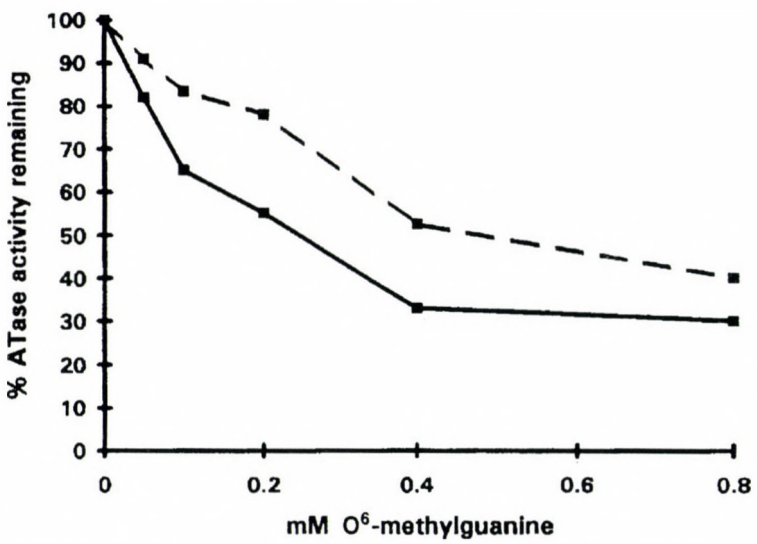


Fig. 6. The effect of O^6-methylguanine on the in vitro inactivation of ATase in extracts from undifferentiated (solid line) and differentiated (dashed line) 3/20 transfectant cells

Acknowledgements

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MOLECULAR GENETIC STUDIES IN MONOGENIC AND POLYGENIC HUMAN DISEASES

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The main goal of this study was to determine and characterise the types of mutations in two monogenic human disorders: cystic fibrosis (CF) and Duchenne/Becker muscular dystrophy (DMD, BMD) and the susceptibility allele frequency in a polygenic disease: type I insulin-dependent diabetes mellitus (IDDM). After analysing 220 chromosomes for mutations in the CF (Cystic Fibrosis Transmembrane Conductance Regulator = = CFTR) gene, ΔF_{508} mutation was most abundant (41%) and out of the non-ΔF_{508} CF mutations 5% was identified as $G_{542}X$, $G_{551}D$, $R_{553}X$, $N_{1303}K$ and $W_{1282}X$. The CF haplotype analysis by using linked markers to the CFTR gene revealed that the CF "B" haplotype occurred in 66.7% of patients, and this haplotype was 57.2% in patients carrying the ΔF_{508} mutation. Prenatal genetic diagnosis for CF was performed in 10 fetuses: 3 were affected, 6 were carriers, and 1 without any CF mutation.

Fifty % of 66 patients with DMB/BMD muscular dystrophy had one or more exon deletions in the dystrophin gene. Eighty-five % of the deletions occurred at the 3' and 15% at the 5' end of the gene. Out of the three prenatal diagnosis in one case DMD was substantiated.

Thirty-six % of 50 patients with IDDM possessed four, 44% three and 20% two susceptibility markers in the HLA-DQA1, -DQB1 region. The onset of the disease correlated with the number of susceptibility alleles.

Keywords: Molecular genetic diagnosis — prognose — carrier detection — prenatal diagnosis

Introduction

Cystic fibrosis (CF) is the most common autosomal recessive single gene (7q31) lethal disorder, having 1 in 32 carrier frequency and 1 in 4000 newborn incidence in the Hungarian population /9/. The cloning of the CFTR gene /10, 21, 22/ made possible the characterisation of the mutations. It

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was found that a deletion of three nucleotides (CTT) results an omission of a phenylalanine at position 508 from the cystic fibrosis transmembrane conductance regulator (CFTR) protein (ΔF_{508} mutation). The overall proportion of ΔF_{508} in a worldwide survey was 68% with a range of 30-80% /24/. The number of other CF mutations is very high /27/ so in some cases the closely linked markers (J3.11, XV-2c, KM-19) would give marker information from proposites and parents /1/. The frequency of $G_{542}X$, $G_{551}D$, $R_{553}X$, $N_{1303}K$ and $W_{1282}X$ mutations is 1-5% in other populations /18/.

The Duchenne and its milder allelic form Becker muscular dystrophy (DMD, BMD) are X-chromosome-linked (Xp21) recessive, lethal muscle wasting disorders affecting approx. 1 of 4000 male newborns (DMM). The 2.5 million base pair gene consists of more than 70 exons and has high mutation rate. In one third of all patients the disease is the result of new mutations. About 65% of patients possess deletions of exons within the gene. By the use of 3 multiple polymerase chain reaction the most frequently deleted 18 exons (3, 4, 6, 8, 12, 13, 17, 19, 43—45, 47—52, 60) and the muscle specific promoter could be detected /2, 4/. We planned to screen our 66 DMD/BMD patients to know the frequency of deletion prone exons in our population and to help genetic counselling and prenatal diagnosis.

Type I (insulin dependent) diabetes mellitus (IDDM) is a complex, polygenic, multifactorial autoimmune disease. The main pathological feature is the selective destruction of the insulin producing β cells in the pancreas. IDDM has a strong association to haplotypes of the HLA class II region, particularly with alleles of the DR_β (DRB1) and DQ_α (DQA1), DQ_β (DQB1) subregions /11, 20, 26/. We analysed the DRB1, DQA1, DQB1 allele frequencies in 50 Hungarian IDDM affected children in order to further characterise the geographic variation of the susceptibility alleles in European population subgroups.

Patients and Methods

1/a. Analysis of ΔF_{508} mutation in 110 cystic fibrosis affected patients has been carried out by the application of polymerase chain reaction (PCR) /15/.

1/b. $G_{542}X$, $G_{551}D$, $R_{553}X$, $N_{1303}K$ and $W_{1282}X$ mutations in 32 non ΔF_{508} hetero- and homozygotic patients were detected in collaboration with Dr. Krisztina Németh, II. Pediatric Clinic, Budapest and Dr. Annamária Stenger, Genova, as described by Ober et al. /18/.

1/c. In 40 CF patients and their parents the CF haplotype analysis was carried out with XV-2c/Taq I and KM-19/Pst I RFLP after PCR amplification /6, 23/.

1/d. Prenatal diagnosis was performed in 10 fetuses from DNA, isolated from chorionic villi samples.

2. Deletion analysis of 66 DMB/BMD patients and prenatal diagnosis of 3 fetuses was carried out with multiple polymerase chain reaction as described by Beggs et al. /2/ and Chamberlain et al. /4/.

3. Fifty IDDM affected patients were tested for HLA-DRB1, -DQA1, -DQB1 genotype as previously published /16, 19, 28/.

Results

1/a. The distribution of ΔF_{508} mutation and other mutations of 110 CF affected patients was:

$\Delta F_{508}/\Delta F_{508}$ = 30 patients
$\Delta F_{508}/non\Delta F_{508}$ = 31 patients
$non\Delta F_{508}/non\Delta F_{508}$ = 49 patients

The frequency of ΔF_{508} mutation on 220 CF chromosomes was: 41%, this is in the range of other populations reported previously (30-80%) /24/.

1/b. The frequency of $G_{542}X$, $G_{551}D$, $R_{553}X$, $N_{1303}K$ and $W_{1282}X$ mutations is 5% on 101 $non\Delta F_{508}$ CF chromosomes.

1/c. The CF "B" haplotype (without Taq I restriction site on XV-2c sequence and with Pst I restriction site on the KM-19 marker) on CF chromosomes was detected in 66.7%, as found by Németi et al. /17/ in their examinations.

The haplotype "B" occurred in 57.2% of CF chromosomes carrying the ΔF_{508} mutation. This is lower, than that reported by Lemna et al. (96%) /14/.

1/d. Prenatal diagnosis in 10 fetuses revealed 7 healthy (6 heterozygotes and 1 without any CF mutation) and 3 CF affected ones.

2. Figure 1 shows that 33 of 66 patients with DMB/BMD muscular dystrophy were found to have one or more exon deletions (50%). Five patients' deletions were extended toward the 5' end of the gene (15% of the deletions), while 28 patients' deletions (85% of all of the deletions) were extended toward the 3' end. Three (9%) BMD patients were found in the deletion groups, versus 10 (30%) in the non-deletion group. There was a correlation between the geno- and phenotype (BMD phenotypes with in frame deletions) /12/. Three prenatal diagnoses were performed in DMD affected families. Only one male fetus of an exon deletion patient's sister was found to have DMD.

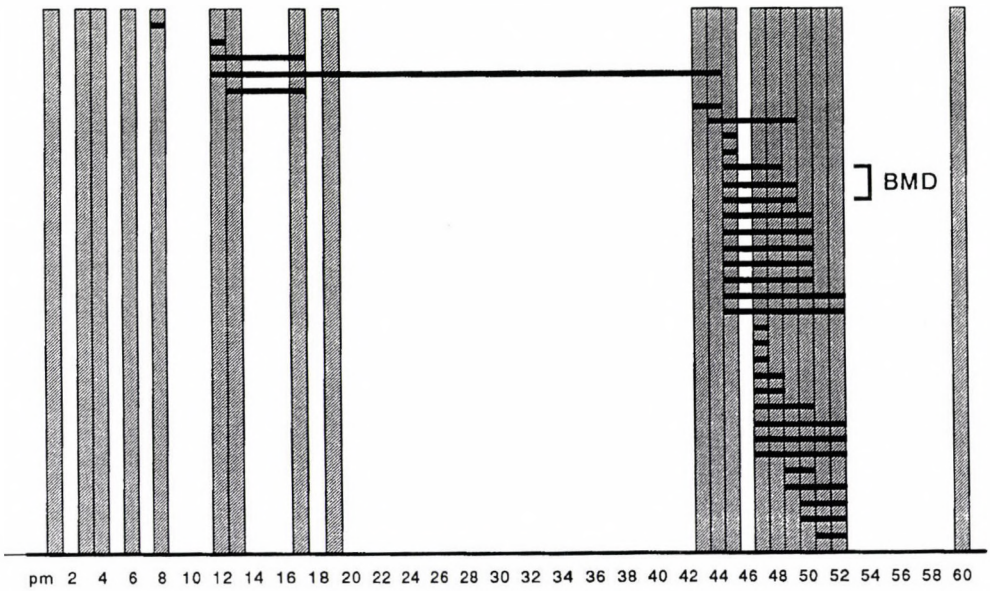


Fig. 1. Muscle specific promoter (pm) and 18 exon deletion examinations of the dystrophin gene (Xp21) in 33 Duchenne (DMD) and milder allelic Becker (BMD) muscular dystrophic patients

3. In 50 IDDM affected children the 0301 and 0501 DQA1 alleles (having arginine at position 52 of the protein) were detected with significantly higher frequency in the patients ($p < 0.001$ at both alleles) than in the controls.

The 0201 and 0302 DQB1 alleles (having no aspartic acid at position 57 of the protein) proved to be "susceptible alleles" ($p < 0.01$; $p < 0.001$, respectively) in the patients compared to the controls.

The constitution of the DQA1/DQB1 haplotypes in the diabetic group correlated with the onset of the disease. In the "early onset" group (onset under the age of 8) in 12 out of 15 patients 3 or 4 susceptibility alleles constituted the heterodimer (47% of the patients of this group harboured 4 and 33% 3 susceptibility alleles), whereas in the "late onset" group 28 from 35 patients possessed this characteristics (31% carried 4 and 49% 3 susceptibility alleles) (Table 1).

Also significantly higher DR3 and DR4 allele frequencies were found in IDDM affected children, as compared to the controls ($p < 0.001$).

Table 1

Distribution of patients with different number of susceptibility loci according to the age of onset of the IDDM (n: nonsusceptible, s: susceptible DQ α/β alleles)

	Onset	DQ α/β heterodimer	n	%
I.	< 8 years	4 (ssss)	7	47
	n = 15	3 (nsss)	5	33
		2 (nnss)	3	20
II.	> 8 years	4 (ssss)	11	31
	n = 35	3 (nsss)	**17**	**49**
		2 (nnss)	7	20
III.	Summary	4 (ssss)	18	36
	n = 50	3 (nsss)	22	44
		2 (nnss)	10	20

Discussion

In order to characterise the distribution of mutations of two monogenic, and that of the susceptibility alleles in one complex human disease, molecular genetic analysis was performed.

As far as cystic fibrosis was concerned the frequency of the most abundant mutation (ΔF_{508}) in our study was 41%. This is lower than that published by Fekete et al. in a previous study (52%) /5/.

The distribution of CF haplotypes showed that 57.2% of the patients carrying the ΔF_{508} mutation belonged to the "B" CF haplotype (disagreement

with Lemna et al. /14/), while 66.7% of all CF patients had this haplotype. This latter is in agreement with previously published data of Németi et al. /17/. The value of the prenatal diagnosis for CF has recently been argued, because the available antibiotic treatment significantly prolongs the life expectancy of the patients. There were 3 fetuses detected to have CF. In these cases the parents decided to have abortion.

The frequency of DMD and BMD patients with deletion was 50%. This number is lower than the previously published data /2/. Unfortunately, at present there is no adequate therapy for these muscular dystrophies, but there is hope that the method of myoblast transplantation at least will slow down the progression of this devastating disease /13/. Therefore in these cases the value of prenatal genetic diagnosis is great to prevent the disease.

Type I insulin dependent diabetes mellitus is a multifactorial autoimmune disease. In our study it has been found that, as in the case of other Caucasian populations, in the Hungarian patients there is also a strong association between the presence of DQA1 0301, 0501; DQB1 0201, 0302 alleles and the disease. Lower number of patients carried all four susceptibility alleles (36%), this is lower than that for patients reported from Belgium (47.7%), France (53.9%), Spain (64.7%) and it is similar to the Italian data (35.3%) /3, 8, 25/.

Our data on the significance of the number of susceptibility alleles and the onset of disease are in agreement with the previously published results /7/.

Our results offer a molecular-biological approach to a better understanding of autoimmune mechanism of IDDM and might involve a possibility for the development of preventive therapy. These investigations may contribute to a more exact and differentiated diagnosis of diabetes mellitus, providing a useful tool for the screening and follow up of children at risk.

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BOOK REVIEWS

DIET AND CANCER: MOLECULAR MECHANISM OF INTERACTIONS

Edited by American Institute for Cancer Research.
Washington, D.C., 1995, pp. 220

The fifth annual conference of the American Institute for Cancer Research was held September 1—2, 1994 in Washington. The topic was: Diet and Cancer: Molecular Mechanism of Interactions.

The subtropics for the three sessions were:

- Retinoids, Vitamin A, Vitamin D, Prevention and Therapy,
- Choline and Lipids: Signal Transduction, Gene Expression and Differentiation,
- Dietary Factors and Regulation of Oncogenes, Growth and Differentiation.

The book contains 220 pages, divided into 13 chapters, a section with 54 abstracts and 60 figures.

The authors describe how retinoids may have benefical effect in cancers where viral factor plays a role, what is the role of the Vitamin A in chemoprevention of lung cancer, and how vitamin D appears to lead to growth inhibition in MCF-7 cells and to stimulation of prostate specific antigen, thus indicating potential therapeutic utility in prostate cancer.

Choline-deficiency leads to disturbances of molecular mechanisms, thus promoting hepatocarcinogenesis in rats. In choline-deficient rats, activators of PKC (protein kinase C) is increased. PKC targets many proteins involved in control of gene expression.

Methionine deprivation indicated that the deficiency causes alteration of the C-mic gene. The alterations result in an inbalance of the production of C-myc 1 protein, which inhibits cell growth, and of C-myc 2 protein, which is involved in tumorgenesis.

An interesting discussion is that tumor growth was approximately 30% slower in rats on fish-oil rich diet when compared to rats fed a diet rich in sunflower oil.

There is a correlation between dietary factors and colon carcinoma. Certain short-chain fatty acids produced by fermentation of dietary fiber could induce cell differentiation.

In conclusion, dietary factors may be implicated in carcinogenesis. The knowledge of these mechanisms may help in the therapy and prevention of cancer.

The book provides valuable and useful information for those, who would like to know the molecular relationships between certain dietary factors and carcinogenesis.

J. Rigó
(Budapest)

PURINE AND PYRIMIDINE METABOLISM IN MAN VIII

In: ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY, Vol. 370.

Edited by Amrik Sahota (Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis IN 46202) and Milton W. Taylor (Department of Biology, Indiana University Bloomington, IN 47405). Plenum Press, New York and London, pp. 831, with Subject and Author Index

Main Chapters: The Biochemistry of Gout, Diagnosis and Treatment of Inherited Diseases, Gene Therapy, Nucleotide Metabolism and Cancer, Nucleotide Metabolism and Ischemia, Regulation of Endogenous Adenosine Level, Enzymes of Nucleotide Metabolism, Gene Structure and Molecular Biology, Differentiation, Methods

The book records the presentations made at the VIIIth International Symposium on Purine and Pyrimidine Metabolism in Man held at Indiana University, Bloomington, USA, May 1994. These meetings have started at 1973 with the idea of bringing clinicians and basic scientists together, resulted in cross-fertilization of ideas. The papers presented in the volume are dedicated to Dr. H. Anne Simmonds (Purine Research Laboratory, UMDS Guy's Hospital, London, GB), whose contributions to inborn errors of purine and pyrimidine metabolism have been exemplary and outstanding. The number of such disorders has been an exponential increase in the last twenty years, they now total 22, but many are little known in the clinic or laboratory. The emphasis has shifted to gene structure and molecular genetics with the beginnings of gene therapy of Adenosine Deaminase (ADA), the first efforts in this field.

Most of purine and pyrimidine disorders are associated by neurological abnormalities which molecular mechanisms are yet unknown and untreatable. Immunodeficiency symptoms are dominating in Adenosine Deaminase (ADA) and Purine Nucleotide Phosphorylase (PNP) inborn errors. The first human gene therapy has been performed in several ADA deficient children, but all have initially or eventually been given purified enzyme, because the gene was eliminated. The potential to apply knowledge derived from purine and pyrimidine disorders to the more common killers of mankind is a rapidly developing field, particularly in heart and brain diseases, organ transplantation and treatment of malignancies.

Capillary Electrophoresis may become important in diagnosis, because it has a higher sensitivity and a smaller sample requirement than HPLC.

Maria Staub
(Budapest)

TISSUE RENIN-ANGIOTENSIN SYSTEMS

Current Concepts of Local Regulators in Reproductive and Endocrine Organs

Edited by Amal K. Mukhopadhyay and Mohan K. Raizada. — Advances in Experimental Medicine and Biology, Vol. 377. Plenum Press, New York and London, 1995, pp. 458, 120,- USD

The book is a Congress Proceedings of the Satellite Symposium on "Tissue Prorenin-Renin-Angiotensin Systems" hold in Hamburg, Germany, July 15—16, 1994. It is subdivided into three main sections. Section A deals with the more recent results on reproductive endocrinology and biology, while Section B covers recent advances in biology of the renin-angiotensin system. Finally, Section C contains short communications (6-8 pages each) of somewhat more different aspects of the two main subjects.

In Sections A and B, among more general survey papers, specific modern aspects of reproductive biology (e.g. autocrine and paracrine regulations both in females and males, the role of interleukins in the regulation of ovulation), and of the nature and function of the renin-angiotensin system (e.g. angiotensin receptors, the role of the renin-angiotensin system in the female and male reproductive processes) are extensively discussed.

Since the topics of each paper are fairly different, it would be difficult to summarize all the main subjects of this book even in outlines.

Each paper is followed by a detailed bibliography of the recent literature.

It is a pity that some of the papers do not contain any documentation (figures, tables, etc.). Figures and tables are numbered in the individual papers starting always from 1, so it would be hard to estimate the total number of figures and tables, however they are not very abundant.

The design of this hard cover-book is elegant.

This volume might be useful for everybody interested in reproductive endocrinology or in neuroendocrinology. Medical or veterinary doctors, biologist as well as researchers or postgraduate students can benefit by studying this valuable volume.

B. Mess
(Pécs)

FETAL ISLET TRANSPLANTATION

Edited by C. M. Peterson, L. Jovanovic-Peterson and B. Formby.
Plenum Press, New York, 1995, pp. 159, 50,- USD

Diabetes mellitus is a common disease of our age, a growing number of population in all ethnic groups suffers from it. The book provides an outstanding overview of the ethiopathogenesis, genetics and — first of all — the treatment of diabetes mellitus.

Recently many approaches are available for the treatment. This book focuses on a particular one, the islet cell transplantation. In this book both xeno- and isotransplantation of pancreatic cells is introduced. In 15 chapters the reader may be familiar with all relevant technologies, their immunological, genetic and surgical aspects. There is a great challenge today for the transplantation, since due to new compounds and manoeuvres the immunopharmacology of tolerance induction and immunosuppression is rapidly developing.

The editors of the book selected various articles generating a complete summary from both scientific and medical point of view. The reader finds a whole set of methodological approaches including immunological, genetic, molecular biological and pharmacological technologies reflecting the necessity for multidisciplinarity of diabetes mellitus research.

Hungarian readers are provided by domestic experiences in a chapter written by Gyula Farkas.

The authors finally create a chapter including the description of consensus criteria and treatment of the disease.

The book contains fifteen chapters, 67 figures and 31 tables, all are of fair or good quality.

This book provides a good review for all who are interested in pancreatic endocrinology and diabetes mellitus.

A. Falus
(Budapest)

NAGASAKI SYPOSIUM ON CHERNOBYL: UPDATE AND THE FUTURE

Edited by S. Nagataki, Proc. of Nagasaki Symposia on Chernobyl, 1994, Amsterdam, Elsevier, ISBN 0-444-81953-3

International Congress Series Volume 1074

This volume consists of three parts. The first part contains the session of Nagasaki Symposium on Chernobyl with the title Chernobyl Update devoted to the scientific data concerning the health effects of the Chernobyl accident. According to the main goal of the symposium, scientists from the former Soviet Union, the European Community, USA, Japan, as well as experts of WHO discussed and analysed the health consequences of the nuclear accidents trying to exclude influence of any other factors such as psychological, social, economic and political. Experts in radiomedicine and radiobiology, radiobiophysics collected all the data available and summarized them in order to provide more accurate information and to establish scientific basis for the treatment of the victims of nuclear accidents.

The Chernobyl nuclear plant is located in Ukraine, however, the accident in it involved also the people in the Republic Belarus and the Russian Federation. People injured by the accident of the nuclear reactor are to be divided into three groups: 1) several hundreds of people working at the moment of the accident in the station, firemen and others, 2) several thousands or hundred thousands of people who died from late effects of the accident, 3) several millions of people injured by the radioactive fallout. Personal of the power plant and firemen belong to the first group, were exposed to large doses of radiation and many of them died of radiation injury. Second and third groups are affected both by external and internal radiations due to radioactive contamination. The investigated patients are from these two latter groups. Different types of thyroid disorders have been found in these groups as leading health damage induced by the radiation, suffered in 1986. In this part of the book, national specialists (Tsyb et al., Demidchuk et al., Tronko et al.) from all the three republics (Russian Federation, Belarus, Ukraine resp.) report on the 131-J contamination level, estimate the thyroid dose and evaluate the thyroid cancer incidence before and after 1986 in the population affected. A dramatic increase in the cancer incidence, especially among children, has been found in all three countries, however, in rates they differ: in the Russian Federation the attributive risk has been found about 12% in the affected regions, while in Belarus and Ukraine a 4.7 and 6.5-fold increase was detected. In WHO report on the International Programme on the Health Effects of Chernobyl (IPHECA) a survey is given on the aims of the international studies and criteria of the clinical, ultrasound and laboratory findings are described in order to standardize the diagnostics of thyroid cancer. As a further assistance to the correct evaluation of radiation contribution in cancerogenic effects, specialists of thyroid diseases from the United States, United Kingdom, Japan compare the incidence rate, the sex distribution, clinical and histological picture etc. of their own "spontaneous" thyroid cancer cases with those, provoked by radiation in the Chernobyl territory.

Second part of the book, i.e. Chernobyl in the Future, is devoted to establishing a wide international co-operation of the future investigations on the health effects of the Chernobyl accident. WHO's International Programme on the Health Effects of the Chernobyl Accident (IPHECA) contains several research projects, like dose reconstruction, hematology project, epidemiological investigations, study of psycho-social effects and of late effects of radiation, oncological and cytogenetical research, moreover study of the general health status of the population in the contaminated

areas. — The European Community research project relates to the radiation-induced thyroid cancer: to its optimal treatment in childhood and to its molecular epidemiology. United States co-operates in cohort studies of the thyroid disorders intending the reconstruction of the 131-J doses in the thyroid. — Japanes governmental and private organizations co-operate with the specialists of the three republics in several projects assisting them to perform multilateral approaches for detecting and screening disorders, like blood disorders, particularly leukemia, thyroid disorders, moreover to perform measurements and evaluations of radiation dosimetry of the whole body. These contributing organizations and projects are as follows: Chernobyl Sasakawa Health and Medical Co-operation, Radiation Effects Research Foundation, National Institute of Radiological Sciences, Radiation Effect Research Association, Hiroshima International Council for Health Care of Radiation-Exposed, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Nagasaki Association for Hibakusha's Medical Care, Atomic Disease Institute Nagasaki University.

Third part of the book contains the proceedings of the Japan-NIS Chernobyl Thyroid Symposium, held in December, 1993. Papers give detailed reports on the thyroid cancer cases before and after the Chernobyl accident, their ultrasound diagnostics, estimation of the radioactive contamination due to plutonium fallout of atomic bomb in Nagasaki.

This book is in about 250 pages a very important contribution to our knowledge on the health effects of radiation damage. The book is completed with an author index. It may be interesting for a large scale of specialists involved in radiation protection, radiation epidemiology, nuclear medicine, radiation dosimetry, public health, endocrinology. I suggest to study it for my Ph.D. students working and studying in the topics "biologic effects of ionizing and non-ionizing radiations".

György Rontó
(Budapest)

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Acta Biologica Hungarica 48 (2), pp. 135—144 (1997)

SEASONAL DYNAMICS OF THE TISSUE LEVELS OF TOTAL PROTEIN, FREE AMINO ACID AND RIBONUCLEIC ACID IN AN INDIAN AIR-BREATHING TELEOST, CHANNA PUNCTATUS

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(Received: 1995-11-08; accepted: 1996-05-21)

The tropical freshwater air-breathing teleost, Channa punctatus, reveals a seasonal metabolic reorganization with reference to its biochemical tissue compositions. The summer-adapted and winter-adapted C. punctatus seem to be two biochemically distinct populations. Besides, the different reproductive phases viz. pre-spawning, spawning, post-spawning, preparatory-I and preparatory-II are also biochemically distinct.

Keywords: Seasonal — protein — free amino acid — RNA

Introduction

While most of investigations have been conducted on the effects of seasonal variation on the levels of biochemical tissue composition in marine and freshwater fishes of temperate and of polar regions, as summarized by Shulman /16/ and Love /5/, little is known about the tropical freshwater fishes, more particularly about the air-breathing fishes. The Indian air-breathing fishes undergo for summer estivation during the summer period when the ambient temperature enhanced to 38 ^{o}C or more. Whatever reports are available, with regards to biochemical tissue compositions, of Indian air-breathing teleosts /2, 9, 11, 12/, the data recorded have been expressed per unit of tissue wet weight. In the present investigation an attempt has been taken to explore the dynamics of tissue protein, free amino acid and ribonucleic acid i.e. RNA (expressed as per unit of DNA) of the male murrel, Channa punctatus during an annual cycle.

The present investigation has been aimed at answering the following questions:

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(i) Is there a rhythmic annual cycle of alteration of biochemical tissue compositions (expressed per unit of DNA) of C. punctatus?

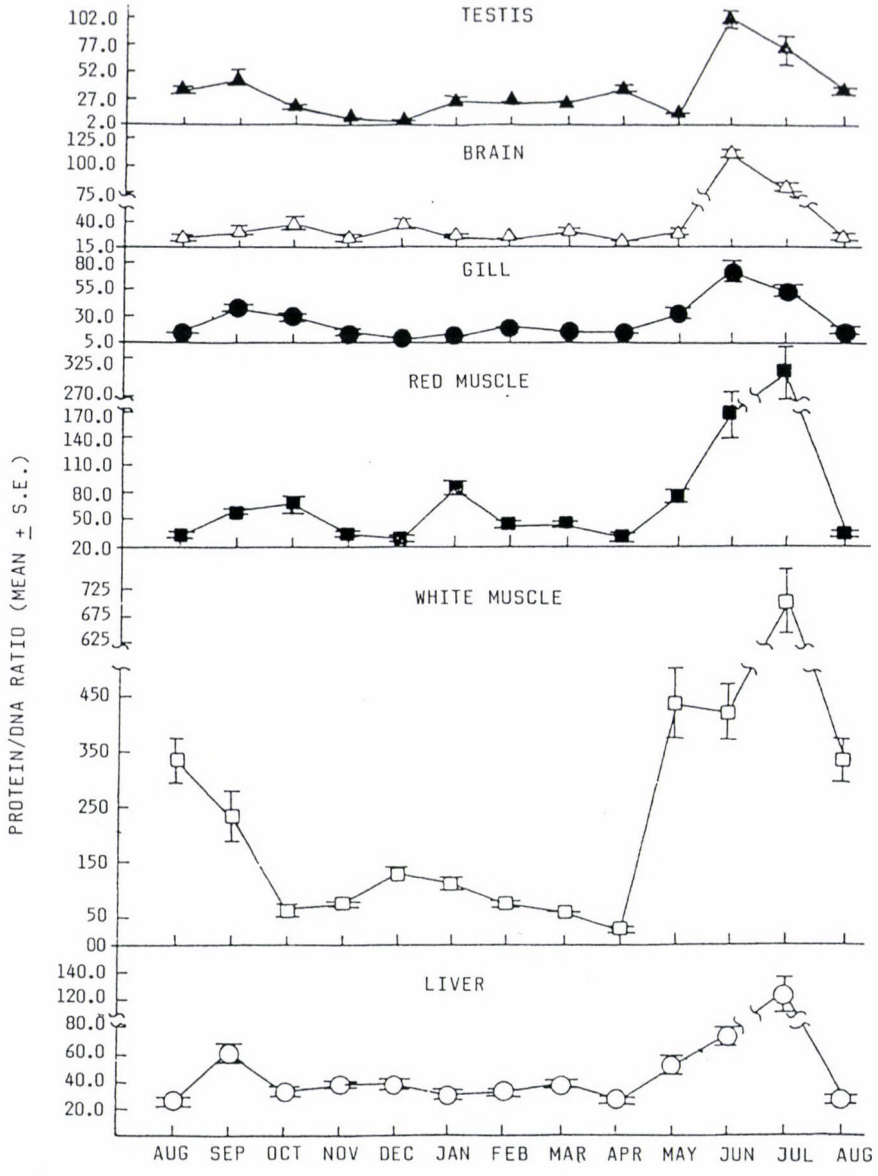
(ii) Can such an annual cycle be broken down into specific periods, which are quantitatively distinct stable states through which populations pass in the course of a year /16/?

Materials and Methods

Male Channa punctatus, of 30.0-40.0 g weight groups were collected during the second week of each month in an annual cycle from the near by villages of Santiniketan, W. Bengal, India. The total protein, free amino acid, RNA and DNA were extracted from the different tissues viz. liver, white and red muscles, gill, brain and testis. The extraction was started with homogenization in 5% ice cold perchloric acid and then subsequent extraction of free amino acids, lipids (not reported here), RNA, DNA and protein as per the experience of Roy et al. /15/. In brief, the free amino acid was collected from the first supernatant after centrifuging at 800 x g for 20 min. at room temperature. Before hydrolyzing the RNA in 0.3 N sodium hydroxide at 37 ^{o}C, all the lipid fractions were washed out with proper organic solvents. DNA was collected by hydrolyzing the residue in 10% perchloric acid at 90 ^{o}C. Finally, the protein was collected and dissolved in 1 N sodium hydroxide. The quantification of total protein was done with folin ciocalteau reagent /6/, of free amino acid with ninhydrin reaction /10/, of RNA with orcinol /8/ and of DNA with diphenylamine reagent /4/ with slight modification as per the experience of Das and Prosser /3/. All the results were statistically analysed (analysis of variance, linear and multiple correlation, "t"-test etc.) with the help of FORTRAN IV computer language.

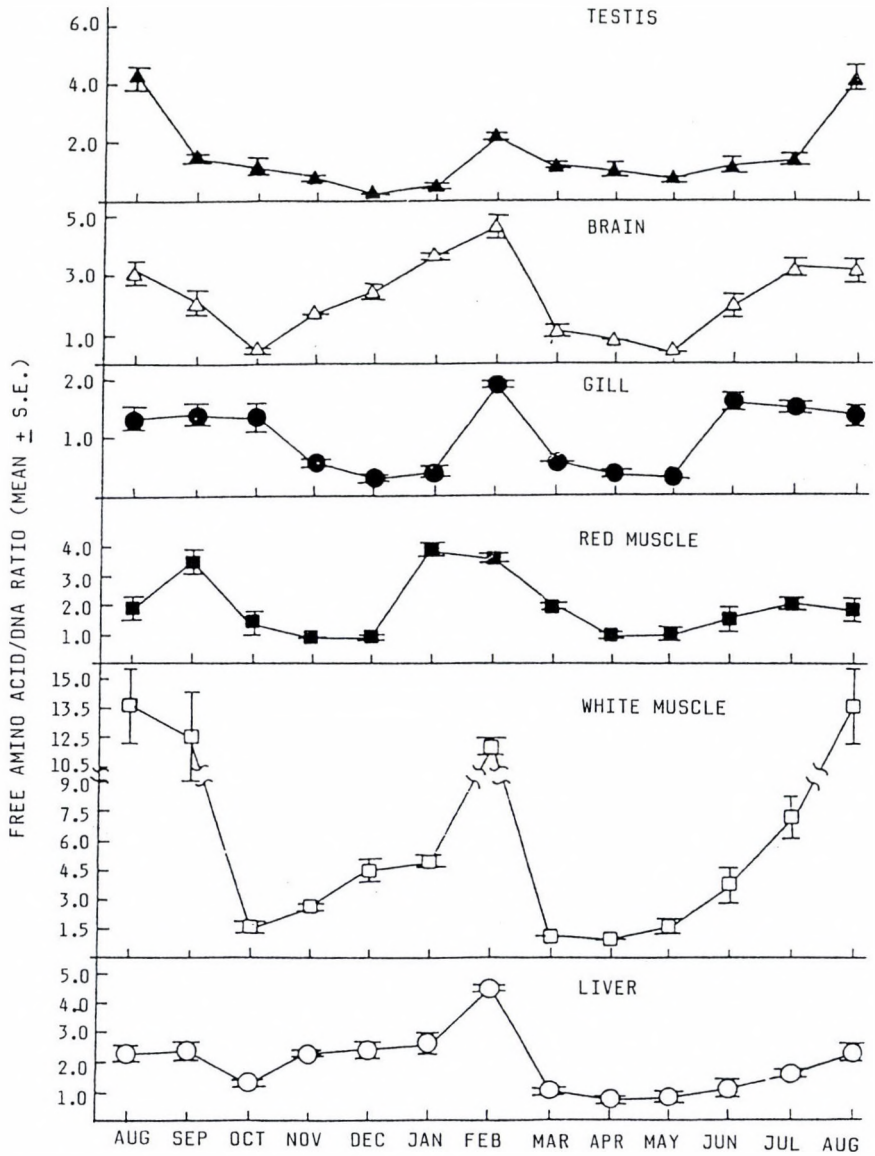
Results

There is a significant alteration of the water content in each tissue of C. punctatus during seasonal variation /2/ but the tissue level of DNA content has been found to be more or less constant (except in the gonadal tissue) during the an annual cycle of this fish (data not shown here). Hence it is misleading to express any biochemical tissue composition with respect to another variable like wet weight of tissue. The data is expressed here with terms of per unit DNA which is almost stable through the annual cycle. Figures 1 to 3 represent the annual (monthly) variation of the value of total protein, free amino acid, RNA (expressed per unit DNA) were statistically significant ($p < 0.001$). There is a positive linear correlation between the total protein content (per unit DNA) and free amino acid concentration (per unit DNA) in white muscle, gill and testis and between total protein concentration (per unit DNA) and RNA content (per unit DNA) in red muscle and testis.



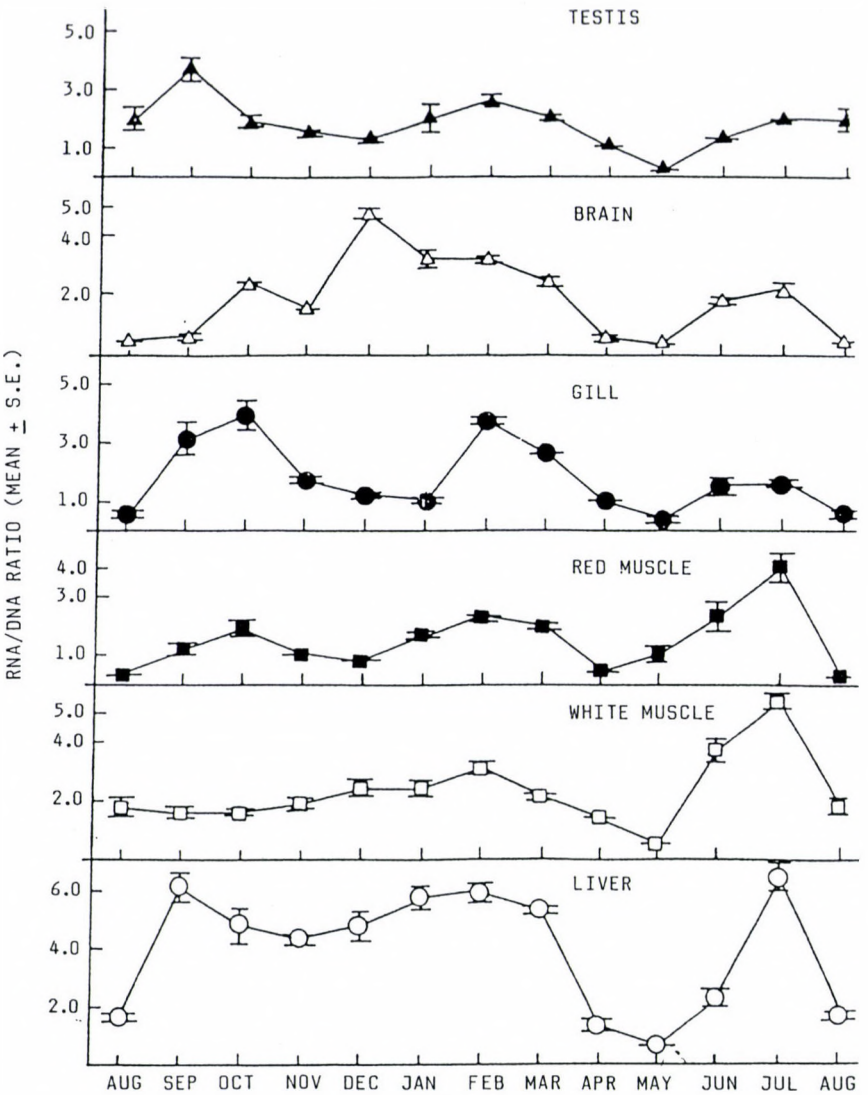
	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG
Aver.Max.Temp.(°C)	32.0	32.2	31.0	28.5	23.0	20.0	24.5	35.4	38.6	40.9	34.2	32.2	32.0
Aver.Min.Temp.(°C)	24.4	26.3	22.2	15.0	11.2	10.9	11.5	18.7	23.8	30.6	25.6	25.4	24.4

Fig. 1. Seasonal variation of the protein concentration (expressed as per unit DNA) in different tissues of Channa punctatus (Mean ± S.E. of six fishes)



Aver.Max.Temp. (°C)	32.0	32.2	31.0	28.5	23.0	20.0	24.5	35.4	38.6	40.9	34.2	32.2	32.0
Aver.Min.Temp. (°C)	24.4	26.3	22.2	15.0	11.2	10.9	11.5	18.7	23:8	30.6	25.6	25.4	24.4

Fig. 2. Seasonal variation of the free amino acid concentration (expressed per unit DNA) in different tissues of Channa punctatus (Mean ± S.E. of six fishes)



	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG
Aver.Max.Temp. (°C)	32.0	32.2	31.0	28.5	23.0	20.0	24.5	35.4	38.6	40.9	34.2	32.2	32.0
Aver.Min.Temp. (°C)	24.4	26.3	22.2	15.0	11.2	10.9	11.5	18.7	23.8	30.6	25.6	25.4	24.4

Fig. 3. Seasonal variation of ribonucleic acid (RNA) concentration (expressed per unit DNA) in different tissues of Channa punctatus (Mean ± S.E. of six fishes)

Discussion

A scrutiny of the Figs 1 to 3 reveals that most of the changes are in the form of oscillation from a theoretical basal line value /16/, which may be drawn round the year. This theoretical basal line value is tabulated in Table 1. The tissue specificity of the level of protein, free amino acid and RNA is quite clear from the data presented.

Table 1

Basal line value of biochemical tissue compositions in Channa punctatus

Parameter	Liver	White muscle	Red muscle	Gill	Brain	Testis
DNA (mg%)	0.236	0.036	0.097	0.209	0.122	0.245
Protein/DNA	47.941	223.488	81.262	27.099	39.431	31.882
Free amino acid/DNA	1.908	5.448	2.202	0.971	2.120	1.317
RNA/DNA	4.117	2.327	1.573	1.863	1.930	1.791

Patterns of rhythmic oscillation in biochemical tissue composition

It is quite evident from the Figs 1 to 3 that the total protein, free amino acid and RNA contents (expressed as per unit DNA) of the tissues of male Channa punctatus undergo distinct seasonal rhythmic fluctuations in an annual cycle. While a mathematical analysis describing the harmonic (periodic) function of the seasonal alteration of whole body protein and fat content of the black sea fishes (Pickerel, Surmullet, Anchevy, Spart etc.) have been conducted by a group of Russian workers /16/, such biometric analysis for the Indian fishes is utterly lacking. This is the first time an attempt has taken to treat the observed data with such harmonic equation. It is noticed that due to such biometric analysis the major "peak" (primary peak) is appearing repeatedly same time (month) in an annual cycle. Thus it is giving strong support about the circa-annual rhythmicity in the biochemical tissue composition of Channa.

Protein

The major noteworthy alteration of the tissue levels of total protein is build-up, characterizing the pre-spawning period viz., June—July (Fig. 1). In the State of West Bengal this fish spawns in late monsoon i.e. August—September. This characteristic rise in the protein level during pre-spawning period has been focused particularly in the graphic representation of harmonic function for the dynamics of protein concentration in different tissues (liver, muscles, gill, brain and testis) of C. punctatus (data not shown here). Augmentation of the protein content (percent yield value) in muscle of another air-breathing teleost, Clarias batrachus /17/, and in Chela cluppeides /18/ or of gonad in Mystus vittatus /1/ during the annual maturation of these fishes have been recorded. This augmentation in the protein content in different tissues may be a preparatory process for meeting the nitrogen demands and energy needs for spawning in this fish. A small spawning elevation in the level of some tissue protein (testis, liver and gill) is noticeable during August—September and this is followed by post-spawning depletion in October—November. This has been attributed to the massive mobilization of protein towards energy requirement for spawning as the level of nitrogen excretion and ammonia quotient are maximum in the month of September /13, 14/. Besides, a significantly high level of tissue protein is maintained during the winter months (December to February), particularly in white muscle, red muscle and testis and to some extent in liver and brain over that of summer months (April—May). The role of environmental temperature causing such a season specific change of tissue level protein, in the same fish is already described by Roy et al. /15/.

Free amino acid

The seasonal variation of cellular total free amino acid concentrations is characterized by a late 'winter peak' in all the tissues of Channa punctatus (Fig. 2). This season specific alteration is amplified by the treatment of the date in the plot of harmonic function particularly in liver, red muscle, and brain (data not shown). It is interesting to notice that in these tissues an inverse correlation depicted between the total protein concentration and free amino acid content during the winter period, suggesting an accumulation of the tissue level of free amino acid apparently at the cost of total protein in these tissues. This has been further confirmed

by the data on the absolute rate of protein synthesis and the rate of protein degradation in liver and red muscle /7/ of Channa punctatus. The characteristic summer depletion in the tissue levels of free amino acid in all the tissues is quite evident from the data. This corroborates the findings regarding augmented level of ammonia nitrogen excretion /14/ as well as of the ammonia quotient /13/ of this fish during the summer months, suggesting enhanced protein mobilization and utilization of free amino acid as the source of energy in summer populations. Besides, a gradual build up in pre-spawning period and a spent up phase during post-spawning period in the dynamics of total free amino acid and total protein in white muscle, gill and testis also reveal a direct relationship between the accumulation of these biomolecules with the breeding activity of this fish Channa.

RNA

A high level of RNA seems to be maintained throughout the winter months (December—February) in almost all the tissues of C. punctatus (Fig. 3). This accumulation is particularly focused when the data were processed with the above-mentioned harmonic equation particularly in liver, gill and brain (data not shown here). A spawning peak in the testis and liver may be correlated with the increase of biosynthesis of nucleic acid and thus the maximal value of gonado somatic and hepato somatic indexes during this time is noticed in this fish (data not shown here) and as well as in Mystus vittatus /1/.

Periods in the annual cycle

It has been possible in many cases to describe a summer adapted and winter adapted population of a particular species /14/, besides the usual reproductive forms. The annual cycle of rhythmic oscillation in the steady-state levels of total protein, free amino acid and RNA in the different tissues of the tropical murrel, Channa punctatus reveal two distinct periods or phases, one in winter (January—February) and another in summer (April—May). While the winter period is characterized by the accumulation of these biomolecules, the summer period is marked by a significant depletion of these biomolecules. Besides, like other seasonal breeders, the five different reproductive phases are also observed in the seasonal dynamics of biochemical tissue compositions in this fish. The reproductive phases viz.

pre-spawning (June--July), spawning (August--September), post-spawning (October--November), preparatory-I (December to February) and preparatory-II (March to May) are distinguishable which have been found to be characterized by the specific reorganization of the cellular biomolecular composition in particular period. The preparatory-I and preparatory-II are coinciding with winter and summer population, respectively, in regards to seasonal dynamics of biochemical tissue composition.

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A COMPARISON OF BLOOD COMPOSITION IN THE COMMON CARP (CYPRINUS CARPIO L.) USING SAMPLES OBTAINED FROM CAUDAL VESSELS AND DORSAL AORTA CANNULAE

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(Received: 1996-01-26; accepted: 1996-02-23)

Blood samples have been taken from chronic cannulae in the dorsal aorta of Carp under anaesthesia and at intervals of 24, 29 and 101 hours later. A few minutes after the 29 hours and 101 hours sampling was completed similar quantities (1 ml) were withdrawn from a caudal vessel. All samples were treated in the same way and determinations made of haematocrit value, total protein, glucose concentration and activity of the following plasma enzymes-acetylcholinesterase (AChE), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and lactic dehydrogenase (LDH).

Comparisons between dorsal aorta and caudal samples at 29 hours and 101 hours showed no significant differences for any of the enzyme activities that were tested. However significant (paired "t"-test, $P < 0.05$) differences were observed for haematocrit and glucose concentration. It is suggested that the higher haematocrit and lower glucose of caudal samples are consistent with the expected differences between arterial and venous blood.

Apart from these differences which reflect normal physiological function, values obtained for samples from the two sites were identical.

It is concluded that blood sampling from caudal vessels is a satisfactory method to obtain blood for biomonitoring purposes at least for those enzymes commonly used in recent surveys.

Keywords: Carp — blood sampling — haematology — blood biochemistry

Introduction

During the last 30 years the value of blood sampling for studies in fish physiology has extended beyond the original aim of investigating the cardiovascular system directly. It has proved particularly helpful in further analyses of the physiological mechanisms involved during adaptation to environmental changes in temperature, gas and ionic composition /9/. More recently they have provided useful methodology for endocrinological studies

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and monitoring toxicological changes in natural waters. The earliest studies depended upon direct sampling from the cardiovascular system with syringes; the most common being by cardiac puncture or insertion into the haemal arch of the vertebral column in order to withdraw blood from the caudal vessels /16, 30/. Caudal sampling has the main advantage of being a relatively easy operation and can be applied to a wide variety of fish species and sizes ranging from a few grams to many kilograms. Furthermore it can be equally useful in the laboratory and under field conditions. More recently cannulation techniques have developed which allow sampling of blood from the same individual over prolonged periods in order to follow changes in blood characteristics over many hours or days. These methods have mainly been developed by respiratory physiologists wishing to characterise the gas exchange functions of fish respiratory systems. Indwelling catheters have been successfully inserted into the dorsal aorta /10, 26/ or other arteries such as the pneumogastric (eel /5/), coeliaco-mesenteric (ray /12/, Torpedo /8/). Venous blood has been sampled from a number of veins including the ductus Cuvieri /25/ but in a few cases has it been possible to obtain venous blood immediately pre-brancial except in the eel /23/. The elongated ventral aorta of the eel provides an especially useful preparation for this purpose and an extracorporeal circulation /9/ enables the monitoring of blood parameters. Extracorporeal preparations have also allowed continuous monitoring of arterial blood in salmonids and sea bass /28, 29/. The caudal vessels have also been successfully cannulated and in some cases both arterial and venous catheters have been inserted /1, 4, 31/.

In spite of this wide usage of blood sampling methods it is perhaps surprising that few comparisons /22/ have been made between results obtained using different methods. In recent years studies have been made on the effects of a variety of pollutants /3, 19/, mainly using samples taken from the caudal vessels, which have shown that they may influence the enzymic composition of the plasma. Cannulation of the dorsal aorta has also recently been applied to these problems /11, 13/ and the opportunity has been taken to compare some results obtained from the same individual fish using the two methods.

Materials and Methods

Thirteen carp (Cyprinus carpio) of mean body weight 1007.3 g (range 750-1415 g) were used in the experiments which were carried out during May 1995 at Szeged. Each carp was kept in a separate aquarium containing 50 litres of unpolluted water which was saturated with air throughout the acclimation and experimental periods.

The general design of the experiments was to cannulate the dorsal aorta of the carp /10/ and to take the first sample (A_0) while the fish was anaesthetised (MS222, 100 mg/l). It was then allowed to recover overnight and the first control sample was taken the next morning (DA_{24}). In the afternoon, approximately 5 hours later, a second control sample (DA_{29}) was taken and immediately the fish was removed from its aquarium and caudal blood sampling (CV_{29}) carried out. The fish was then returned to another aquarium containing clean water where it was left undisturbed for a further period, usually 2-3 days. Another sample was taken from the catheter (DA_{101}) and again the fish was removed from its aquarium followed by caudal sampling (CV_{101}) as before. After this sampling the fish was sacrificed and its body weight determined.

Blood samples of 1.0 ml were drawn into well-heparinised syringes and routinely duplicate haematocrit (Hawksley microhaematocrit tubes and centrifuge) and total protein of plasma (hand held refractometer) were determined for each sample. The major part of the sample was centrifuged (10 min, 6.000 g + 4 ^{o}C, bionik MF-1) and the plasma stored at -80 ^{o}C until the completion of the experiments.

The glutamic oxaloacetic transaminase (GOT, or aspartate aminotransferase: EC 2.6.1.1) ang glutamic pyruvic transaminase (GPT, or alanin aminotransferase: EC 2.6.1.2) activities were measured according to Reitman and Frankel /24/; lactate dehydrogenase (LDH, EC 1.1.2.3) activity according to Annon /2/; acetylcholinesterase (AChE, 3.1.1.7) activity according to Ellman et al. /7/. The plasma glucose level was determined with the glucose-oxidase peroxidase (GOD-POD) method after deproteinizing the plasma with 0.3 N trichloro acetic acid (TCA). All biochemical parameters were assayed using Reanal kits (Hungary).

For the main objective of these experiments two sets of data (DA_{29}, CV_{29} and DA_{101}, CV_{101}) were obtained from each fish in which blood samples were withdrawn from an indwelling dorsal aorta catheter and the caudal blood vessels within a period of less than five minutes. Paired "t"-tests were carried out on these paired data. In addition comparisons (unpaired "t"-test) were made between the blood obtained under anaesthesia and the later control samples.

Cannulation of the dorsal aorta

The basic method has been described by Hughes et al. /14/ and substantially the same procedure was followed here. Because of the relatively great length of the catheter before the bubble (7.9-8.4 cm, according to fish size) it is helpful to mark the side of the bubble which is on the outside at its angle. By ensuring its correct orientation as the steel wire and cannula are inserted, the chance of any accidental withdrawal of the tip of the catheter from the aorta is reduced. This can be especially important at the stage when the catheter is being inserted into the protective catheter already fixed through the roof of the oral cavity.

Caudal sampling

Sampling from the caudal blood vessels of fish is a well-established method described in the literature (e.g. /16, 30/). Under the conditions of the present experiments after sampling from the dorsal aorta the specimens were taken out from the aquaria and placed on a horizontal surface. The head and the tail were then quickly covered by filter papers and held firmly by an assistant during the sampling procedure. The needle (3 cm, No. 14) of a

one millilitre heparinized syringe was inserted into the trunk of the carp at the level of the beginning of the anal fin and 1.5 cm below the lateral line at an angle of 45-55 degrees. In the case of scaled scarp, one scale was carefully removed before the needle was inserted. The blood sampled was withdrawn from the blood vessels (the caudal artery or vein) which pass below the centra of the vertebral column. The vertebral column is about 2-2.5 cm from the point where the needle was inserted, depending upon the size of the fish. After taking 1.0 ml of blood the needle was cautiously removed and filter paper held over the puncture site in order to reduce bleeding. The carp was replaced in an aquarium. The whole sampling procedure took only 2-3 minutes from the time the carp was removed from the aquarium to its return to water.

Results

Although no measurements were made of blood P_{02}, differences in colour of the blood samples was observed routinely. Blood sampled under anaesthesia was dark red but all other blood taken from the dorsal aorta cannulae was pinkish red in colour clearly indicating its well oxygenated condition. This contrasted with the darker colour of most blood taken by caudal sampling. In particular there were clear differences in nearly all cases in which blood was sampled from the dorsal aorta and caudal vessels of the same fish and with delays of only a few minutes. This strongly suggests that the caudal samples were mainly derived from the caudal vein.

A summary of the results is given in Table 1 where mean values and their standard errors are set out for each of the haematological and biochemical measurements. Further analyses of the data from individual samples using Student's "t"-tests are shown in Tables 2A (unpaired) and 2B (paired data).

The haematocrit values showed the expected fall when samples taken under anaesthesia were compared to those following 24 hours recovery. Erythrocyte swelling is well-known under these conditions and there was a further slight reduction in swelling over the next 3 days (Fig. 1) although part of this may have been due to haemodilution. Comparison of haematocrit values for the aortic and caudal samples showed higher values for blood taken caudally. These were not significant when unpaired samples were tested even using all 22 measurements ($P = 0.819$, Table 2A) nor were they significant when each set (29 h and 101 h) was tested separately (Table 2B). However, when all 22 samples (29 h plus 101 h) were compared using the paired "t"-test a significant ($P = 0.043$) difference was obtained. At first sight this result is surprising as the mean values ($DA_{29} + DA_{101} = 23.125$ +/-

Table 1

Mean values ± standard error (SEM) for haematological and biochemical parameters of carp blood sampled at different times from the dorsal aorta (DA) and caudal blood vessels (CV). Thus DA_0 = blood sampled from dorsal aorta under anaesthesia and CV_{29} = blood sampled from caudal vessels 29 hours later. n = number of samples

Sample	Temperature °C SEM	Total protein mg/ml SEM	Haematocrit value % SEM	AChE mU/mg protein SEM	LDH x 10 mU/mg protein SEM	GOT mU/mg protein SEM	GPT x 10^{-1} mU/mg protein SEM	Glucose mg/100 ml plasma SEM
$\frac{A_0}{n = 13}$	16.6 ± 0.4	25.9 ± 1.7	29.846 ± 1.847	2.201 ± 0.233	1.58 ± 0.330	2.254 ± 0.288	3.437 ± 0.66	123.6 ± 6.9
$\frac{DA_{24}}{n = 13}$	16.5 ± 0.4	23.2 ± 1.8	23.500 ± 2.131	2.209 ± 0.244	1.803 ± 0.373	3.19 ± 0.606	3.395 ± 0.619	138.9 ± 11.6
$\frac{DA_{29}}{n = 13}$	16.7 ± 0.4	22.7 ± 1.6	23.288 ± 2.121	2.237 ± 0.264	2.075 ± 0.497	3.332 ± 0.745	3.2 ± 0.624	132 ± 11.8
$\frac{CV_{29}}{n = 13}$	16.7 ± 0.4	23.1 ± 1.7	23.750 ± 2.225	2.092 ± 0.234	2.066 ± 0.477	3.214 ± 0.703	3.172 ± 0.673	128.8 ± 11.5
$\frac{DA_{101}}{n = 9}$	16.6 ± 0.5	22.9 ± 1.5	22.889 ± 2.445	2.208 ± 0.371	2.413 ± 0.760	2.510 ± 0.637	3.004 ± 0.801	89.1 ± 9
$\frac{CV_{101}}{n = 9}$	16.6 ± 0.5	22.5 ± 1.4	23.472 ± 2.312	2.240 ± 0.358	2.339 ± 0.683	2.516 ± 0.631	2.776 ± 0.683	87.1 ± 9.8
$\frac{DA_{29} + DA_{101}}{n = 22}$	16.6 ± 0.5	22.8 ± 1.1	23.125 ± 1.566	2.226 ± 0.212	2.214 ± 0.418	2.995 ± 0.508	3.119 ± 0.482	114.4 ± 9
$\frac{CV_{29} + CV_{101}}{n = 22}$	16.6 ± 0.5	22.9 ± 1.2	23.636 ± 1.581	2.153 ± 0.923	2.177 ± 0.388	2.928 ± 0.485	3.01 ± 0.477	111.7 ± 8.9

Table 2

Statistical analyses of data using Student's "t"-tests. In 2A data for blood haematological and biochemical parameters obtained for different samples are compared using unpaired data. In 2B the comparisons are only made where the two sets of samples were obtained from the same individual carp and hence the data was paired

	Total protein		Haematocrit		AChE		LDH		GOT		GPT		Glucose	
	t	P	t	P	t	P	t	P	t	P	t	P	t	P
Table 2A														
$\frac{A_0}{n = 13,}$ v $\frac{DA_{24}}{13}$	1.0935	0.2850	2.250	0.034*	0.0258	0.9796	0.4474	0.6586	1.3952	0.1757	0.0467	0.9631	1.1351	0.2676
$\frac{DA_{24}}{n = 13,}$ v $\frac{DA_{29}}{13}$	0.2169	0.8301	0.070	0.944	0.0784	0.9382	0.4371	0.6659	0.1474	0.884	0.2221	0.8261	0.4173	0.6802
$\frac{DA_{29}}{n = 13,}$ v $\frac{DA_{101}}{19}$	0.0760	0.9402	0.122	0.904	0.0661	0.9479	0.3899	0.7007	0.7876	0.4402	0.1952	0.8472	2.6665	0.0148*
$\frac{CV_{29}}{n = 13,}$ v $\frac{CV_{101}}{9}$	0.2660	0.7930	0.084	0.934	0.3610	0.7220	0.3390	0.738	0.7000	0.492	0.4010	0.6930	2.5970	0.0170*
$\frac{A_0}{n = 13,}$ v $\frac{DA_{101}}{9}$	1.2572	0.2232	2.312	0.032*	0.0183	0.9856	0.1226	0.2749	0.4066	0.6886	0.4185	0.6801	3.1023	0.0056*
$\frac{DA_{29} + DA_{101}}{CV_{29} + CV_{101}}$ v $\frac{}{n = 22, 22}$	0.0554	0.9550	2.300	0.819	0.2452	0.8028	0.0621	0.9496	0.0934	0.9242	0.1574	0.8726	0.2083	0.8320
Table 2B														
$\frac{DA_{29}}{n = 13}$ v $\frac{CV_{29}}{}$	1.637	0.128	1.404	0.186	1.865	0.087	0.388	0.705	1.305	0.216	0.241	0.813	1.903	0.081
$\frac{DA_{101}}{n = 9}$ v $\frac{CV_{101}}{}$	0.957	0.367	1.650	0.138	0.353	0.733	0.902	0.394	0.084	0.935	1.238	0.251	1.710	0.126
$\frac{DA_{29} + DA_{101}}{CV_{29} + CV_{101}}$ v $\frac{}{n = 22}$	0.385	0.704	2.158	0.043*	1.201	0.243	0.995	0.337	1.112	0.279	1.091	0.288	2.490	0.021*

*Data indicating significant differences

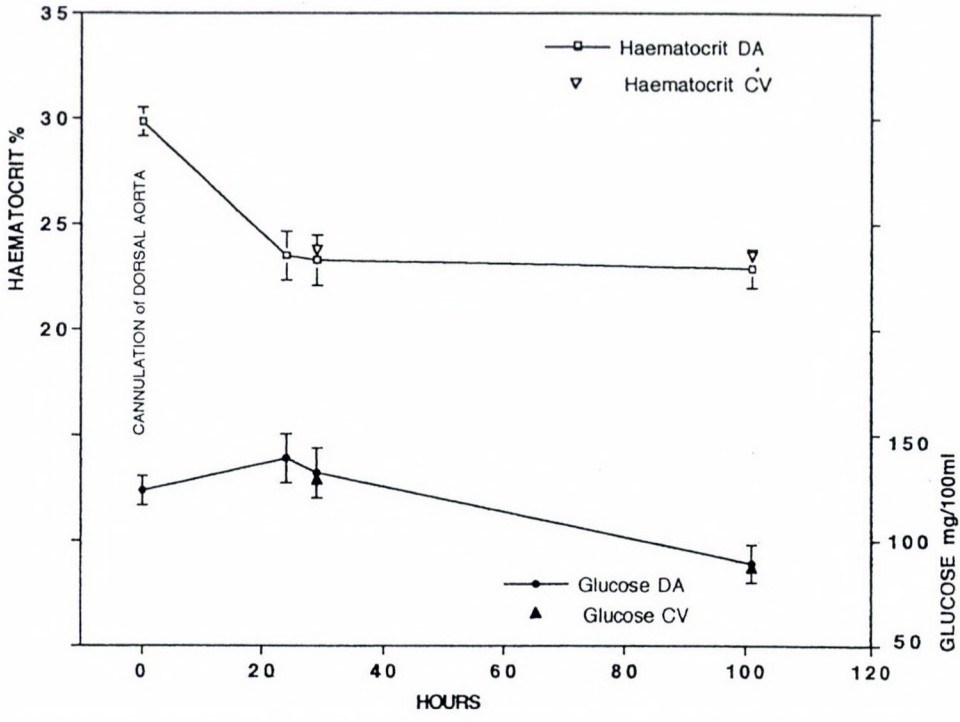


Fig. 1. Plot of mean values for haematocrit value (open symbols) and plasma glucose concentration (closed symbols) for samples taken under anaesthesia (0 hour) and at different times later from the dorsal aorta and caudal vessels of carp. Lines are drawn through points for dorsal aorta samples and the SEM of each mean is shown

+/- 1.566; $CV_{29} + CV_{101} = 23.636$ +/- 1.581) are close but the comparison of a large number of paired samples showed this to be significant.

With the exception of glucose, the biochemical determinations showed no difference between the two sampling methods. Differences between the glucose concentration of the caudal and dorsal aorta samples were only detected by the paired "t"-test (Table 2B) which indicated a significantly ($P = 0.021$) greater glucose level in the aortic samples. Small significant time-related differences can also be observed in the plasma glucose concentration (Tables 1 and 2, Fig. 1). The 3-day-long rest between the samplings (DA_{29}-DA_{101}, CV_{29}-CV_{101}) resulted in a reduced glucose level. The enzymes commonly used for aquatic biomonitoring (AChE, LDH, GOT, GPT /17, 21/) showed relatively high individual differences, which resulted in quite large standard errors (Table 1). However, even comparing these individual values (Table 2B), the two sampling methods did not give differences between samples taken at the same time, so other factors were excluded.

Discussion

The main result of these experiments has been to confirm the usefulness of the caudal sampling method as few differences have been established between those obtained using this method and the indwelling cannulation procedures which are generally recognised to be less stressful. In fact the only differences with any statistical significance may indicate features of the normal physiology of the fish. Thus the higher haematocrit value of the caudal samples is quite possibly related to the lower oxygen levels in this blood which was always darker than the aortic samples indicating its origin from the caudal vein. Correspondingly the lower glucose content of this blood is what is to be expected for blood which has recently perfused active tissues where glucose is utilised for metabolic activities.

Data published in some earlier studies might also be interpreted in this way. For example Oikari and Soivio /22/ compared the composition of blood taken from dorsal aorta cannulae with that from the exposed heart following immobilization with MS222 or stunning with a blow to the head. Their results show mean values for haematocrit of aortic blood of 18.3% (SEM 1.9) and heart blood of 22.8% (1.4) and 20.2% (1.7) for the pike Esox lucius. The corresponding results for glucose were 54.3 mg% (SEM 6.72), 49.4 mg% (3.41) and 47.5 mg% (3.73). Differences between samples of the 3 different types were non-significant using a "t"-test, presumably unpaired as samples were from different fish and numbers varied from 5-13. The differences in values for the aortic and heart samples show similar trends to those observed in the present study with carp and strongly suggest that they are related to the arterial or venous nature of the blood. Had it been possible to carry out paired "t"-tests and with a larger numbers of samples it is probable that statistically significant differences would have been revealed.

Apart from these features which may reflect normal physiological differences between arterial and venous blood, the results for the DA and CV samples were almost identical with respect to the biochemical parameters that were measured. However, it must be remembered that other constituents such as ionic, catecholamine and adrenocorticoid hormones are more sensitive indicators of stress than glucose /6/. Glucose is also known to be relatively slowly-responding as is indicated in the results plotted in Fig. 1 and confirms results of previous studies mainly using salmonid fish /26, 27/. The latter studies have also emphasized the sensitivity to brief periods of removal from water but it is not improbable that carp are less sensitive in

this respect. In this content it seems unlikely that any stress-related response producing an increase in plasma glucose could have been activated in the 5 minute period during which the aortic and caudal samples were taken in the present series of experiments. The raised glucose concentration of the 24 and 29 h samples are probably related to the anaesthesia and cannulation procedures.

Significant time-related differences in glucose level (Table 2A, B) could be the result of the less stressful conditions between the last (DA_{101} and CV_{101}) and the previous (DA_{29} and CV_{29}) samples. In practice this means 72 hours without any disturbance and a consequent reduction in the glucose level of the blood plasma is to be expected. Another factor to be considered is that the fish had not been fed during the entire experiment, and this could have modified the glucose content as well as the stress.

As a conclusion of the comparison, the values measured have shown that caudal blood sampling can be used for experimental purposes involving the biochemical parameters measured almost as well as the cannulation technique, because the composition of caudal blood was similar to that of the dorsal aorta, and the sampling procedure itself did not disturb significantly these particular blood parameters.

Caudal sampling has been applied in field experiments. For example Nemcsók and Benedeczky /17/ used caudal sampling for biomonitoring purposes using carp enzymes to show the effects of phenolic compounds in natural waters. A large cage (3.0 x 1.0 x 1.5 m) containing 10 carp was submerged into the river Maros in Hungary. Blood samples were taken weekly from each fish and biochemical parameters (GOT, GPT, LDH, AChE, plasma glucose level) were measured.

However, taking samples under such field conditions can cause a greater stress for the fish than using the same method in laboratory aquaria. Before sampling the cages are partly lifted out of the river, then the fish are caught with a net, which takes several minutes. The already sampled fish are placed into a separate tank until all fish have been sampled after which they are all returned to the cage. The results of Nemcsók and Benedeczky /17/ have shown a higher glucose level in blood plasma derived from fish under field conditions compared with those from fish kept in laboratory aquaria. This may not be entirely due to pollution of the river, because difficulties during sampling from the caudal vessels might give similar results. Nevertheless, it should be emphasized that caudal sampling is applicable for biomonitoring purposes in spite of its limitations, because

most of the biochemical parameters used in such studies do not show any significant difference compared to those of the dorsal aorta samples. Furthermore, sampling via chronic cannulae is normally impossible in the field for practical reasons, though it is probably a less stressful method. Another advantage of the caudal sampling method is that the fish can continue to feed whereas this is not usually possible for fish with a dorsal aorta cannula. This restriction is not so important in the laboratory, but in the field such long-term effects must be considered.

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STUDY OF THE EFFECTS OF BRIEF EXPOSURE TO AN ORGANOPHOSPHOROUS INSECTICIDE (METHIDATHION) ON BLOOD CHARACTERISTICS OF CARP (CYPRINUS CARPIO)

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1. Carp with the dorsal aorta cannulated have been used to study the effects of a 5 hour exposure to methidathion at concentrations of 2 or 6 mg/litre. Blood samples taken during control, exposed and recovery periods were used to determine plasma acetylcholinesterase (AChE) activity, haematocrit value, mean cell volume, total plasma protein, and filtration time through Nuclepore filters containing pores of 8 μm.

2. A drastic inhibition of AChE activity was observed and this continued during the recovery for at least 5 days. Changes in other blood parameters were less marked and recovered soon after fish were returned to non-polluted water. The increased haematocrit and decrease in filtration time suggests some impairment of gas transfer during exposure but the depletion of AChE activity and associated muscular and neural disturbances are more serious results of pollution with this organophosphate insecticide.

Keywords: Methidathion — acetylcholinesterase — carp — haematology — red cell deformability

Introduction

The increased use of insecticides in agriculture has given rise to many problems associated with their overall effect on the environment. The leaching of long-lasting pesticides into natural waters inhabited by fish may lead to a serious deterioration in fish populations. In this context it is important to assess the possible impact of well-known pesticides on fish from these natural waters. Toxic chemicals can affect fish at all levels of organisation from the morphological structure of organ systems to the molecular structure and activity of important enzymes /11/. Many studies have demonstrated the value of investigating changes in blood parameters in order to assess the impact of water pollutants on fish /8, 13/.

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Organophosphorous insecticides are commonly employed in agriculture and their toxicity to different groups of vertebrates has been widely studied /1, 7, 22, 24, 26, 28, 32, 33/. The pollutant tested during the exposure period in this series of experiments was Ultracid-WP40 which is an insecticide containing methidathion (MD).

Materials and Methods

Carp were acclimated in aquaria at 21 +/- 1 °C (May/June) or 18 +/- 1 °C (October) for several days before being used for experimentation. Cannulation of the dorsal aorta was carried out according to the method of Hughes, Albers, Muster and Goetz /18/ and each fish placed into a separate aquarium containing 50 litres of well-aerated tap water. Twenty-four hours later control blood samples were taken and then 50 millilitres of an insecticide solution (0.25 g Ultracid-WP40 which contains 40% (w/w) active ingredient) was added to the 50 litre aquarium to give a final concentration of 2 ppm active ingredient (2 mg/litre). In some later experiments corresponding increased strengths were used to produce a final concentration of 6 mg/litre. These concentrations did not remain constant during the exposure period but rather mimic the natural conditions, i.e. a large initial dose which decreases with time. After 5 hours' exposure to the insecticide blood samples were again taken after which the fish was then transfered into another aquarium containing 50 litres of clean water. Following a 24 hour period of recovery, blood samples (1.0 ml) were taken in order to monitor recovery from the insecticide and further recovery samples were obtained at times up to 140 hours post-exposure. Almost all fish survived this procedure and were later killed and weighed. The range of body size was 530-1710 g (mean 877.6 +/- 65.1 g SEM, n = 19). Each blood sample was taken into a heparinised one millilitre syringe containing a strong heparin solution so that the final concentration in the blood was 500 IU/ml and was analysed by the following procedures. Duplicate samples were used to determine haematocrit value (Hawksley microcentrifuge), total protein (hand-held refractometer) red cell count (haemocytometer). Most of the sample (0.6 ml) was used to determine the filtration rate through a Nuclepore filter containing pores of 8 µm diameter in a modified apparatus /19/ which made it possible to follow the timecourse of the flow of blood past 17 light beams which were detected by a corresponding series of light dependant resistors. Filtration rate during the flow of the first 0.1 ml of blood was used to determine the filtration rate for a 0.3 ml sample and this value was used to determine the pore passage time (PPT) of single red blood cells which has been used as an index of red cell deformability /20/. Part of the blood sample (0.25 ml) was centrifuged and the separated plasma used to determine the activity of acetylcholinesterase (AChE, EC 3.1.1.7). AChE activity was measured by the method of Ellman et al. /6/. The reaction mixture was as follows: 2.0 ml of phosphate buffer 52.0 mM, pH 7.2 containing 0.26 mM 5,5'-dithio-bis-nitrobenzoic acid (Sigma), 0.050 ml of acetylthiocholine iodide 82.4 mM (Fuka A.G.) and 0.025-0.100 ml of plasma. Measurements were performed using a spectrophotometer (Cecil CE 5501). Activities could be calculated from the enzymatic hydrolysis of acetylthiocholine iodide per unit of time. Enzyme activities were expressed in mU/mg protein (1 standard U: 1 µmol substrate /acetyl-thiocholine iodide/ x min^{-1}). Protein concentrations were determined by a refractometer (see above).

Mean values and standard errors were determined together with probabilities that any differences were significant using Student's "t"-test. Where the comparison was between the blood from the same individual fish, a paired "t"-test was also used.

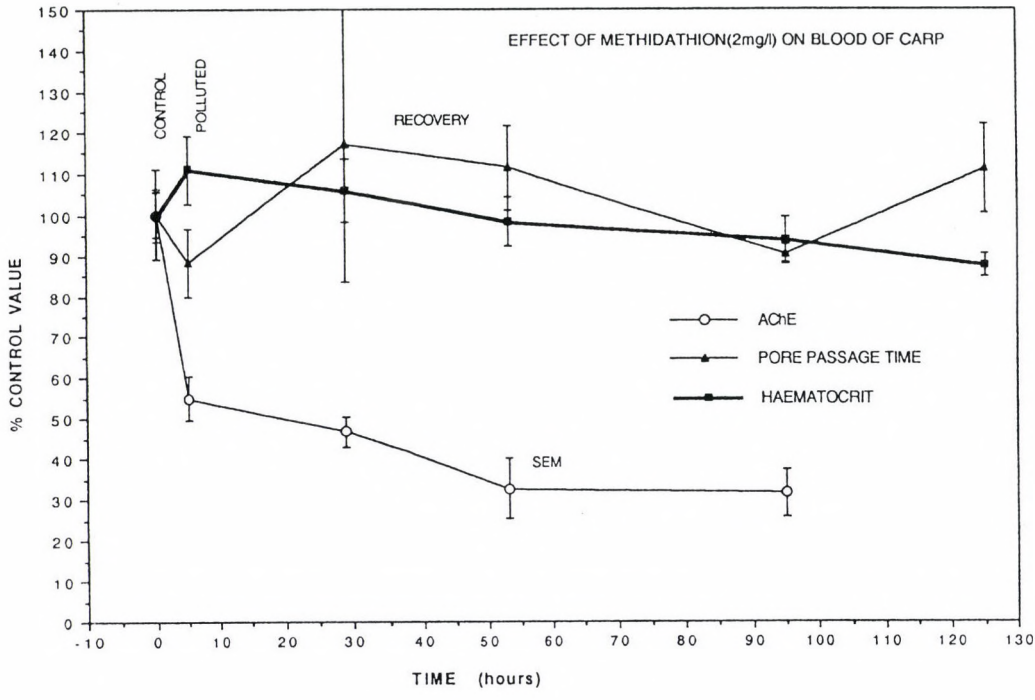
Results

Most of the fish that had been cannulated survived the 24 hour period before the control blood sample was taken. Following this the fishes were treated with MD for five hours and there were very few deaths during the exposure period. This is not surprising as the LC50 /25/ for four days is 5.5 mg/litre, a concentration much stronger than that used (2 mg/litre) in most of the experiments and was determined under similar conditions. Even when this concentration was increased to 6 mg/litre only two fish out of 11 died during the exposure period and one other during the succeeding 24 hour recovery period.

Prior to the treatment with MD most carp were resting in their 50 litre aquarium containing well-aerated tap water (pH 7.7-7.8) and showed the characteristic intermittent ventillation of this species. Following exposure to MD the behaviour of the fish varied over a wide range. Some specimens appeared to be scarcely affected whereas others showed increased ventillation which became a regular rhythm of 50-70 per minute. In addition there were some agitated movements of the fins and, particularly at the higher concentration, tremorous movement of the lower jaw and other parts of the body was sometimes observed. All of these usually became reduced when the fish was placed in clean water. Indeed some of the specimens appearing most agitated by the treatment seemed to have completely recovered although a few specimens continued to display several features over the extended recovery period of several days.

Effects on blood haematology and filtration

Of the measurements made the most consistent finding was an increase in haematocrit value during exposure to MD. This then returned to control levels during recovery (Fig. 1). The consistency of these effects became more clear when paired "t"-tests were carried out. This has been found in previous studies /14, 15/ and is related to the wide range in control values in individual specimens. An increase in mean cell volume was sometimes


EFFECT OF METHIDATHION(2mg/l) ON BLOOD OF CARP
CONTROL
POLLUTED
RECOVERY
AChE
PORE PASSAGE TIME
HAEMATOCRIT
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% CONTROL VALUE
TIME (hours)

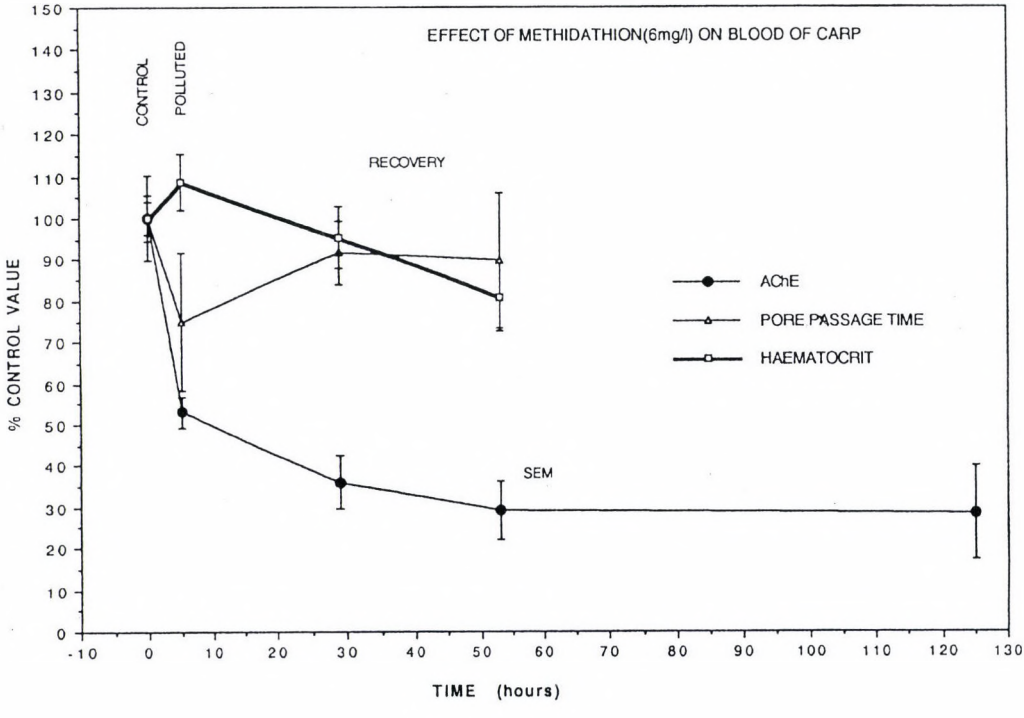

EFFECT OF METHIDATHION(6mg/l) ON BLOOD OF CARP
CONTROL
POLLUTED
RECOVERY
AChE
PORE PASSAGE TIME
HAEMATOCRIT
SEM
% CONTROL VALUE
TIME (hours)

Table 1

Control haematological values for carp treated with methidathion (2 or 6 mg/l)

	2 mg/l n = 12 Mean /S.E./	6 mg/l n = 7 Mean /S.E./	All controls n = 19 Mean /S.E./
Haematocrit value (%)	23.9 /0.761/	21.6 /2.416/	23.048 /1.004/
Red blood cell count (x $10^6/mm^3$)	1.371 /0.051/	1.25 /0.116/	1.3263 /0.053/
Mean cell volume (μm^3)	175.6 /5.472/	172.9 /11.56/	174.58 /5.296/
Total protein g/decilitre	3.058 /0.129/	2.657 /0.296/	2.91 /0.139/
Pore passage time (msec)	0.511 /0.041/	0.477 /0.084/	0.499 /0.039/

Mean control values for blood parameters of 19 carp together with their standard errors obtained from blood samples taken before exposure to water containing methidathion. Results obtained for fish exposed to 2 and 6 mg/l methidathion are shown separately together with combined values for all 19 specimens.

associated with the increased haematocrit values. Another feature found in most specimens was a decrease in the pore passage time (PPT) for individual red blood cells. This effect was statistically significant ($P < 0.05$) in the experiments using the higher concentration of MD (6 mg/litre). There was no consistent effect on the level of total protein determined for the plasma portions of blood used for measuring haematocrit value.

The control values upon which values plotted in Fig. 1a, b are based are summarized in Table 1 where both mean values and the standard error are given. A total of 19 fish were used in these analyses and includes only those specimens from which samples were obtained for control, treated and 24 hour recovery periods. Of the 19 fish analysed twelve had been exposed to initial concentrations of 2 mg/l and seven to concentrations of 6 mg/l. No significant differences were found between the results of experiments carried out in May/June or October.

Fig. 1. Plots of time course for haematocrit value, pore passage time and AChE activity resulting from exposure to insecticide plotted with standard errors as percentages of their control values: a. 2 mg/l methidathion; b. 6 mg/l methidathion

Activities of AChE in blood plasma

The results showed a very marked reduction in activities of AChE following exposure to MD (Fig. 1), from a control value of 1.883 +/- 0.1004 (SEM) mU/mg protein. The sudden decrease in enzyme activity was even greater when fish were exposed to 6 mg/l (Fig. 1b). When the specimens were returned to clean water there was no significant increase in enzyme activity. In fact these levels continued to decrease in many specimens. Thus no recovery was observed in this aspect of carp blood biochemistry during the experimental periods which in some cases extended to five days.

Discussion

Although a number of recent studies have elucidated the nature of some of the effects of MD on AChE activity in the blood and other tissues of carp /21, 25, 29/ most of these have been concerned with long-term exposure of the fish to pesticides many of them for a minimum exposure time of 24 hours. It has been clearly demonstrated that MD has a very serious effect on many carp tissues and that recovery from such exposures at 2 ppm MD persisted for at least two weeks. The time scale of the present investigations was quite different as the fish were only exposed to MD-containing water for five hours. The concentration used (2 ppm) was similar to that used as a maximum in some previous studies but the 6 ppm used in later experiments was much greater than in long-term studies (1-6 days).

This shorter time course and higher MD concentration of the exposure period was chosen in the present experiments in order to produce more definite effects in the shorter time available for sampling using indwelling catheters. In some ways a short exposure time mimics the episodic type of environmental hazard which is known to occur following spillage of chemicals into waters occupied by fish. The longer term studies correspond to the long-term exposure of fish to waters containing toxic chemicals and in such studies concentrations are usually chosen which are comparable with those persisting in the natural environment.

In terms of the effect on the AChE activity the results obtained have been fundamentally the same as those obtained in other studies although the mode of blood sampling was different as it was obtained from the dorsal aorta in the present experiments. With caudal sampling of blood it is not always possible to take samples from the same individual fish and hence to

make direct comparisons between control and experimental samples although a recent study has shown that comparable values are obtaine by both sampling methods /34/.

That relatively few fish succumbed to the MD treatment is in keeping with the relationship between the concentrations used and the 96-hour LC50. Some fish certainly showed signs of distress during exposure to MD but these seemed to disappear when they were returned to clean water. Average activity levels in the blood continued to fall throughout recovery even when periods of up to five days were followed. This strong inhibitory feature of MD agrees with the findings of the Bruijn et al. /3/ and de Bruijn and Hermens /4/. Their studies have shown that MD, amongst others, has a low lethal body burden (LBB), and a low partition coefficient. These values strongly indicate that this compound has a specific mode of action in the fish. Although the fish did not seem to be adversely affected clearly there were very significant effects upon those aspects of neurological function involving cholinergic systems. Presumably such effects can only be observed if the behaviour of the fish during feeding and escape are monitored. For example, salmon with low AChE values have various impairments, such as the inability to maintain position in current, or to avoid predators /27/, which can be unfavourable under natural circumstances.

It is generally accepted that 96-hour LC50 values of organophosphorous compounds are associated with approximately an 80% reduction in AChE activity /10/. However, there are differences amongst the tissues. Previous experiments have shown /23, 29/ that the relative inhibition of AChE is greater in AChE-rich tissues, like brain, heart and muscle than in the blood plasma. (Fish blood plasma contains Acetyl ChE unlike mammals /29/.) Therefore, a strong inhibition of AChE in blood plasma is probably associated with strong blocking of AChE activities in other tissues.

The long-lasting effect of MD in these experiments also agrees with studies on several fish species /10, 27, 29/ that recovery of AChE to control levels takes several weeks. This recovery is considered to be derived from the resynthesis of AChE /10/ rather than from a slow reactivation of the phosphorylated enzyme /5, 31/. Nevertheless, there are suggestions /2, 21, 25/ that the resynthesis can also be blocked and cause an even longer lasting effect of organophosphorous compounds.

The effects on haematology and filtration properties of the red blood cells contrast with those discussed above both in their extent and time course (Fig. 1a, b). The changes in haematocrit, pore passage time and other

parameters either showed no change at all or very small changes some of which were masked because of variability between individuals. In some cases it was only by use of the paired "t"-test that such changes could be substantiated. This of course is one of the main advantages of the possibility of comparing samples from each cannulated specimen throughout the control, experimental and recovery periods.

An increase in haematocrit value from control levels was found in most specimens and it had returned to pretreatment levels during the 24 hour recovery period. Such increases in haematocrit have been observed in carp and other fish during a number of stressful treatments /16, 30/ and often seems associated with hypoxic conditions produced either directly by lowering the water P_{O2} or following treatments which damage the ability of the fish to transfer oxygen across the gill water/blood barrier /14/.

Effects on pore passage time, which is indicative of greater deformability of the red blood cells, has also been shown to be a common feature of such environmental change /12, 15/. recovery from these changes is almost immediate following return to clean water and again is in marked contrast to the continuing effects on plasma AChE activity.

Following treatment with some toxic chemicals the extensive effect on morphological and physiological characteristics of the gas exchange organs can be the most damaging to a fish in the short term although underlying biochemical effects may be more long lasting. In the case of MD it is clear that the effects at the biochemical level are the more significant at least in the case of carp. The drastic AChE inhibition with no recovery during the period of observation, contrasts with the rapid recovery of haematological and deformability properties recorded during the same period.

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IN VIVO INDUCTION OF SISTER CHROMATID EXCHANGES (SCE) IN A TROPICAL FISH, ETROPLUS SURATENSIS (BLOCH)

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Sister chromatid exchange (SCE) test was applied to a tropical cultured fish, Etroplus suratensis by exposing to known mutagens. Intramuscular injections of methyl methane sulphonate (MMS) and cyclophosphamide (CP) for an exposure period of 96 h resulted in significant increase in the frequency of SCE in gill tissues. MMS induced dose dependent increase in SCE. The findings revealed that the test species in the present study may be used in mutagenicity assays for screening environmental pollutants.

Keywords: Fish — mutagens — sister chromatid exchanges — genotoxicity — microsomal activation

Introduction

The current status of the potential toxic chemical in the environment is no doubt a threat to human health. These chemicals can be mutagenic or carcinogenic as well as toxic to a broad range of organisms. There have been several short-term tests developed to detect genotoxic potential of chemical pollutants in the environment. Among them sister chromatid exchange analysis is very sensitive and simple. The standard protocols are mainly based on mammalian systems /3, 4, 6, 15, 19/. In fish the pioneering attempt /13/ had been made to develop a suitable in vivo system for studying SCE. Kligerman /11/ explored the possibility of using U. limi as an in vivo aquatic model for such study in measuring the mutagenicity of water borne chemicals. This innovative approach was followed by several other studies demonstrating SCE bioassay using aquatic animals as test organisms /2, 9, 17, 21, 22/. The

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present study reports the applicability of SCE test to a tropical brackish water fish E. suratensis using two known mutagens.

Materials and Methods

E. suratensis, a tropical cichlid inhabiting the brackish waters of India, Pakistan and Sri Lanka, was used as the test animal. Its karyotype consists of 48 large acrocentric chromosomes, almost uniform in size. The laboratory maintenance of the species is simple, and it is available throughout the year. The economic importance of the species lies in its value as cultured fish in the Subcontinent.

Young specimens were collected from brackish water ponds and acclimatised for at least two weeks in a salinity range of 5-10‰. Methyl methane sulphonate (MMS), John Backer, Inc. USA and cyclophosphamide (CP), Khandelwal, Bombay, India were used as test chemicals. MMS is a direct acting mutagen whereas CP requires metabolic activation to become functionally active and therefore, is a promutagen. Doses were selected on the basis of tolerance limits for an exposure period of 96 h through preliminary experiments. Three dosages of each chemical were tested. The maximum tolerated dose in 96 h, that produced no mortality, was the highest dose and subsequent doses were fractions at the highest dose. At 0 h, intramuscular injection of chemicals were given in the dosage of 50, 100 and 150 µg/g body weight for MMS and 25, 50 and 100 µg/g body weight for CP. At 72 h, animals were given one injection of 5-Bromodeoxyuridine (BrdU) at the rate of 0.5 mg/g body weight. Control animals received injection of solvent at 0 h and 5-BrdU at 72 h. For chromosome preparation, gill tissues were processed following the solid tissue technique of Kligerman and Bloom /14/ with modifications. Animals were administered with 0.05% colchicine intramuscularly α 1 ml/100 g body weight for 2-2.5 h. Gill tissue was removed, hypotonised in 0.8% sodium citrate and fixed in methanol-acetic acid (3:1). Cell suspensions were prepared in 40-50% acetic acid (in water). The suspensions were dropped on warm slides using a Pasteur pipette. After a few seconds the drops were withdrawn with the pipette leaving behind rings of cells. For differential staining, FPG (Fluorescent plus Giemsa) technique modified by us /14/ was used. Experiments starting from BrdU injection till slide preparation were carried out in subdued light due to the photosensitive nature of BrdU. Slides were stained in 4% Giemsa solution for 20 min and screened without mounting with the help of an Olympus microscope. At least 10 metaphases displaying second round of replication /10, 12/ were screened per anim al. Likewise, data were collected from four animals in each dose and the cell populations were pooled for each dose of chemical and value expressed as SCE/cell. An equality of proportion test (Z-test) was used for statistical analysis of the results.

Results

Significant increases in SCE in gill tissues were recorded by both the test chemicals against a control value of 1.82 exchanges/cell (Table 1 and Figs 1, 2). The highest dose (150 ug/g) induced the highest number of SCE

Table 1

Analysis of SCE induced by methyl methane sulphonate (MMS) and cyclophosphamide (CP) in *E. suratensis*

Chemical	Dose (μg/g)	No. of animals	No. of SCD_2 cells	Total No. of SCEs examined	SCE/cell ± SE
MMS	50	4	95	385	4.05 ± 0.360*
	100	4	111	999	9.00 ± 0.805*
	150	4	61	732	12.00 ± 1.471*
	Control	6	104	190	1.82 ± 0.120
CP	25	4	47	147	3.12 ± 0.376*
	50	4	47	195	4.14 ± 0.527*
	100	4	65	240	3.69 ± 0.391*
	Control	6	104	190	1.82 ± 0.120

*Significant ($Z \geq 1.96$)

(12 SCE/cell) followed by subsequent dose levels depicting a typical dose dependent response. On the other hand, CP induced the highest number of SCE (4.19 SCE/cell) by the middle dose (50 μg/g) followed by 3.69 and 3.12 SCE/cell in the highest and lowest doses, respectively. The trend was lowest dose < highest dose < middle dose. Generally, long chromosomes were found to



Fig. 1. Control metaphase from gill tissue of fish demonstrating sister chromatid differentiation and SCE. Bar = 10 μm

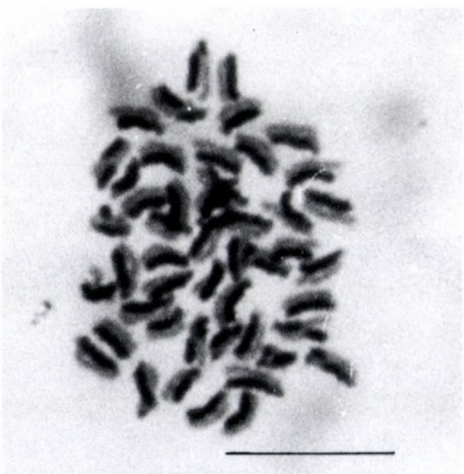


Fig. 2. Metaphase from gill tissue of fish treated with MMS showing multiple SCE. Bar = 10 μm

have more exchanges than short chromosomes. It was also noted that cyclophosphamide treated animals displayed relatively less divisional metaphases and consequently lower number of second division cells than MMS treated ones.

Discussion

The SCE induced by 3 different dose levels of MMS are in agreement with the reports of others demonstrating a clear dose dependent response. At lowest dose (50 µg/g) MMS showed 4.05 exchanges per metaphase in the present study which is 8.3 exchanges/cell in Umbra limi reported by Bishop and Valentine /5/. At 100 µg/g, E. suratensis showed an exchange rate of 9 exchanges/cell while U. limi showed 8.1 exchanges/cell. The differences in the SCE rate could easily be explained taking several factors, such as source of fish, differential species responses, experimental limitations with respect to BrdU, and mutagen treatment protocols into account.

Cyclophosphamide, a water soluble promutagen, needs metabolic activation to become active. Significant increase in the induction of SCE was observed indicating the ability of the test species to convert promutagen into its active metabolites. The lowest and the middle doses (25 and 50 µg, respectively) showed dose dependency but the highest dose showed a fall in the SCE rate. This decline in SCE incidence despite increase in CP dosage is likely due to selective killing of cells /20/. Though a correlation between SCE induction and doses is not indefinite but is limited by cytotoxicity responses. Consequently the highest doses always do not show the highest frequencies.

With regard to the relative genotoxicity, CP induced a slightly higher SCE per cell than MMS in the present study when compared on a µg/g basis. This finding is interesting against the backdrop of reports by others /5, 12/ that CP is significantly more potent inducer of SCE than MMS. A low response of E. suratensis in the present study could be explained assuming a variant microsomal activation system of the test species /12/. It is evident that different species of fish possess different types of cytochrome P-450 with resultant differences in the metabolism of carcinogens and related compounds /1/. So, high species and strain variability along with pronounced differences in hepatic cytochrome P-450 and mixed function oxidase (MFO) levels associated with sex, season, temperature and diet are important factors /8, 16, 18/. Hence, the apparent departure in the present study may be

indicative of a weak or a moderate microsomal activation system of the test species. However, significant induction of SCE by both the mutagens indicates that E. suratensis may be a useful tool for detecting genotoxic potential of pollutants in water. Further study on the species would give additional information on the action of promutagen in this fish.

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ULTRASTRUCTURAL PATHOLOGY OF DEGENERATING "DARK" GRANULE CELLS IN THE HIPPOCAMPAL DENTATE GYRUS OF ADRENALECTOMIZED RATS

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Adrenalectomy-evoked delayed degeneration and death of granule cells in the hippocampal dentate gyrus (DG) of the rat brain were studied by means of electron microscopy and a recently elaborated silver method that selectively stains the "dark", collapsed neurons in a Golgi-like manner. At the light microscopic level, the silver technique revealed degenerating granule cells located exclusively in the dentate gyrus; other glucocorticoid receptor-containing regions of the brain were not affected. The silver-stained cell bodies were shrunken, most of the dendrites had a beaded appearance, and the stained axons could be traced along their route to the CA3 pyramidal neurons of the hippocampus. The analysis of 2.5 μm thick Epon-embedded sections stained with toluidine blue revealed hyperchromatic, dark granule neurons and their remains and a heavy glial activity in the vicinity of collapsing neuronal profiles. At the ultrastructural level, early and late stages of neuronal degeneration were observed. The early phase was characterized by markedly increased electron density, a massive shrinkage of the whole somato-dendritic domain, vacuolization of mitochondria, swelling of the nucleolus and condensation of the nuclear chromatin. In the late stage, subcellular organelles were hardly recognizable due to the extremely high electron density and dramatic shrinkage of the cytoplasm. These profiles exhibited disintegration of the cellular organelles and loss of their afferents. Concomitantly, disintegration of granule cell dendrites (clasmatodendrosis) and lifting of "dark" mossy fibers from cell bodies and dendrites of CA3 pyramidal neurons were observed. In the latter cells, this partial denervation caused no apparent signs of ultrastructural alterations. Proliferation of astrocytes and microglial cells was also obvious as they engulfed and eliminated the degenerating neuronal elements. Degenerating neurons frequently occurred adjacent neurons with normal morphology. These morphological features indicate that the delayed degeneration of hippocampal granule cells following adrenalectomy might proceed through a cytoskeletal collapse terminating in cell death.

Keywords: Adrenalectomy — dark neurons — dentate gyrus — electron microscopy — silver intensification

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Introduction

The hippocampal formation participates in the regulation of neuroendocrine cascades /31/ and in processes associated with memory and learning /38/. The cells of the dentate gyrus and the different sectors of the hippocampus receive humoral messages /6, 36/ in addition to neuronal afferents /2/. Both units contain glucocorticoid receptors /5, 17/ and their function is known to be influenced by circulating steroids. Alterations in the glucocorticoid milieu result in changes of the anatomical architecture /29, 32/, electrophysiology /35/ and operation /30/ of the hippocampal formation. Sloviter et al. /32/ have recently reported a selective and delayed degeneration of granule cells in the dentate gyrus of adult rat brain in long-term adrenalectomized animals. The observation was reinforced by other groups with the following complementary findings: 1. The cell damage starts to develop shortly after adrenalectomy (ADX) and is sustained for several months /10, 13/. 2. The degeneration occurs in a highly characteristic pattern in which both septo-temporal and medio-lateral differences exist /13/. 3. The cell loss can be prevented by corticosterone supplementation /32/ and local delivery of dexamethasone /13/. 4. The activation of type I and type II glucocorticoid receptors /26/ gives differential protection against the cell death in ADX-animals /39/. 5. Granule cell degeneration is manifested only if plasma corticosterone levels are less than 2 ng/mL in the adrenalectomized animal /19/. 6. Sulfated glycoprotein-2 expression is associated with the death of granule cells /19/, and 7. The degenerating neurons can be selectively visualized with silver intensification /13/.

Recently, the collapse of cytoskeleton has been hypothesized /9/ to precede the neuronal degeneration and cell death evoked by different pathological stimuli. For the selective and reliable demonstration of neurons affected by cytoskeletal damage, a new silver method was elaborated /8/. By this novel technique and electron microscopy, we analyzed the cellular peculiarities of the delayed neuronal degeneration caused by adrenalectomy in the hippocampal dentate gyrus of the rat brain.

Materials and Methods

Animals

Twenty Wistar male rats (200-250 g) kept under standard environmental conditions (12:12 h light/dark cycle, 20 °C) were surgically adrenalectomized under Nembutal anesthesia (40 mg/kg b.w., i.p.). After recovery, they had access to both tap water and 0.9% physiological saline. Five rats were sham-operated to serve as controls.

Fixation

Both the adrenalectomized and the control animals were reanesthetized 1, 3, 9, 27 or 81 days later, 4 adrenalectomized and 1 control rats at a time, and were perfused through the ascending aorta, first, with 30 ml of a rinsing buffer solution (pH 7.5) containing 5.9 g $CaCl_2 \times 2\ H_2O$, 9.0 g NaCl and 1.0 g sodium cacodylate in 1000 ml distilled water. Then, 500 ml fixative consisting of 2% paraformaldehyde and 2.5% glutaraldehyde in the above cacodylate buffer was perfused slowly through the vasculature. The brains remained for 24 h in the skull and, thereafter, gentle autopsy was performed. The tissue was fixed further with the same fixative for 7 days.

Specimen processing for light microscopy

Serial eighty micrometer thick vibratome sections were cut from the brains in the coronal plane. Every second section was silver-stained as described earlier /8/, and the rest were saved for electron microscopy. The progress of silver staining was monitored with the light microscope. Optimally processed sections had yellow-light brown background and intensely labeled black neurons. After dehydration in 1-propanol, the sections were cleared with xylene and mounted with Canada balsam.

Preparation for electron microscopy

Based on the light microscopic evaluation of silver-stained sections of the adrenalectomized animals, corresponding areas suspected to contain degenerating, collapsed neurons were dissected out for electron microscopy. Similar areas were cut out from sections of control animals. All the specimens were postfixed with 2% osmium tetroxide, dehydrated in ethanol and embedded into Epon /23/. Serial sections of 1 to 2.5 μm thickness were cut and stained with 1% toluidine blue for 2 min. After locating sufficient numbers of hyperchromatic and shrunken neurons in semithin sections of the dentate gyrus, series of ultrathin sections were cut from the same region, placed on copper grids (75 x 300 mesh) and contrasted with uranyl acetate and Reynold's lead citrate.

Specimen evaluation

The light microscopic preparations were analyzed and photographed on a Zeiss Axiophot microscope. For ultrastructural evaluation a Zeiss 10 CR transmission electron microscope was used.

Results

Silver staining of collapsing neurons

In control animals no silver-stained neurons were demonstrated in the hippocampal formation or other parts of the brain. On the contrary, in adrenalectomized rats, silver-stained neurons appeared symmetrically in the dentate gyrus. The labeled cells were located exclusively in the granule cell layer (Fig. 1a) occupying both the dorsal and ventral blades. Other neuron populations of the hippocampus (Fig. 1a—f) and other regions of the brain contained no silver-stained cells. The silver-stained neurons had a Golgi-like appearance allowing easy tracing of their dendritic arborizations (Fig. 1c) and their axons running to the pyramidal cells of CA3 and CA4 areas (Fig. 1a, f). All the affected cell bodies were considerably shrunken, and several of them stained homogeneously (Fig. 1c, d), while others contained granular silver deposits (Fig. 1b, e). Most dendrites of the homogeneously stained cell bodies appeared continuous and had tortuous segments (Fig. 1c); whereas, those belonging to granularly stained cell bodies displayed the features of clasmatodendrosis (Fig. 1b, e). Most axons were beaded or fragmented (Fig. 1f). The number of affected cells progressively increased with survival time. The relative number, distribution pattern and cellular morphology of silver-stained granule cells varied from animal to animal within the same experimental group. However, in most animals the majority of the degenerating granule cells occurred in the hidden blade of the middle and temporal thirds of the dentate gyrus. In four adrenalectomized animals the symptoms of degeneration did not develop at all.

Evaluation of toluidine-blue stained semithin sections of DG

Toluidine blue staining of Epon-embedded semithin sections revealed numerous hyperchromatic, pycnotic cells intermingled with non-affected, more lightly stained granule cells (Fig. 1g). At higher power, differences were noted in different sectors of the granule cell layer. Certain spots seemed to be less (Fig. 1h), others more (Fig. 1i), affected. In the former case, the granule cells looked healthy, and only occasional dark, degenerating profiles were present (Fig. 1h). Contrarily, severe granule cell diminution and an increase in numbers of astrocytes and microglial cells characterized other, sometimes neighbouring sectors (Fig. 1i). The remains of decomposing granule cells were found in processes of astrocyte cells (Fig. 1j).

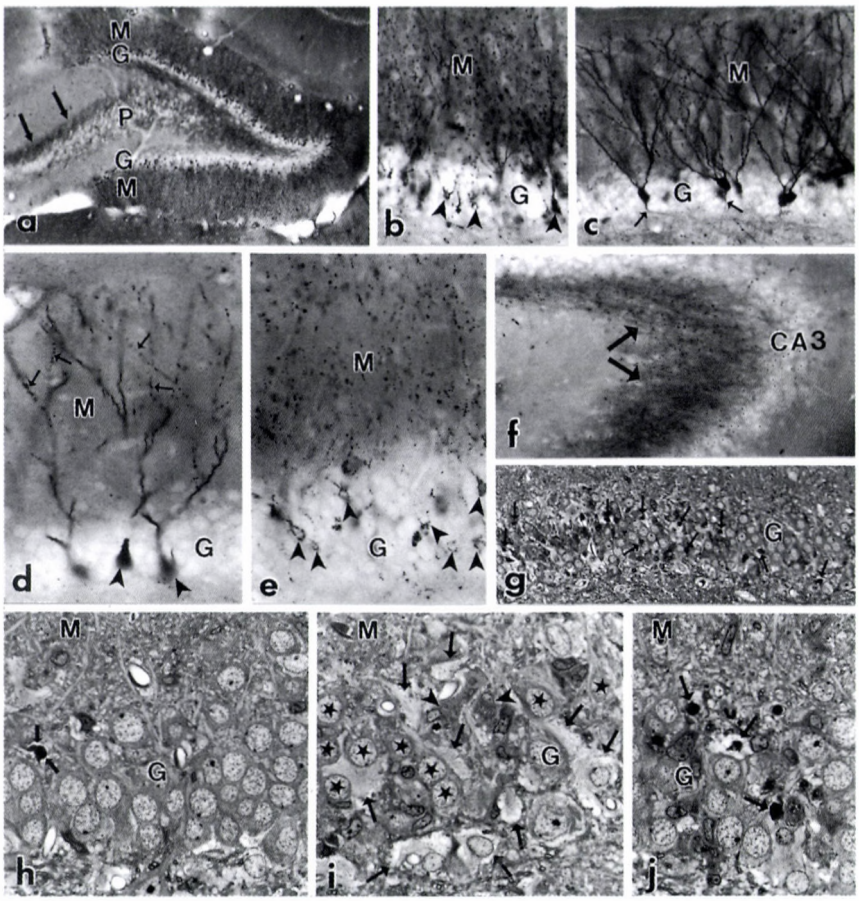


Fig. 1. Light-microscopic characteristics of degenerating granule cells in the dentate gyrus of long-term adrenalectomized rats revealed by means of silver (a—f) and toluidine blue (g—j) stainings. M: molecular layer; G: granule cell layer; P: pyramidal cell layer

a) Low power micrograph of the dentate gyrus demonstrates silver-stained "dark" neurons in the granule layer of both the dorsal and ventral blades. The molecular layer contains heavily stained dendritic trees of the "dark", granule cells. Arrows point to mossy fibers en route to pyramidal cells. x 54. b) Degenerating granule cells with dendritic varicosities and varying amounts of silver deposits in their cytoplasm (arrowheads). x 220. c) Silver-stained degenerating neurons possess dendrites with corkscrew-like and tortuous segments. Arrows indicate the axons originating from granule cells. x 220. d) Axon-like beaded appearance of some degenerating dendrites (arrows) of granule cells (arrowheads). x 340. e) Silver granules indicate the disintegration of dendrites in the molecular layer; the cell bodies (arrowheads) are still recognizable with granular silver deposits. x 340. f) Silver-stained mossy fibers (arrows) reaching pyramidal cells in the CA3 area of the hippocampus (CA3). x 110. g) Semithin section taken from a non-intensified dentate gyrus demonstrates several hyperchromatic and shrunken pycnotic cells (arrows) within the granule cell layer of the dorsal blade. x 180. h) Healthy-looking granule cells with a solitary decomposed (arrows) neuron. x 570. i) Paucity of granule cells (asterisks), abundance of astrocytes (arrows) and presence of microglia cells (arrowheads) characterize the pathology of the granule cell layer. x 570. j) Pycnotic profiles (arrows) engulfed by astrocyte processes. x 570

Ultrastructural properties of degenerating granule cells

In accordance with the light microscopic finding (Fig. 1h), several subpopulations of granule cells did not demonstrate signs of cell death after adrenalectomy (Fig. 2a). The affected neurons displayed an increased electron density (Fig. 2b—j). However, differences in electron density were noted among degenerating neurons. Granule cells in the early phase of degeneration demonstrated a very dense, prominent nucleolus and a slightly less dense nuclear matrix in which the chromatin seemed to clump and glycogen-like particles appeared (Fig. 2b). Some of the mitochondria were vacuolated; other organelles seemed to be intact. Cells in the late phase of degeneration were so highly electron dense that their intracellular architecture was indiscernible (Fig. 2c). The cells shrank considerably during collapse and displayed bizarre shapes due to the invagination of astrocyte processes onto their cytoplasm (Fig. 2d). Astrocytes almost completely encircled and isolated the degenerating profiles from the surviving tissue (Fig. 2d). Some of the afferents to deteriorating granule cells were detectable even at advanced stages of degeneration (Fig. 2e).

The above-described pathological alterations were also manifested in dendrites and axons of granule cells. The dendrites first darkened (Fig. 2f), then became varicose (Fig. 2g) and, later, fell apart as astrocytes engulfed them like other remains of the degenerating neurons (Fig. 2h). The mossy fibers terminating on pyramidal cells of CA3 also demonstrated a dense, dark appearance (Fig. 2i). The terminals displayed highly electron dense vesicles and dark mitochondria. The postsynaptic targets, the pyramidal cells, were not altered morphologically (Fig. 2i). The debris of granule cell axons were also noted separated from pyramidal neurons and buried in astrocyte processes (Fig. 2j).

Features of degenerative plaques in the granule cell layer

At certain loci the dentate gyrus demonstrated a significant loss of granule cells and proliferation of glial cells, as shown in Fig. 1i. By taking such samples for electron microscopy, the final acts in the death of granule cells and reorganization of the granular layer were revealed. The reactive plaques contained the remains of degenerating granule cells and glial elements. The decomposed structures were surrounded by astrocyte processes (Fig. 3a—e) and microglial cells. The astrocytes exhibited rich

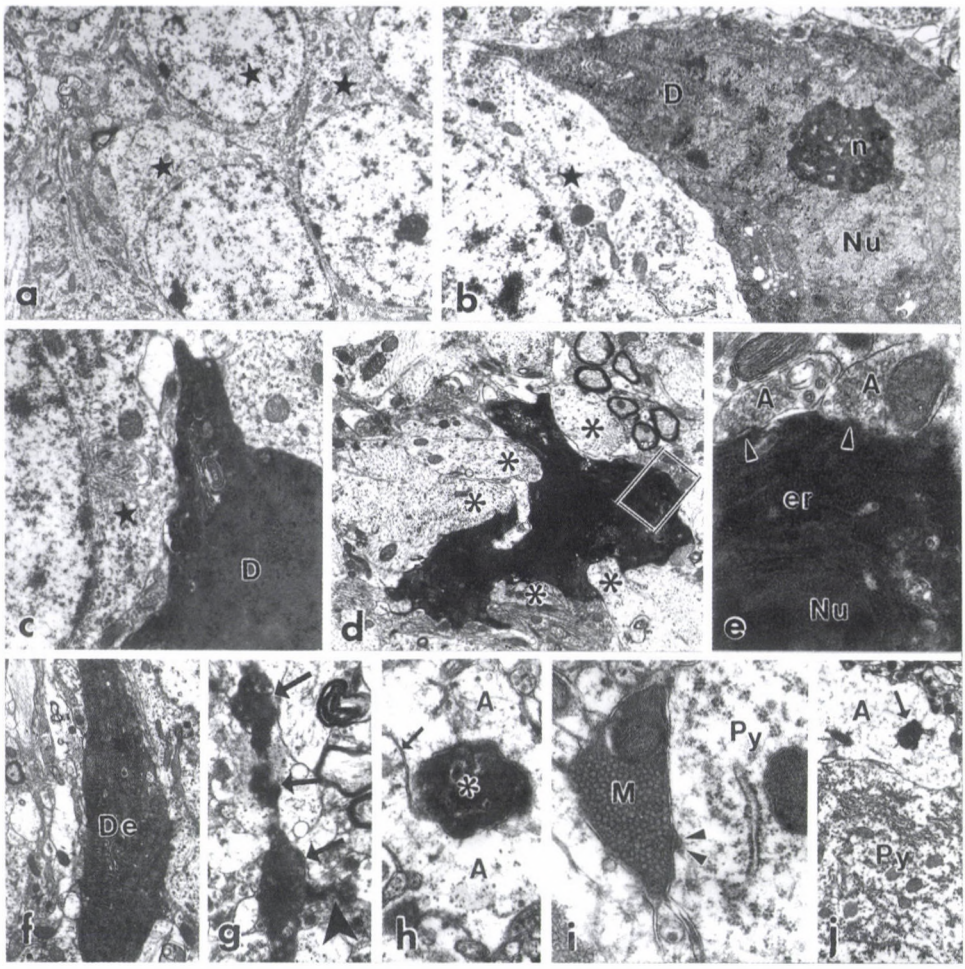


Fig. 2. Ultrastructural detection of degenerating granule cells in the hippocampal formation of adrenalectomized rats. a) Granule cells (stars) without obvious signs of degeneration. x 3,100. b) Intact (star) and deteriorating (D) granule cells in juxtaposition. The degenerating neuron exhibits a prominent, enlarged nucleolus (n) and increased electron density of both the nucleus (Nu) and cytoplasm. The organelles can still be recognized. x 9,000. c) A dark collapsed neuron (D) adjacent a granule cell (star). x 11,500. d) Bizarre profile of a degenerating granule cell surrounded by astrocyte processes (*). x 4,800. e) Higher power of the enframed area in Fig. d. demonstrates two terminating (arrowheads) axons upon the collapsed neuron. Nu.: nucleus; er: endoplasmic reticulum. x 35,500. f) A proximal granule cell dendrite (De) with dark appearance. x 6,000. g) Decomposing dendrite demonstrates varicosities (arrows) in the molecular layer. Arrowhead points to a dendritic spine. x 11,500. h) Hardly identifiable degenerating profile (*) deeply invaginated (arrows) into an astrocyte (A). x 17,800. i) A dark, degenerating mossy fiber (M) synapses (arrowheads) on a pyramidal cell (Py). x 35,500. j) Astrocyte process (A) juxtaposed to a pyramidal cell (Py) contains the debris of a mossy fiber (arrow). x 4,800

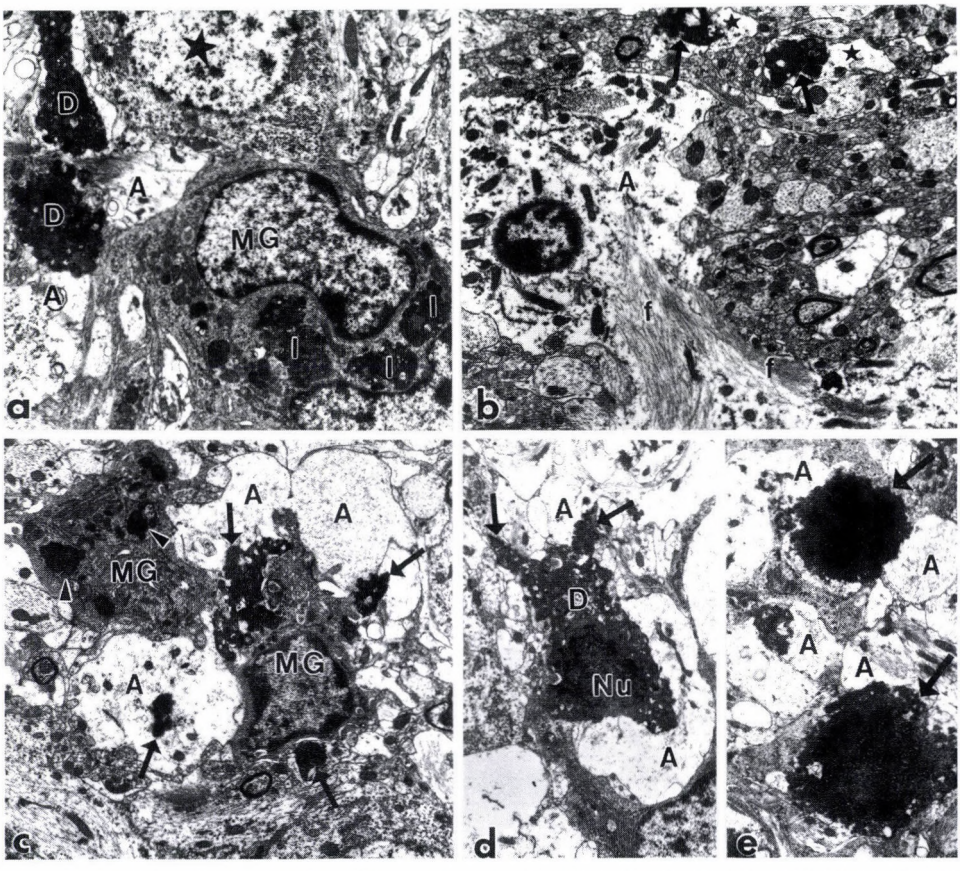


Fig. 3. Participation of glial cells in the reorganization of the granule cell layer of the dentate gyrus in adrenalectomized rats. a) Degenerating profiles (D) surrounded by astrocyte processes (A) and inclusion bodies (I) in the cytoplasm of a neighbouring microglia cell (MG). x 4,800. b) Astrocyte (A) containing bundles of filaments (f) in its cytoplasm and two astrocyte processes (stars) enclosing degenerating neuronal elements (arrows). x 6,000. c) Fragments of degenerating granule cell(s) (arrows) engulfed and surrounded by a cluster of astrocytes and microglia cells (MG). Arrowheads indicate secondary lysosomes. x 4,800. d) Shrunken, dark granule cell (D) with recognizable nucleus (Nu) and bifurcating main dendrite (arrows) is isolated by astrocyte processes (A). x 3,800. e) The perikaryal remains of two degenerating granule cells partly encompassed by processes of astrocytes (A). x 4,800

bundles of filaments (Fig. 3b), and their processes engulfed larger parts (Fig. 3d) and small fragments (Fig. 3b) of the degenerating neurons. The proliferation of microglia (Fig. 3a, c) was also an obvious sign of the reorganization. These cells contained numerous inclusion bodies (Fig. 3a) and secondary lysosomes (Fig. 3c). Sometimes the shrinking degenerating cells maintained their shape (Fig. 3d); in other cases only the perikaryal contour

was sustained (Fig. 3e). The thin, finger-like processes of astrocytes invaginated into the decomposing profiles imparted a frondose appearance (Fig. 3d, e). At this final stage of degeneration the nuclear chromatin of the cells was clustered into larger aggregates (Fig. 3e).

Discussion

The hippocampal formation is one of the main targets /6, 16/ of glucocorticoids secreted by the adrenal gland. The ligands entering the cells activate specific receptors /34/ that undergo conformational changes and translocation to the cell nucleus where they can influence the genetic machinery of the neurons /3/. The effects mights be different upon different cell types of the hippocampal formation. Circulating glucocorticoids can potentiate ischemic injury /28/ and exacerbate neuronal damage associated with aging /31/ by affecting mainly pyramidal neurons. While glucocorticoids seem to potentiate the cell loss of the Ammon's horn, they protect and maintain the structural integrity of the dentate gyrus /13, 32/. Sloviter et al. /32/ have reported that adrenalectomy of young adult male rats resulted in a significant loss of the granule cells 3 to 4 months after adrenalectomy. The response proved to be selective because pyramidal neurons were not affected. Corticosterone replacement /32/ or local administration of dexamethasone into the dentate gyrus /13/ prevented the granule cell loss and the attenuation of the electrophysiological response of the cells /32/.

Until recently, conventional stains have been used for the detection of degenerating granule cells that visualized the pycnotic cells with characteristic hyperchromatic cytoplasm and condensed nuclear chromatin /11, 18, 32, 39/. The number of pycnotic cells seemed to correlate with the time elapsed after adrenalectomy /11/. Golgi impregnation is also a useful tool in detecting affected neurons provided they are numerous in the area studied. Golgi-impregnated samples from the dentate gyrus revealed a decrease in the cross-sectional cell body area and numbers of dendritic branch points one week after adrenalectomy /11/.

The detailed morphological analysis of degenerating neurons requires techniques that are highly selective for the decomposing cells and capable of detecting not only the cell bodies but also the processes of the neurons. Using a modified /22/ silver intensification technique /7/, Jaarsma et al. /13/ have recently described the pattern and time course of somatic and

neuritic degeneration of granule cells in the dentate gyrus of adrenalectomized Wistar rats of both sexes at the light microscopic level. Our intention has been to study the adrenalectomy-induced alterations of granules cells and the healing process of the dentate gyrus at the electron microscopic level. To obtain light microscopic correlates and samples from the most desired areas of the hippocampal formation we applied a new silver technique /8/ for the tracing of adrenalectomy-induced dark neurons. The advantage of the technique is that it stains selectively both the early and advanced forms of "dark neurons", together with their dendritic arborizations and axonal projections. By means of this method granule cells became stained exclusively and displayed a Golgi-like image after adrenalectomy. Neither other hippocampal structures nor other loci of the brain known to exhibit receptors for glucocorticoids, e.g., the hypothalamic paraventricular nucleus /5, 24/, regulator of several neuroendocrine functions, demonstrated silver-stained degenerating neurons. Concerning the spatial distribution of the degenerating cells our finding confirms that observed 1 to 3 weeks after adrenalectomy /13/, i.e., the majority of the degenerating cells occurred in the hidden blade of the middle and temporal thirds of the dentate gyrus. In some animals, however, degenerating granule cells were sporadic; in others they were absent from the dentate gyrus supporting the view that residual corticosterone levels (15 ng/ml) of accessory adrenal origin /27/ are sufficient to protect the cell loss /19/. The scale of dendritic damage is wide varying from slight configurational changes to total disintegration. Beaded processes and scattered multiple argyrophil pearls are the most common phenotypes of the pathological dendrites. The intensity of the silver staining of the molecular layer and the mossy fiber bundle depended on the number of degenerating neurons. Jaarsma et al. have reported /13/ that the maximal response of the dendrites occurred by the end of the first postoperative week followed by a decline in the dendritic and an increase in the somatic labelling. The technique used in this paper detects different parts of the degenerating neurons homogeneously. Thus, both corkscrew-like dendrites belonging to heavily stained and varicose or fractured dendrites of granularly labeled perikarya did exist even 5 weeks after adrenalectomy in our material.

As anticipated, toluidine blue staining of semithin sections was useful for the demonstration of the pycnotic cell bodies but not the dendrites and the axons of dark granule cells. Astrocytes, microglial cells and degenerating neurons were distinguishable in toluidine blue-stained specimens.

The analysis of series of semithin sections showed that the morphological alterations were not uniform even in severely injured areas of the dentate gyrus. Damaged and non-affected sectors appeared randomly. Whether this phenomenon reflects differences among granule cells in sensing glucocorticoids or having distinct protective mechanisms is unknown.

In concert with the light microscopic finding, degenerating cells intermingled with non-affected neurons /21/ at the ultrastructural level. The increasing electron density and shrinkage of the entire cell are the leading symptoms of the early phase of degeneration. Accompanied by cytoplasmic vacuolization and chromatin condensation the cells collapse further and astrocyte processes appear around them gradually resulting in the loss of their neuronal afferents and, later, in a total disintegration of the cells. This process is pronounced in the dendrites that gradually fall apart producing swollen, dark debris remaining lined up for a time, giving the characteristic beaded appearance at the light microscopic level, before glial processes engulf and remove them from their original position.

By finding degenerating mossy fibers in synaptic connection with pyramidal neurons of the C3 area of the Ammon's horn and remains of degenerating axons around the pyramidal neurons, we provide evidence for the denervation of pyramidal cells by granule cell axons after adrenalectomy. The potential fine and late consequences of this partial deafferentation have to be elucidated. The pyramidal neurons receiving degenerating mossy fibers do not display pathological features at the ultrastructural level 4 weeks after the removal of the adrenal glands.

The degenerating spots also have glial constituents. Decomposing granule cells, astrocytes and microglial cells form the pathological plaques. The remains of the cells are often seen in astrocyte processes and in microglia as inclusions. The total disintegration of granule cells and decomposition of their debris by glial cells unequivocally indicate that the absence of glucocorticoids results in cell death and reorganization of neuronal circuits of the hippocampal formation.

The putative mechanisms by which glucocorticoids might influence the survival of hippocampal neurons have been reviewed /18/ recently. Loss of nerve growth factor protection /1/, altered electrophysiological activity /25, 37/ and damage from lipid peroxidation /12/ are among the candidates for detrimental effects of absence of glucocorticoids. After finding the trigger, further studies should explore the molecular basis /18/ of cellular cascade mechanisms /9/ that finally result in the death of granule cells /33/.

The adrenalectomy-induced degeneration of granule cells of the hippocampal formation possesses four unique features. 1. The vulnerability seems to be selective for this subpopulation of glucocorticoid receptor-expressing neurons of the brain. 2. The morphopathological process is extremely delayed. 3. The onset of the events is not synchronized. 4. The degenerating cells have a random distribution within the hidden blade of the dentate gyrus. There is a considerable number of other pathological conditions like cerebral ischemia /10, 14/, hypoglycemia /15/, experimental epilepsy /20, 23/, and cell poisoning /38/ that result in cell death with the above characteristics /8/. Therefore, it is reasonable to suppose that the different noxious stimuli that affect neurons via different channels initiate a common cellular cascade mechanism that starts with a cytoskeletal collapse and terminates in an irreversible damage of the cells. The condensation of ultrastructural elements both in the perikaryon and the cell nucleus with concomitant increase in electron density of the affected granule cells favors the concept that a sudden cytoskeletal /9/ collapse might be responsible for the pathogenesis of "dark neurons" after adrenalectomy. The activation of the apoptotic machinery has also been proposed /34/ to take part in the adrenalectomy induced granule cell loss.

In summary, this paper introduced a novel light microscopic silver staining method for the selective detection of degenerating, "dark granule" cells in the dentate gyrus of adrenalectomized animals and provided the ultrastructural correlates of the pathological alterations. All parts of the affected cells undergo degeneration and decomposition resulting in diminution of the granule cells, proliferation of glial cells and denervation of CA3 and CA4 pyramidal neurons.

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SUBSTANCE P IMMUNOREACTIVE ELEMENTS IN THE NERVOUS SYSTEM OF EARTHWORM (LUMBRICUS TERRESTRIS)

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Substance P immunoreactive elements were detected in the nervous system of the earthworm, Lumbricus terrestris using peroxidase-antiperoxidase method. Each part of the central nervous system contains immunoreactive perikarya. The central neuropil shows no stained fibers. Immunopositive nerve cells were found in the stomatogastric ganglia as well as nerve cells and fibers in the wall of the alimentary canal. In the body wall, epidermal sensory cells and fibers surrounding the chaetae display immunoreactivity. The results are compared with those described previously by other authors.

Keywords: Lumbricus terrestris — nervous system — substance P — immunohistology

Introduction

Mammalian neuropeptides have been shown to occur in many invertebrate species /6, 22, 30–32, 35/. Among these peptides, substance P (SP) is one of the longest history, and it has been proved to be a sensory neurotransmitter in mammals /12, 27/.

The presence of SP has been demonstrated in various annelids /7, 8, 14, 17/, though the results in the earthworm Lumbricus terrestris are contradictory. Aros et al. /1/ found small SP immunoreactive cells in the cerebral ganglion giving a strong or medium dense reaction and sending their processes to the neuropil. No immunoreactive cells or fibers were demonstrated in the subesophageal ganglion. Other parts of the nervous system were not studied. By means of a radioimmunoassay technique Kaloustian and Edmans /13/ demonstrated the presence of a SP-like peptide in the intestinal and body wall tissues, ventral nerve fibers and seminal vesicles of the

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earthworm. The authors measured the greatest SP-like immunoreactivity (SPLI) in intestinal tissues where it was shown to stimulate the rate of spontaneous contraction. Using an indirect immunofluorescent technique, Curry et al. /6/ surprisingly found no SPLI in the tissues of the earthworm, that, as they pointed out, did not necessarily indicate the absence of SP. In Eisenia fetida, another earthworm species, Lkhider et al. /17/ observed only a few, faintly reacting perikarya in the cerebral ganglion.

Considering these contradictory data, it seemed to be tempting to investigate the presence of SPLI in the nervous system of the earthworm using immunohistochemistry. In a preliminary study our laboratory reported the presence of SP in the nervous system of the earthworm /16/, further contribution is given to these results in the present study. By means of an image-analysis technique, reconstruction of the immunoreactive elements was achieved, giving a better demonstration of the distribution of the cell groups stained by the antiserum.

Materials and Methods

Adult specimens of the earthworm, Lumbricus terrestris were collected locally during spring and summer periods. The animals were maintained in fresh moist foil supplemented with leaves until required. Prior to decapitation the animals were quickly anesthetized in 10% ethanol. For fixation, the first 6-8 body segments were removed, and fixed in Zamboni's fixative /36/. Penetration of the fixative was promoted by treating the specimens with microwave irradiation /21, 25/. Excess amount of fixative was removed by washing the specimens in phosphate buffered saline solution (PBS) followed by dehydration in graded ethanol and embedding into paraffine (Paraplast, 52 ^{o}C melting point). Serial frontal, sagittal or horizontal 10 μm sections were cut from the embedded materials.

Immunohistochemistry was performed on mounted sections after treating the sections with trypsin solution (0.12%). Unspecific background staining was inhibited by incubation in 1% normal sheep serum (20 min). Subsequently the sections were stained according to the unlabeled peroxidase-antiperoxidase (PAP) method /33/. Anti-SP serum was obtained from Sigma. The incubation time was 48 h at 4 ^{o}C, at a dilution of 1:10 000. The rest of the staining procedure was carried out at room temperature. The sections were then incubated in anti-rabbit-gamma globulin (ARGG, sheep, 1:300) for 10 min, and in PAP (1:600) for 10 min. ARGG was raised in our laboratory and PAP was obtained from Arnel. The entire procedure starting from the treatment with ARGG was repeated (double PAP method). Thorough washing in PBS was performed after each step of staining procedure. Bound PAP was visualized by 0.25% 3,3-diaminobenzidine in 0.05 M Tris buffer at 7.5 pH containing 0.01% H_2O_2 (20 min). The diaminobenzidine end product was intensified in osmium tetroxide vapor.

Specificity of the anti-substance P antiserum was tested by preabsorption of the primary antiserum with substance P (Sigma, 10 mg/100 μl staining dilution). Method controls were performed by omitting primary antiserum.

Pseudoperoxidase axtivity was inhibited in control sections by 0.3% H_2O_2 in saline. Comparison of these control as well as native sections with the immunostained sections is especially important in the case of Lumbricus terrestris since in the immunostained but otherwise not counter-stained sections the natural color of the pigment of the animals in the cuticula and in the dorsal body wall muscles resembles to the color of the diaminobenzidine end product.

Preparations were examined and photographed using a Nikon photo microscope Microphot-FXA. Reconstruction of the immunopositive cell groups was achieved by using the software NIH Image 1.55 for Macintosh on digitalized pictures captured by MMCCD72 video camera attached to the microscope. Following graphical processing and the appropriate dimensional orientation of horizontal sections of the ventral cord ganglia, consecutive slides were projected in transparency mode on the coronal plane.

Results

No immunoreactivity was observed following preabsorption or omission of the primary antiserum. The distribution of immunoreactive cells showed no difference in control sections where endogenous peroxidase activity was inhibited by H_2O_2. The color of the natural pigment of the animal can be distinguished from the diaminobenzidine end product. The appearance of the natural pigment is homogeneously black in contrast to the granular brown color of the immunostaining. This distinction was reinforced by comparing the native and the immunostained sections.

Each part of the earthworm central nervous system contains SP-like immunoreactive perikarya. Most of the immunopositive cells show medium dense reaction. The staining appears granular in the cell somata around the nuclei. No processes of these cells are visible, and the neuropil mostly shows no immunoreactivity. SP immunoreactivity is found outside the central nervous system as well.

The cerebral ganglion contains 80-110 SP-like immunoreactive nerve cells. These are most abundant in the dorsal part of the ganglion, and are distributed in medial, dorsomedial and dorsolateral groups (Fig. 1). Cells are scattered in the central part as well, while at the ventral boundary of the ganglion small immunopositive nerve cells are aligned in a row. These latter are found only in the caudal part of the ganglion, where the connectivum already appears. At the emergence and even in the initial part of the circumpharyngeal connectivum small lateral cells are found. The smallest immunoreactive cells are the medial (Fig. 2), ventral and the lateral cells, which measure between 10-15 μm. The dorsal and the central (Fig. 3) cells are very variable in size, they are between 15-30 μm. The distribution

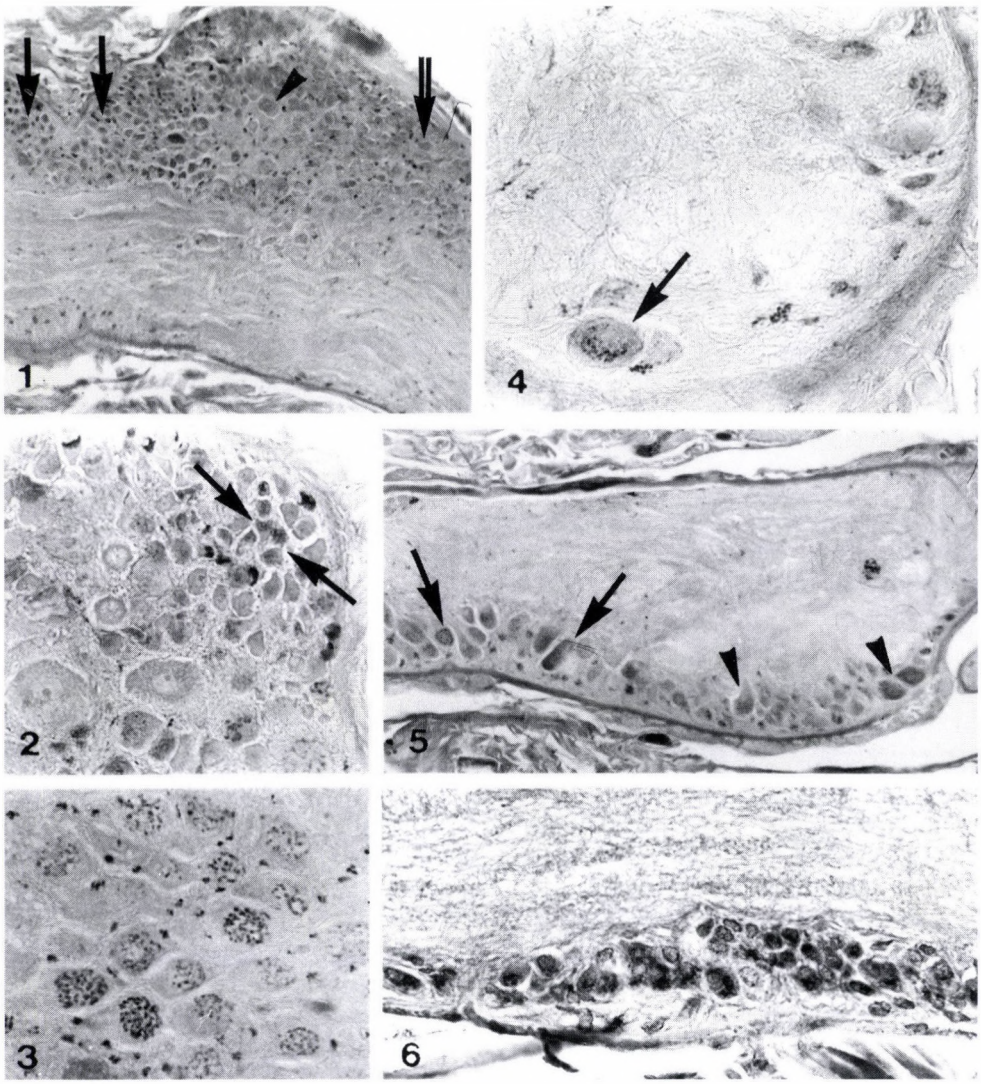


Fig. 1. Medial (arrows), dorsomedial (arrowhead) and dorsolateral (double arrow) immunoreactive cell groups in the cerebral ganglion. x 75. Fig. 2. Small immunoreactive medial cells (arrows) in the cerebral ganglion. x 250. Fig. 3. Immunoreactive cells of the central cell group in the cerebral ganglion. x 250. Fig. 4. Medial immunoreactive cell in the rostral part of the subesophageal ganglion (arrow). x 250. Fig. 5. Immunopositive ventromedial (arrows) and ventrolateral (arrowheads) cells in the subesophageal ganglion. x 125. Fig. 6. Ventral immunoreactive cell layer in the subesophageal ganglion. x 150

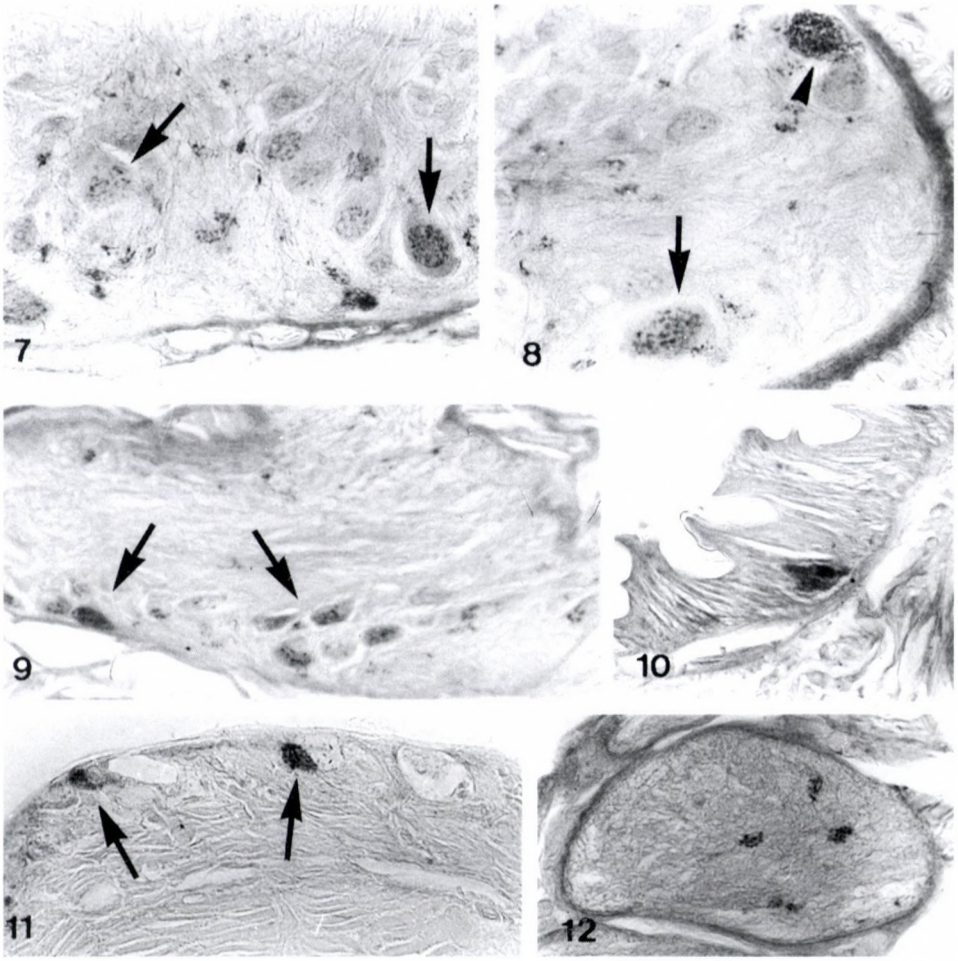


Fig. 7. SP immunoreactive medial cells in a ventral cord ganglion (arrows). x 250. Fig. 8. Solitary ventrolateral (arrow) and lateral (arrowhead) immunoreactive cells in the ventral cord ganglion. x 250. Fig. 9. Immunoreactive cells in the stomatogastric ganglion (arrows). x 250. Fig. 10. Immunoreactive cell in the wall of the foregut. x 250. Fig. 11. Epithelial sensory cells displaying SP immunoreactivity (arrows). x 250. Fig. 12. Prostomial nerve showing SP immunoreactivity. x 250

pattern of the immunoreactive cells in the cerebral ganglion is shown in Fig. 13a. The central neuropil shows faint immunoreactivity, the processes of the cells cannot be detected.

In the most rostral part of the subesophageal ganglion the immunoreactive nerve cells are found bilaterally, close to the midline (Fig. 4). Caudal to these cells the stained perikarya are distributed in ventromedial

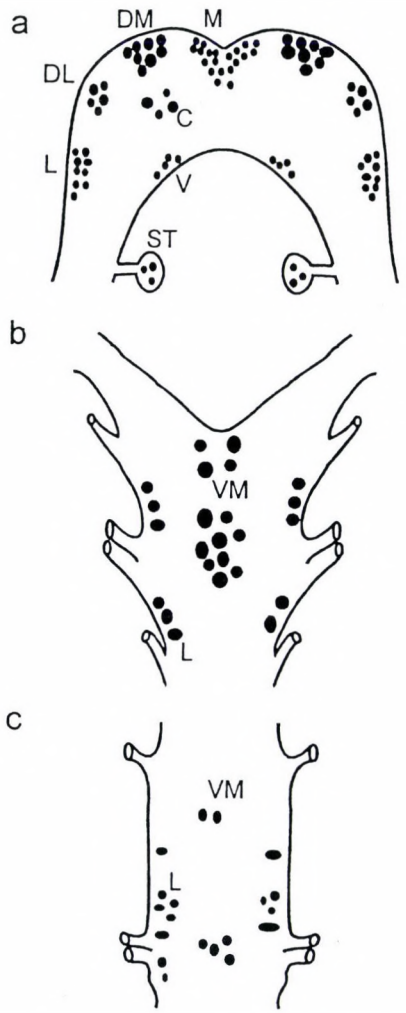


Fig. 13. Distribution pattern of SP immunoreactive cells in the cerebral (a), subesophageal (b) and ventral cord (c) ganglia. (C: central, DL: dorsolateral, DM: dorsomedial, L: lateral, M: medial, V: ventral, VM: ventromedial cell groups, ST: cells in the stomatogastric ganglia.) (Only location of cells and not their exact numbers are given.)

and ventrolateral positions (Fig. 5). Most of the cell bodies are pear-shaped, a few round somata are found in the lateral part. They are evenly distributed in the length of the ganglion (Fig. 13b), forming a ventral immunoreactive cell layer (Fig. 6). The average number of the immunostained cells is 40-50.



Fig. 14. Distribution of SP immunoreactive cells in a ventral cord ganglion. Summation of immunostained sections of a representative ganglion in the transverse plane. NIH image based reconstruction, the white areas represent immunostained cells. Note the distinct lateral (L) and ventromedial (VM) cell groups (GF: giant fiber, SN: segmental nerve)

The ventral cord ganglia contain 20-30 SP-like immunoreactive cells which do not form a continuous cell layer in the ganglia studied (Fig. 13c). Solitary somata or groups of 4-5 cells are found in ventromedial (Fig. 7), ventrolateral and lateral (Fig. 8) positions (Fig. 14). Lateral immunostained cells are most numerous at the origin of the segmental nerves. The cells are medium sized (20-25 μm), pear-shaped, only a few larger lateral cells are found (30-35 μm). The central neuropil shows faint immunoreactivity in the ventral ganglia.

In the peripheral nervous system, constant staining is detected in the stomatogastric ganglia and in the intestinal wall. The stomatogastric ganglia contain 20-30 perikarya which stain for the SPLI (Fig. 9). Many solitary nerve cells are found scattered in the wall of the foregut throughout the studied segments (Fig. 10). Fine fibers are also detected in the wall of foregut.

Some epithelial cells display immunoreactivity in the body wall (Fig. 11). Small cells and a fine plexus were detected around the chaetae. The prostomial nerves show SPLI as well (Fig. 12).

Discussion

In the present study SPLI was shown in the nervous system of the earthworm. Neurons in both the central and in the peripheral nervous system showed SPLI, while nerve fibers displayed faint immunoreactivity in the intestinal wall, in the prostomial nerves and in plexuses around the chaetae. The lack of immunoreactivity in axons in other parts of the nervous system is probably due to the low concentration of SP-like material. The reason for the granular appearance of the immunostaining in most of the neurons is not known. Similar granular appearance was observed using other antisera in Lumbricus terrestris /16/. Similarities and differences between our findings and those previously described by other authors regarding occurrence and distribution of SPLI in Lumbricus terrestris are discussed below.

In contrast to the results of Aros et al. /1/, SPLI was detected not only in the cerebral ganglion, but in all ganglia, including the subesophageal ganglion, where they found no immunoreactivity. The distribution of immunoreactive perikarya in the cerebral ganglion is similar to the observation mentioned /1/, however not only small cells, but larger cells stained as well. Unlike these workers /1/, we found only faint immunoreactivity in the central neuropil. Lkhider et al. /17/ found far less number of immunoreactive perikarya in Eisenia fetida than we observed in Lumbricus terrestris. Curry et al. /6/ detected no immunoreactivity using an immunofluorescent technique. This, as they pointed out, did not necessarily indicate the absence of SP, and reexamination was suggested by these authors as well.

Our results show a closer correlation with radioimmunoassay data of Kaloustian and Edmands /13/. Besides the central nervous system, we also found consequent staining in the stomatogastric ganglia and in the wall of the alimentary canal. These authors detected significantly lower level of immunoreactivity in the ventral nerve cord than in the intestinal tissue sections. This is also in accordance with our findings, considering the small number of immunoreactive cells in the ventral cord ganglia. Kaloustian and Edmands suggested that the source of the peptide might be primarily from specialized endocrine-type cells rather than neuronal tissues.

The stomatogastric system of Lumbricus originates either from the brain or from the subesophageal ganglion, and the nerves reach the gut directly, or through the stomatogastric nerve cells /26/. Besides the stomatogastric system, nerves innervating the muscle layer of the gut may originate from the ventral cord ganglia /4, 20/. The enteric nervous system shows lack

of ganglionic arrangement in the earthworm, only solitary cells and nerve fibers are found /10/. Since we detected SP-like immunoreactive cells in the wall of the foregut, it is suggested that SP-like immunoreactive nerve fibers may be partly of intrinsic origin, as Kaloustian and Edmands /13/ suggested. This is further supported by the fact, that the gut of annelids shows coordinated motility patterns that are independent of central nervous system activity, and are likely to be controlled by local nerve cells /5/. However, extrinsic origin of SP-like immunoreactive fibers cannot be excluded, since the stomatogastric ganglia contain a large number of immunoreactive cells. Our results also support the dual localization of SPLI in earthworm intestinal and nerve tissues, a pattern characteristic of peptide hormones in vertebrates /2/. Kaloustian and Edmands /13/ detected the lowest level of immunoreactivity in the body wall tissues, which, as they assumed, might be due to residual intestinal tissues. This is in accordance with our results, since we found no immunoreactivity in the body wall, expect in some epithelial cells.

SP belongs to a structurally related peptide family, the tachykinins /19, 28/. Widespread occurrence of SP has been shown in many invertebrates, including annelids as well /22, 23/. However, most of these findings are based on immunocytochemistry or radioimmunoassay. The major problem with these data is that immunoreactivity does not necessarily represent the presence of SP but may be due to other, structurally related tachykinin peptides /34/. Several tachykinin-like peptides have been shown to occur in the same species /24/, furthermore, one neuron may contain more than one tachykinin-like peptide /9/. These could explain the differences between our findings and those described by the above-mentioned authors in Lumbricus terrestris.

The most established roles of the tachykinin-like peptides are mainly the contraction of smooth muscles and sensory neurotransmission /12, 29/. The significance of tachykinins was shown in the stomatogastric and enteric system of many invertebrate species /3, 11, 18/. Goldberg et al. /11/ pointed out that there are several differences in the distribution of SPLI even in closely related species. This could also explain the differences between our results and those found in Eisenia fetida /17/. Recently, Blitz et al. /3/ have shown, that the locusta tachykinins account in part for the substance P-like immunoreactivity in the crab reported by Goldberg et al. /11/. There is evidence that SP stimulates the rate of intestinal contraction in the earthworm, inhibited by a SP antagonist /13/. However, it cannot be ex-

cluded that in earthworm as well, other members of the tachykinin peptide family are also included in the innervation of the enteric system. Further histochemical experiments with antisera to different tachykinin-like peptides are planned to be carried out to specify the distribution of these peptides in the earthworm, which may be partly responsible for the SPLI found in the present study.

As far as other functions of the tachykinins are considered, the wide distribution of immunoreactivity in the nervous system, in sensory epidermal cells and in plexuses surrounding the chaetae of Lumbricus, it is suggested that members of the tachykinin peptide family may be involved in sensory processes as well. The majority of the immunoreactive cells in the cerebral ganglion are most probably interneurons. Further investigation is needed to prove these assumptions, for which immunohistochemical descriptions give a useful starting point.

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Acta Biologica Hungarica 48 (2), pp. 201—207 (1997)

CHROMATOGRAPHIC SCREENING OF GROWTH INHIBITORS FOR CLADOSPORIUM HERBARUM IN THE EXTRACTS OF CALCAREOUS RED ALGAE

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The thin-layer chromatographic screening of growth inhibitors for an imperfect fungus was carried out in two types of calcareous red algae, Corallina pilulifera (Corallinaceae) and Lithothamnium pacificum (Corallinaceae) and Crathromorchum circumscriptum (Corallinaceae). Several substances having positive activities on Cladosporium herbarum were detected in the alcoholic extracts of the marine plants. The observed differences among the substances can be attributed to the morphological characteristics of the sea weeds.

Keywords: Growth inhibitors — red algae — black mould — chromatography

Introduction

A vast variety of calcareous algae is known to be responsible for "ISOYAKE" (rocky-shore). Where "Isoyake" once occurred, useful sea plants such as Laminaria japonica, L. religiosa, etc. cannot grow fully and thereafter the culturing banks are wasted gradually. "Isoyake" is now growing more steadily and more seriously on a worldwide scale. Although a wealth of information /1, 3, 4/ has been supplied and certain indicative evidence /2/ is ready in our hands, real aspects of the alarming source remain largely unknown. We assume that noxious substances might be excreted from the cal-

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Abbreviations: BWA: 1-butanol/acetic acid/water (4:1:2, v/v); ETA: ether/toluene/acetone (60:15:2, v/v); CTHA: chloroform/toluene/hexane/acetone (70:13:15:4, v/v); THE: toluene/hexane/ether (70:2:0.5, v/v); TLC: thin-layer chromatography; VIS: visible-light; UV: ultraviolet-light.

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careous algae and through the algae-excreted process the substances restrict the healthy growth of sea plants belonging to other genera.

In this study, it is shown that alcoholic extracts from two morphologically distinguishable types of calcareous algae, namely, thallus with the erect portions articulated (Corallinaceae) and thallus not articulated (Corallinaceae) contain physiologically active substances which inhibit the growth of a black mould.

Material and Methods

Plant materials

Corallina pilulifera (Corallinaceae) (approx. 1000 g wet mass) and Lithothamnium pacificum Foslie (Corallinaceae) and Crathromorchum circumscriptum (Kjerrman) Sosrie (Corallinaceae) (approx. 24 000 g wet mass, with pebble fragments) were collected on 22 July, 1990 at Usujiri, Hokkaido. A culture of Cladosporium herbarum Link ex Fr. was derived originally from a stock line which is maintained at Department of Applied Microbiology, Faculty of Agriculture, Hokkaido University (AHU). It was placed on an agar medium, which contains powdered potato extracts (4 g), glucose (20 g) and agar (15 g) in 1000 ml water (pH 6.0). The mould culture was done at 27 °C in the dark. After about a week, the grown mould culture was kept at 5-10 °C for 2-3 months in the dark. The stocked culture was used throughout this experiment to evaluate the intensity of the growth inhibiting substances.

Chemicals

Vanillin, anisaldehyde and phosphomolybdic acid were obtained from Wako Pure Chemical (Osaka, Japan). Silica gel TLC plates, $60F_{254}$ No. 5751 with a fluorescent indicator (20 x 20 cm, 0.25 mm thick) were purchased from E. Merck (Darmstadt, Germany). All other reagents used in this study were of analytical grade of purity obtained from several commercial sources. Solvents were glass-distilled prior to the use for extraction and/or chromatographic operations.

Instruments

An electric densitometer, Dual-Wavelength Flying-Spot Scanner (Shimadzu, model CS-9000) was used. The bio-grams were photographed using a Nikon F2 camera with a Fuji Film, Neopan SS. An UV lamp (PAN UV lamp, PUV-LA, Topcon, Tokyo) was used for examining the substances on air-dried chromatograms.

Extraction of growth inhibiting substances

Carollina pilulifera, thallus with the erect portion articulated, and Lithothamnium pacificum and Crathromorchum circumscriptum, thallus not articulated, which stick to pebble fragments, were immersed separately in freshly rectified ethanol (1800 and 21 700 ml, respectively) and extracted

at room temperature of about 22 °C three times at intervals of one week, and at each of the three tests, the solvent was replaced with new one (1800 and 21700 ml, respectively). The extracts were concentrated to a small volume with a rotary evaporator below 35 °C, and the concentrate was extracted exhaustively with ethylacetate. The combined ester extracts were evaporated under reduced pressure after treatment by $Na_2SO_4 \cdot 10\ H_2O$. Aliquots of the concentrate were used during the chromatographic operation. The alcoholic extracts were occasionally treated with hexane and the pooled hexane layer was dehydrated in the presence of $Na_2SO_4 \cdot 10\ H_2O$. After removing hexane, the concentrate was applied to the chromatographic process in several solvent systems as shown below.

Chromatography

Five or six milligrams of the extracts were spotted on silica gel TLC plates (10 x 10 cm or 5 x 10 cm) and chromatographed ascendingly in a chamber using following solvent systems: (A) BAW, (B) ETA, (C) CTHA, (D) THE. The resultant chromatograms were air-dried for several days at room temperature of 22 °C in the dark to remove traced solvent completely.

Bio-test

A black mould incubation technique was here applied, for the first time, to detect growth inhibiting substances in alcoholic extracts of red algae; it is superior to usual techniques, in which isolated and partially purified compounds are sprayed directly on test organisms, in following three points mainly -- (1) active substances can be localized on chromatograms by their specific migration distances, (2) relative activity levels of the inhibiton are comparable directly on chromatograms by apparent mould growth, (3) inoculation of the black mould on chromatographed plates is simple and easy. The air-dried chromatograms were placed over silica gel in a vacuum desiccator and dried for several hours by continuous evacuation using an oil rotary vacuum pump (Shinku Kiko, model ULVAC, GVD-050A). Before conducting the bio-test, spores of Cladosporium herbarum were suspended in a spray medium (6 ml) containing mineral salts (μg/ml): NaCl (1), $KH_2PO_4 \cdot 2\ H_2O$ (7), $Na_2HPO_4 \cdot 12\ H_2O$ (3), KNO_3 (4), $MgSO_4 \cdot 7\ H_2O$ (1), Tween 80 (0.2 μl) and glucose (300 mg/ml). The spore suspension was sprayed equally over TLC plates (60-120 $\mu l/cm^2$ of TLC plate) which were kept in a moisture box for 3-4 days at 27 °C in the dark. Occasionally, the bio-grams were surveyed by a Shimadzu electric densitometer to evaluate indirectly the intensity of the growth restriction of the black mould inoculated. To record the inhibitor distribution, the plates were photographed finally.

Location of growth inhibiting substances on TLC plates

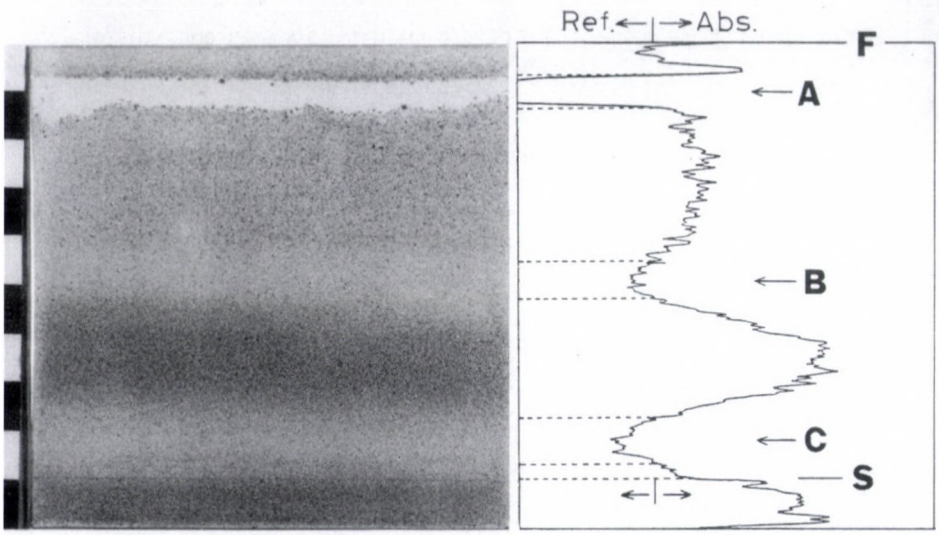
To locate growth inhibiting activity from algal extracts, the TLC plates with no spraying black mould were viewed closely in VIS or under an UV-lamp and marked with a pencil, where they were often compared with those of bio-grams visualized by inoculating Cladosporium herbarum as a bio-indicator.

Tentative characterization of active substances

For diagnosing the growth inhibitors on the chromatograms, three spray reagents, anisaldehyde/sulphuric acid, vanillin/sulphuric acid and phosphomolybdic acid, were used separately /5/. After heating at 100-110 °C for given intervals, the plates were observed in VIS or under UV-lamp and evaluated the coloration mainly on the marked positions.

Results and Discussion

Alcoholic extracts from two morphologically distinguishable types of red algae were subjected to the bio-assay test to examine growth regulating activity on an eukaryote. By using our currently devised technique for inoculation of a black mould, we are now able to accurately show that physiologically active substances are contained in the crude extracts. Figure 1a and b presents typical results from the chromatographic studies. In total, three main bands, A, B and C were found on the bio-gram of Fig. 1a. These whitish bands are all caused by the restriction of the mould growth from algal inhibitors extracted. Among these band-A compound(s) with R_f-values of 0.89 hinders the mould growth most prominently. Whether or not it restricts spore germination and/or hypha elongation is not clear. Judging from the migration pattern on the bio-gram, band-A is obviously hydrophobic. To characterize the compound(s) with growth inhibiting activity on band-A, diagnostic reagents were sprayed as described above. The colour thus visualized was violet-blue, which is known to be characteristic of terpenoids /5/. The band-A compound(s) presumably contributes to the physiological process through interaction with such membrane-bound substances as lipoprotein and related components of the test mould. Other compounds corresponding to B and C with R_f-values of 0.45 and 0.08, respectively are also seen to be efficient inhibitors. They are rather hydrophilic and may exert their effects by mechanisms different from that of band-A compound(s). The activities are both far lesser. Slight difference of compounds are seen on the bio-grams in Fig. 1a and b, which were obtained for the extracts from morphologically different algae, Corallina pilulifera (Corallinaceae) and Lithothamnium pacificum plus Crathromorchum circumscriptum (Corallinaceae), respectively. In particular, band-B compound(s) with R_f-value of 0.45 visualized in Fig. 1a is not seen in Fig. 1b. This suggests that there is an appreciable difference in qualitative distribution between the algal extracts.



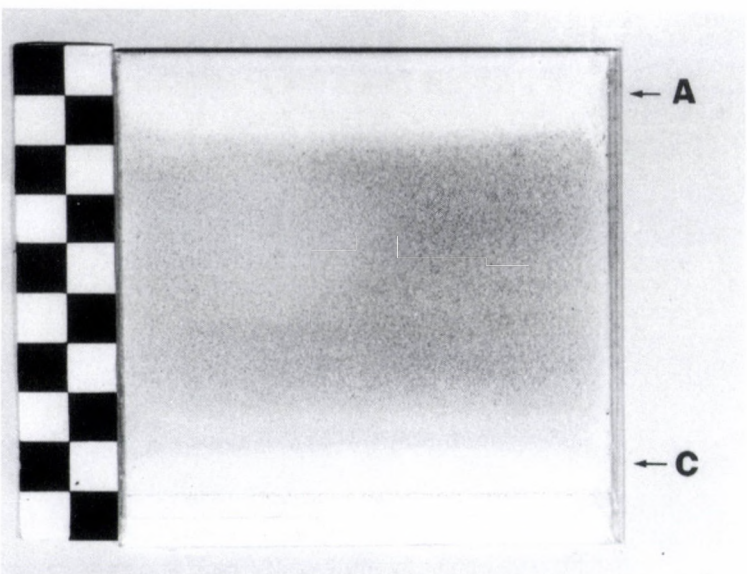


Fig. 1. TLC profiles of alcoholic extracts from calcareous red algae. Gummy residue of ethyl-acetate layer from the ethanol extracts of calcareous sea weeds was spotted on TLC plates, followed by chromatography in solvent (A). (a): Corallina pilulifera. F: front, S: starting point, Ref.: reflection of light, Abs.: absorption of light. (b): Lithothamnium pacificum plus Crathromorchum circumscriptum. For further details of experimental conditions, see Material and Methods

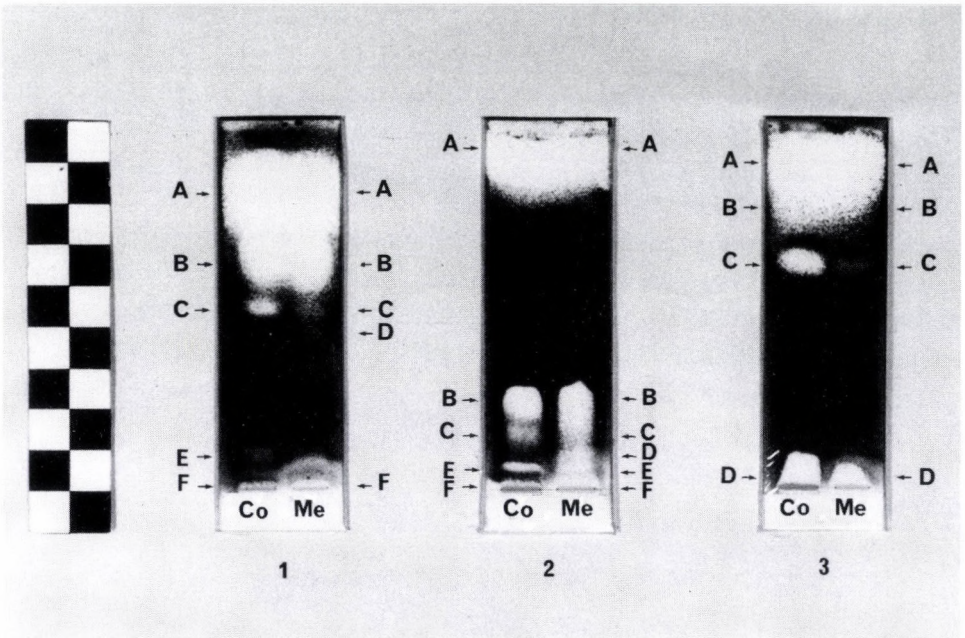


Fig. 2. TLC profiles of alcoholic extracts from calcareous red algae. Darkgreen concentrate of hexane layer from ethanol extracts of calcareous sea weeds was spotted on TLC plates and chromatographed separately in solvent (B)-1, solvent (C)-2 or solvent (D)-3. Co: Corallina pilulifera, Me: Lithothamnium pacificum plus Crathromorchum circumscriptum. For further details of experimental conditions, see Material and Methods

To confirm the above suggestion more precisely, we conducted further chromatographies with different extracts in different solvent systems. The condensed residue from alcoholic extraction was treated with hexane and, after removing the solvent, the organic layer was chromatographed in three different non-polar solvents. The results are given in Fig. 2 (1-3). The photographs reveal that in addition to the qualitative difference, quantitative divergency exists. As seen in Fig. 2 (1), 5 positive inhibitors of the mould growth are present in both Corallinaceae extracts. Band-E compound(s) is contained in Corallina pilulifera, while it is devoid of Lithothamnium pacificum and Crathromorchum circumscriptum. Spot-D in Lithothamnium pacificum plus Crathromorchum circumscriptum extracts, on the contrary, is missing in Corallina pilulifera. This is supported by Fig. 2, showing that spot-D in the extracts from Lithothamnium pacificum plus Crathromorchum circumscriptum is not found at the same position on the chromatogram obtained using Corallina pilulifera. Quantitative variation can be recognized by the

shade of colour on the photograph. Spot-C in corallina pilulifera is largely and clearly accentuated, while it is faint and weak in other Corallinaceae sp. (see Fig. 2 (1)). Similar results indicate the possibility that each alga produces compounds peculiar to the species at varied levels.

In this study, we perhaps provided the first and direct evidence that these sea weeds contain metabolites affecting the normal growth of an organism that belongs to other division. It was observed in a culture by Masaki et al. /2/ that the germlings of Laminaria japonica did not grow well on the living crustose coralline alga, Lithophyllum yessoense. The growth inhibition of the germlings may be caused by physiologically active substances detected in this study. Bio-assay and characterization of the active substances are now in progress in our laboratory.

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FACTORS AFFECTING TRANSIENT EXPRESSION OF VECTOR CONSTRUCTS IN WHEAT PROTOPLASTS

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Direct uptake of reporter gene constructs with the bacterial β-glucoronidase (GUS) gene fused to various promoters was achieved to embryogenic cell suspension culture-derived protoplasts of GK Ságvári winter wheat (Triticum aestivum L.) with polyethylene glycol (PEG) treatment. Based on GUS specific activity values, it was found that Mg^{2+} with PEG at 20% final concentration can significantly increase the transient expression in wheat protoplasts in comparison to the Ca^{2+}-containing medium. The optimum incubation time in transformation mixture was 5-10 min at 25 °C. Transient GUS expression as detected by spectrofluorimetry was positively correlated with the time elapsed after DNA uptake (with maximum activity at 48 h), and the incubation time in GUS reaction mixture. It was also found that the protoplast culture medium plays an important role in the efficiency, and the treated wheat protoplasts cultured in KM medium showed a higher GUS activity than those kept in GM medium. Among the five plasmid constructs 6-16-fold higher promoter activity has been achieved with pKM794 driven by CaMV 35S promoter plus two enhancer elements than with the other constructs tested.

Keywords: Protoplast — Triticum aestivum — reporter gene — vector

Introduction

Genetic transformation of higher plants had already led and will probably lead to a further development of plant breeding and better understanding of basic mechanisms involved in plant gene regulation /27/. Gene transfer techniques for transformation of cereal plants progressed consi-

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Abbreviations: RECS: embryogenic cell suspension culture with regeneration capability; GUS: bacterial β-glucoronidase repoter gene; MU: 4-methyl umbelliferone (7-hydroxy-4-methyl coumarin); PEG: polyethylene glycol.

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derably in the recent years /20, 21/. The direct uptake of free DNA by protoplasts can be considered as a generally applicable gene transfer system /20, 21, 23, 26/. The most extensively used experimental approach involves DNA uptake into protoplasts by chemical treatment with PEG or electroporation. The PEG-mediated transformation is a relatively simple, inexpensive and reliable technique, which can be developed into a routine method in gene technology of cereals /19/.

Various important parameters influencing gene transfer with PEG have been identified: (1) Physiological state of target protoplasts, culture medium and culture conditions /3, 8, 11/, (2) PEG brand/molecular weight /10/, (3) PEG concentration /3, 11, 17, 18/, (4) the transformation protocol, e.g. buffer composition, Mg^{2+} or Ca^{2+} plasmid construction, form and concentration, carrier DNA, incubation time and conditions, dilution and washing /3, 10, 11, 15, 17, 18, 29/.

Transient gene expression is an extremely refined method through which gene activity can be assayed within a few hours to several days after DNA uptake. In this case, the introduced DNA remains extra-chromosomal, resulting in an expression of transient nature /18/. This is an important technique for rapid testing of modified gene constructs on the promotor efficiency without disorder of position effects and for supporting the time-consuming experiments for stable transformation /8, 12/.

Although PEG-mediated, transformation method is very simple and widely utilized in cereal species, PEG was used only in few cases for transformation of wheat /6, 13, 28/. Therefore, the objective of this paper was to characterize and optimize the transient expression of β-glucoronidase (GUS) reporter gene in embryogenic protoplasts of wheat using PEG.

Material and Methods

1. Preparation of protoplasts

Protoplasts were isolated from RECS of KSV-90A and KSV-91A cell lines of GK Ságvári winter wheat, essentially as described previously /1/ with little modification. The protoplasts were washed with MaCa washing solution ($CaCl_2 \cdot 2\ H_2O$ 80 mM, mannitol 0.2 M, MES = 2-N-morpholino-ethanesulfonic acid 2.56 mM, pH 6.0, autoclaved at 121 °C for 15 min). Purified protoplasts were suspended in MaCa washing solution and collected in a test tube to give a final volume of 10 ml MaCa, counted using a Bürker chamber, then centrifuged (1000 rpm for 3 min), and resuspended in MaCa solution to give final density of 7×10^6 protoplasts/ml.

2. Plasmid constructions

Five different plasmids containing the bacterial β-glucoronidase repoter gene were used (Fig. 1). Plasmids A, C and D have been described more in detail by Stefanov et al. /24/ and Omirulleh et al. /19/.

A. pIDS211, carrying the GUS gene fused with the cauliflower mosaic virus (CaMV) 35S promoter, plus the Agrobacterium tumefaciens nopaline synthetase (NOS) polyadenylation signal. Final size is 5.6 kb.
B. pH4g, carrying the GUS gene fused with the wheat H4 histone promoter, plus the NOS polyadenylation signal. Final size is 5.5 kb /25/.
C. pKM794, carrying the GUS gene fused with truncated (up to -90 bp) CaMV 35S promoter, doubled enhancer sequences of CaMV 35S promoter (-208 to -46), plus the NOS polyadenylation signal. Final size is 5.2 kb.
D. p142, carrying the GUS gene fused with the truncated (up to -389 bp) wheat α-amylase promoter, two elements of CaMV 35S enhancer (-208 to -46), and the NOS polyadenylation signal. Final size is 5.5 kb.
E. pEIG, containing the CaMV 35S promoter, the exon 1 and intron 1 of the maize Shrunken-1 gene and fused with in frame GUS-coding sequence, plus the NOS polyadenylation signal. Final size is 6.3 kb /15/.

All genes were cloned in pUC19 vectors. Plasmids were sterilized by ethanol precipitation and dissolved in sterile TE buffer /16/ at 1.0 mg/ml.

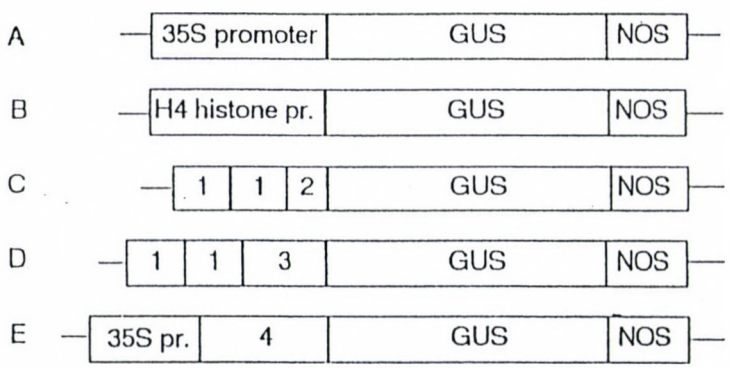


Fig. 1. Schematic maps of the chimetric GUS gene constructs used for transient expression in wheat protoplasts.
A: pIDS211, B: pH4G, C: pKM794, D: p142, E: pEIG.
35S promoter, 35S pr.: CaMV 35S promoter; H4 histone pr.: wheat H4 histone promoter; NOS: nopaline synthetase polyadenylation signal; 1: 35S enhancer (-208 to -46); 2: truncated (up to -90) 35S promoter; 3: truncated (up to -389) wheat alpha-amylase promoter; 4: exon and intron from maize Shrunken-1 gene

3. General protocol for DNA uptake and culture of protoplasts

Aliquots (0.3 ml) of the protoplast suspension (containing 2.1 x 10^6 protoplasts) in 16 x 125 mm round-bottom centrifuge tubes were mixed with plasmid DNA (20 µl) and 0.32 ml of PEG solution (MW 4000, 40% w/v) was added dropwise with gentle shaking (final PEG concentration: 20%). The mixture of protoplasts, DNA and PEG was incubated at 25 °C for 10 min. The transformation mixture was diluted stepwise with 2 ml aliquots of MaCa washing solution to a final volume of 5 ml. Protoplasts were then pelleted by centrifugation (1000 rpm, 3 min), and an additional washing step was made before culturing with liquid KM or GM medium /1/ at a density of 1 x 10^6/ml in 35 x 10 or 60 x 15 mm diameter plastic Petri dishes. Cultures were incubated at 26 °C in darkness. No carrier DNA was used in the transformation. Control treatments were included: protoplasts + PEG alone, protoplasts + plasmid DNA without PEG, non-treated protoplasts.

3.1. Effect of PEG solvent medium and PEG concentration

By using the transformation protocol (described above), two different PEG solvent media: F medium /9/ and MaMg /17/ were tested for their effect on transient gene expression in wheat protoplasts. For studying the effect of PEG concentration on the efficiency, protoplasts were incubated with PEG/F at 10%, 20% or 40% and with 20 µg of pKM794 plasmid for 10 min at 25 °C. To take 40% PEG in F or MaMg medium, 40 g PEG 4000 (Merck) was dissolved in 100 ml or F or MaMg medium, filter-sterilized and stored at -20 °C.

3.2. Effects of incubation time in transformation mixture

The transformation mixtures (protoplasts, pKM794 plasmid DNA and PEG) were incubated at room temperature for 1, 5, 10 and 20 min, then diluted with MaCa solution, washed and cultured as above. Protocol to study the effects of other factors, i.e. protoplast culture medium, culture period after treatment, and activity of different plasmid DNA constructs will be described in the Result section.

4. Fluorogenic assay of the transient expression of GUS gene in treated protoplasts

GUS assay were performed according to Jefferson /7/, after culturing the protoplasts for 15, 20, 24, 48 and 72 hours. The 4-methyl umbelliferone (MU) concentration was determined with a Perkon-Elmer MPF-3 spectrofluorimeter at 365 nm excitation and 455 nm emission wavelengths. One unit of GUS activity was defined as 1 nmol of MU formed per mg protein per hour of incubation at 37 °C.

Results

Detectable transient gene expression has been obtained in GK Ságvári wheat protoplasts with plasmid DNA and 20% PEG. Several factors have been found to affect strongly the level of transient gene expression after PEG-mediated DNA uptake into wheat protoplasts such as PEG solvent, PEG concentration, incubation time of transformation mixture, type of promoter in different plasmid constructs, protoplast culture medium and length of culture time after treatment.

1. Effect of PEG concentration on the efficiency of DNA uptake

Different concentration of PEG (10%, 20%, 40%) in F medium were tested to optimize DNA uptake into KSV-90A protoplasts. Figure 2 shows that transient expression of GUS gene could be observed at all PEG concentrations

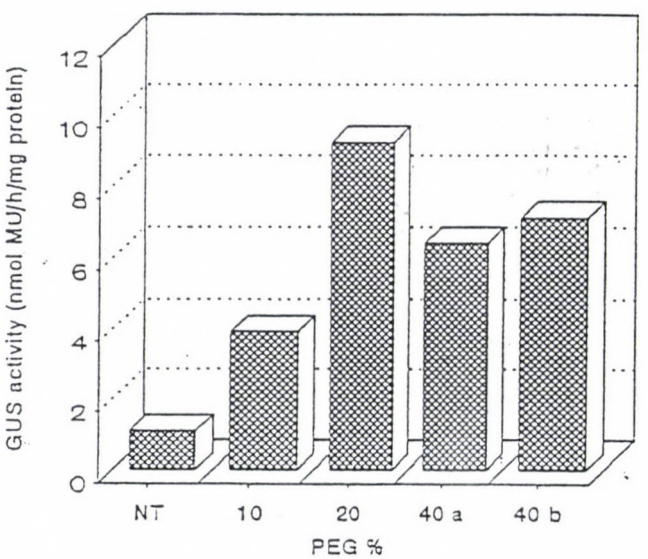


Fig. 2. Effect of PEG concentration on transient gene expression in KSV-90A protoplasts. Protoplasts were washed and suspended before DNA/PEG treatment in MaCa solution. 20 µg pKM794 construct was added to 0.3 ml aliquots (2.1×10^6 protoplasts) followed by PEG 4000 in F medium /9/ at a final concentration of 10%, 20%, 40%. After 10 min incubation at 25 °C, the transformation mixture was diluted with MaCa solution (in case of 40% a, b concentrations, 3x and 5x MaCa volume were used, respectively). Treated protoplasts were cultured in KM medium. GUS activity was assayed 48 h after transformation. NT: plasmid-treated protoplasts without PEG solution

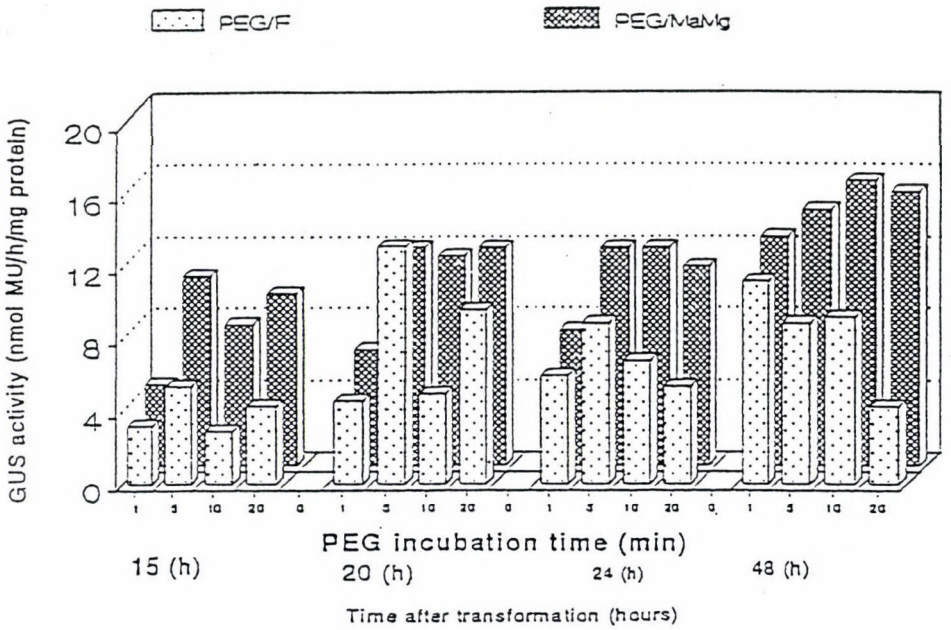


Fig. 3. Effect of PEG solvent medium, incubation time of transformation mixture and culture period after transformation on transient gene expression in KSV-90A protoplasts. Protoplasts were washed and suspended before DNA/PEG treatment in MaCa solution. PEG was dissolved in either F medium (PEG/F /9/) or MaMg medium (PEG/MaMg /17/) and added at a final concentration of 20% to 2.1×10^6 protoplasts and 20 µg pKM794. Transformation mixture incubated for 1, 5, 10, 20 min at 25 °C. Treated protoplasts were cultured in GM medium and GUS activity was assayed 15, 20, 24, 48 h after transformation

tested, and fluorescence intensity was 3.5-8.4-fold higher than the control. The optimum PEG concentration was found to be 20%. Protoplasts treated with 40% PEG followed by dilution in 3 and 5 volumes of MaCa washing solution produced 6.4 and 7.1 nmol MU/mg protein/h, respectively. PEG-treatment induced aggregation of treated protoplasts (5-45 cells) in the culture medium (not shown).

2. Effect of PEG solution and protoplast culture medium on transient expression

Considering the results of higher number of experiments (Figs 3, 4), we can conclude that the PEG/MaMg medium was more effective than the PEG/F medium at majority of incubation times. GUS activity values in transformed protoplasts cultured in GM medium ranged from 4.9 to 9.9 with a mean of 6.9

units in PEG/F vs. 6.8-12.4 with 8.9 units as a mean in PEG/MaMg (Fig. 3). With protoplasts cultured in KM medium, GUS level ranged from 2.9-13.2 with mean of 6.8 units in PEG/F vs. 4.5-15.8 with 10.9 units as a mean in PEG/MaMg (Fig. 4), respectively.

Protoplast cultured in KM medium showed a higher level of transient GUS expression than protoplasts cultured in GM medium (at 24 and 48 h after transformation, Fig. 4, vs. Fig. 3). GUS activity of transformed protoplasts cultured in KM medium increased with the time elapsed after transformation. However, no highly significant differences were observed in GUS activity of protoplasts cultured in GM medium for 24, 48 or 72 h as compared with that of protoplasts cultured in KM medium.

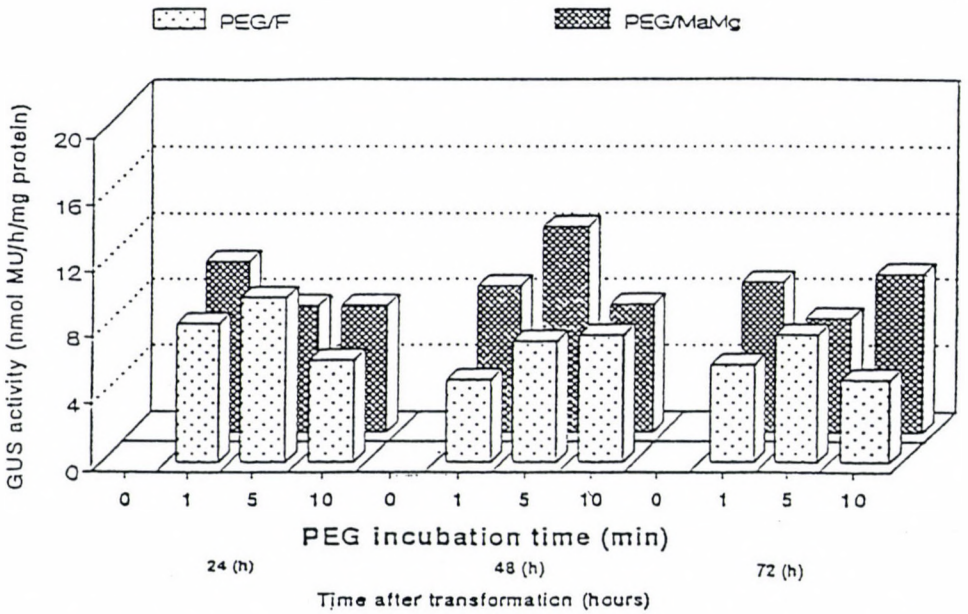


Fig. 4. Effect of PEG solvent medium, incubation time of transformation mixture and culture period after transformation on transient gene expression in KSV-90A protoplasts. Protoplasts were washed and suspended before DNA/PEG treatment in MaCa solution. PEG was dissolved in either F medium (PEG/F /9/) or MaMg medium (PEG/MaMg /17/) and added at a final concentration of 20% to 2.1×10^6 protoplasts and 20 µg pKM794. Transformation mixture incubated for 1, 5, 10 min at 25 °C. Treated protoplasts were cultured in KM medium and GUS activity was assayed 24, 48, 72 h after transformation

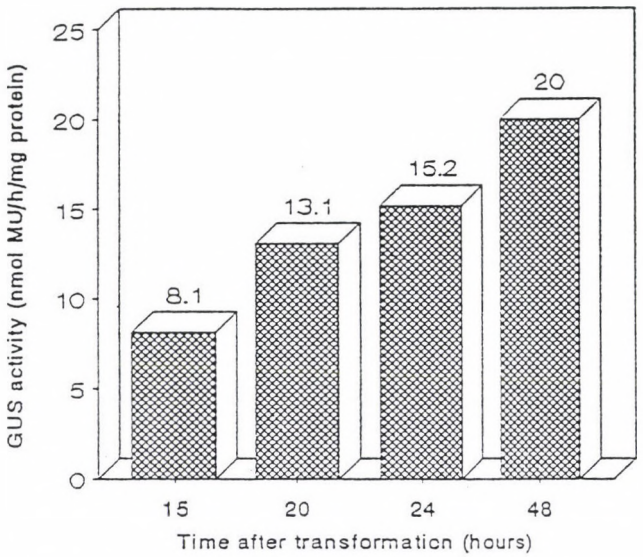


Fig. 5. Effect of culture period after transformation on transient gene expression in KSV-91A protoplasts. Protoplast were washed and suspended before DNA/PEG treatment in MaCa solution. 20 µg pEIG construct was added to 0.3 ml aliquots (2.1×10^6 protoplasts) followed by PEG/MaMg /17/ at a final concentration of 20%. After 10 min incubation at 25 °C, the transformation mixture was diluted with MaCa solution. Treated protoplasts were cultured in KM medium and GUS activity was assayed 15, 20, 24, 48 h after treatment

3. Effect of incubation time in transformation mixture on transient GUS expression

Transient GUS expression was detected at each incubation time and the GUS activity ranged from 2.9 to 15.8 units (Figs 3, 4). The optimum incubation time was 5 or 10 min. Significantly improved transformation activity could not be detected with a longer incubation period. The level of GUS expression in KSV-91A wheat cell line was followed over period of 48 h, after incubation for one min with plasmid DNA and PEG/F solution. Significant increase in GUS activity correlated with the time elapsed after transformation, with maximum activity of 20 nmol MU/mg protein/h at 48 h (Fig. 5).

Table 1

β-glucoronidase activity, 24 hours after DNA uptake, in lysates of protoplasts of KSV-90A and KSV-91A from regenerable embryogenic cell suspension cultures of GK Ságvári wheat. The treatment was carried out with five constructs (Fig. 1) in the presence of 20% PEG/MaMg at 20%, 20 μg plasmid DNA per 2.1×10^6 protoplasts and incubation at 25 °C for 10 min. GUS activity was assayed 24 h after transformation. Control: protoplasts treated with PEG, without plasmid DNA

Plasmid construct Exp. No.	Gus activity (nmol MU/mg protein/h) I	II	III	IV	V	VI	Mean
Control	0.44	1.6	1.7	0.9	—	1.9	1.3
pH4G	0.52	—	1.9	—	—	—	1.2
pIDS211	—	—	7.3	1.9	3.2	9.2	5.4
pEIG	2.2	1.6	4.7	3.7	2.4	12.6	4.5
p142	—	2.0	5.1	—	1.7	8.1	4.2
pKM794	20.8	—	23.4	5.4	19.3	27.5	19.3

4. Effect of plasmid DNA constructions on transient GUS expression

To investigate the function of the different DNA vectors, the plasmid constructs describe in Materials and Methods section were tested in PEG-transformation of KSV-90A and KSV-91A protoplasts, PEG/MaMg at 20%, μg plasmid DNA per 2.1×10^6 protoplasts and incubated at 25 °C for 10 min. GUS activity was assayed at 24 h after treatment.

Table 1 shows that GUS activity in treated protoplasts was dependent on the components of transformation vectors used. Ranging from 5.4 to 27.5 with 19.3 units as a mean, the highest level of GUS activity was detected with pKM794 (two enhancer elements/CaMV 35S promoter/GUS reporter/NOS terminator) being 6-16-fold higher than that with the other four constructs studied. In contrast, H4G construct (wheat H4 histone promoter/GUS reporter/NOS terminator) was inactive in the tested cell system. Rest of the constructs transmitted approximately the same level of GUS activity, with a fluorescent intensity fourfold higher than the control, with a range from 1.6 to 12.6 and a mean of 407 units.

Discussion

Regenerable embryogenic cell suspension culture (cell lines KSV-90A and KSV-91A) of the GK Ságvári winter wheat reproducibility yielded good protoplast populations and gave high plating efficiency suitable for direct gene transfer /1, 2/. However it was found during the course of experiments that several factors strongly affect the efficiency of the methodology and therefore, these were studied further, in order to optimize the transformation process.

The optimum PEG concentration was found to be 20%. This PEG concentration produced higher transformation frequences with tobacco /17/, maize /3/ and rice /5/, too.

GUS activity in DNA-treated GK Ságvári wheat protoplasts was higher for PEG/MaMg than for PEG/F medium, suggesting that Mg^{2+} with PEG can significantly increase the transient GUS expression in wheat protoplasts compared to the Ca^{2+} medium. In tobacco /17/, rice /29/ and in maize /14/, pretreatment in the Mg^{2+} medium was better for gene transfer efficiency over another media.

The treated wheat protoplasts cultured in KM medium produced higher level of GUS activity than in GM medium, and the activity increased with the time elapsed after transformation only if the protoplasts were cultured in KM medium. This emphasizes the importance of protoplast culture medium in gene expression studies /3/.

We observed transient GUS expression between 1 and 20 min incubation times and found the optimum to be between 5 and 10 min. Longer incubation time did not improve the GUS activity significantly. Actually, one min incubation time with PEG/F was sufficient to DNA uptake and presented higher transient GUS expression at 48 h after DNA treatment than other incubation time (Fig. 5). In model plants, Negrutiu et al. /17/ reported that 2-5 min incubation with PEG was optimum for both transient and stable transformations.

Protoplast culture medium also affects the activity of the reporter gene at the same incubation time. Protoplasts treated with PEG/MaMg at 10 min incubation time produced the highest level of GUS activity at 48 h after transformation when cultured in KM medium, but they produced the lowest level when kept in the GM medium (Figs 3, 4). The level of GUS expression in treated protoplasts of GK Ságvári wheat cell lines positively correlated with both the time elapsed in culture medium after treatment (up to 48 h) and with the incubation time in GUS reaction mixture.

In the extracts of treated KSV-90A protoplasts, high GUS activity could be found when GUS gene was under the control of CaMV 35S promoter, but relatively low transient expression has been obtained when the GUS gene was controlled by the wheat-derived promoters (Table 1). The 35S promoter was the most active among the different promoters tested in transformed protoplasts of maize, rice and barley. Nevertheless, expression of a foreign gene depends on the tissue specificity, too /4, 22/.

Under optimized conditions as described above, a routine and efficient transient GUS expression can be achieved in embryogenic wheat protoplasts.

Acknowledgements

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EFFECTS OF DIETARY L-CARNITINE SUPPLEMENTATION AND PROTEIN LEVEL ON PERFORMANCE AND DEGREE OF MEATNESS AND FATNESS OF BROILERS

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Feeding high-protein diets to broilers is a known means for improving performance and carcass composition and quality, however, the combined effects of dietary protein (CP) level concurrent with L-carnitine (CN) supplementation are not known. Performance and carcass traits of broilers fed CN-supplemented diets of different CP-levels were studied from 18 to 53 days of age. Three isocaloric diets containing 18, 20 or 22% CP were formulated, with or without added CN (50 mg/kg), and used. Apart from CP level, supplemental CN attained significantly higher body weight gain (BWG) and feed conversion (FC), means of BWG were 351 vs. 332 and 371 vs. 353 g, and those of FC were 1.25 vs. 1.33 and 1.73 vs. 1.84 for the first and second week of the experimental period, respectively. Amount and percentage of abdominal fat and ether extract (EE) contents of breast meat of 53-day-old broilers were significantly reduced (means of AF were 43.5 vs. 51.5 g, equivalent to 1.98 vs. 2.39%, those of EE were 1.82 vs. 2.24%) in response to CN supplementation. A significant interaction between added CN and CP level was noted on BWG and FC of broilers from 18 to 32 days of age.

Keywords: L-Carnitine — protein level — performance — fatness — broilers

Introduction

A wealth of data has been reported on the effects of dietary protein level on the performance and body composition of broiler chickens. Recently two main questions were addressed: (1) the possibility to reduce dietary protein in broiler feed with attaining optimum performance, in order to reduce N excretion which is a source of pollution and for economic consideration, and (2) the extent to which nutrient manipulation (e.g. increasing dietary protein level) can maximize the performance in terms of growth rate and feed conversion efficiency, and provide the consumer with a product containing more lean and less fat.

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It is generally considered that protein and amino acid requirements of broiler chickens vary with age, leading to a common practice of decreasing levels of crude protein (CP) during the entire growing period. The National Research Council /26/ suggests three levels of crude protein (23, 20 and 18%) for three growing periods: starter (0-21 days), grower (21-42 days) and finisher (42-56 days), respectively.

Since genetic selection of broiler chickens over the last few decades has been made for live weight under ad libitum feeding, the resultant increase in growth rate was accompanied by an increase in carcass fatness. This is potentially due to the fact that selection has been for appetite and fast maturing rather than for maximum lean deposition.

Consisting with an increasing concern over excessive fat deposition (which is undesirable for producer, processor and consumer), the interest has been focused on the possibility of producing a more desirable carcass through dietary manipulations. In this regard, it has been reported that narrowing the calorie: protein (C:P) ratio of the diet enhances lean tissue accumulation and reduces abdominal fat deposition /13/. The C:P ratio may be altered by increasing the crude protein (CP) at constant energy or decreasing the energy at constant CP with equal effectiveness. The latter approach can reduce performance when the nutrient content of the diets becomes inadequate and the former can substantially increase the total cost of the feed. However, increasing the dietary CP level often improves feed efficiency even in absence of a growth response /17, 27/. In addition, less expensive protein sources have been utilized effectively to incrase the CP level of the diets for reducing abdominal fat deposition /7, 8/.

Moreover, the recent consumer trends have encouraged the production of broiler chickens specifically for sale as portions rather than whole birds. Consequently, it has become economically significant to formulate diets for improving the yield of specific carcass parts rather than for optimizing body weight gain. Pertaining to this developmental changes in human consumption patterns for broilers, several reports have been shown that carcass yield and composition were improved, with variable responses, by increasing dietary CP and essential amino acid levels, particularly lysine and methionine /14, 16, 32, 37/. In the light of the fact that both dietary carbohydrates and protein are convertible to fats but the reverse is not true, and that broilers, like other animals, have a very limited capability to store surplus carbohydrates and protein from the diet whereas they possess adipose tissues to store the extra energy. In addition, it has been reported

that the requirements of chicks for lysine and methionine increase with increasing dietary protein level beyond that is needed to maximize growth rate or food efficiency /24, 25/. Therefore, we hypothesized that L-carnitine /β-hydroxy-(γ-N-trimethylamino)-butyrate/, which is synthesized in vivo from lysine and methionine, may help in improving the efficiency of energy utilization of broilers when they are fed on diets of different protein levels.

L-Carnitine is a water soluble quaternary amine which occurs naturally in microorganisms, plants and animals /6/. Its concentrations in animals vary according to species /38/, tissue type /6, 30/ and nutritional status of the animal /21/. Dietary lysine and methionine are the exogenous precursors for L-carnitine biosynthesis, in the presence of ferrous ions and a number of vitamins: ascorbate, niacin and pyridoxine, which are required as cofactors for the enzymes involved in the metabolic pathway of L-carnitine /12, 22, 28, 34/. However, little L-carnitine has been reported to be found in cereal grains and their by-products /5/, at the same time these feed ingredients constitute the major portion of poultry diets.

L-Carnitine serves two major functions. The well-established role of L-carnitine is facilitating the entry of long-chain fatty acids into mitochrondria for energy-generating processes via β-oxidation. L-Carnitine also facilitates removel from mitochondria of short-chain and medium-chain fatty acids that accumulate as a results of normal and abnormal metabolism /29/.

Several studies on pigs, fish, quail, foals and broiler chickens have been shown that growth performance was significantly improved by feeding dietary L-carnitine /15, 23, 35, 39, 40/. However, it has been reported that egg hatchability increased from 83% to 87% and from 82.4% to 85.3% when broilers were fed on diets supplemented with L-carnitine at levels of 50 and 100 mg/kg diet, respectively /22/. In contrast, some investigators failed to observe favourable responses to added dietary carnitine /2, 9/.

Based on the voluminous literature, feeding high-protein diets to brolier chickens has become a proved means of improving performance and carcass yield concurrent with reducing abdominal fat content. Since L-carnitine is synthesized in vivo from lysine and methionine, its addition to poultry diets may contribute in improving boriler performance and/or carcass quality, particularly when the birds are given diets of different crude protein levels.

The purpose of the present study was to evaluate the response of broiler chickens fed diets of different levels of CP to supplemental dietary L-carnitine during the growing and finishing periods from 18 to 53 days of age.

Materials and Methods

Birds and diets

A total of 180 day-old Hybro broiler chicks were used in this study. The chicks were fed on a commercial started diet from day-old to 18 days of age and then switched to experimental diets. Three iso-energetic diets (metabolizable energy content of 13.5 MJ/kg) containing CP levels of 22, 20 and 18% were formulated (Table 1), and unsupplemented or supplemented with a level of L-carnitine of 50 mg/kg diet in the form of carniking (LONZA Ltd.), and thus, six experimental diets were prepared and used.

Apart from CP level, these diets were formulated to meet or slightly exceed the nutrient requirements of broilers specified by the NRC /26/. Recently, a dietary CP level of 18% to 18.7% has been shown to support performance and carcass fat deposition of finishing broilers equal to diets containing 20.4% or 21.5% CP, respectively /19/, when sulphur amino acid content was adequate. Therefore, in the present study, a 18% CP diet was considered to serve as control diet. All diets were offered to the birds in mash form.

Housing and management

During the starter period, chicks were kept at conventional wire-floored brooding batteries (placed in an open-sided growing house equipped with gas-heating system) and provided with feed and water on ad libitum basis.

At 18 days of age, all the chicks were wing-banded, weighed individually, randomly distributed into six experimentel groups and translocated to 3-tier, wire-floored rearing batteries equipped with nipple drinkers (waterers), with continuous lighting throughout the course of experiment. Each battery consists of 12 compartments. Each experimental group of 10 birds was replicated three times and housed in battery compartments, each measuring 60 x 90 cm. Birds had free access to feed and water throughout the experimental period which lasted from 18 to 53 days of age.

Measurements of responses

A. Production traits

The performance of broiler chickens was evaluated in terms of weekly feed intake, live body weight, body weight gain and feed conversion, as influenced by dietary L-carnitine supplementation to isocaloric diets of different CP contents during the growing and finishing periods.

Individual live body weights of the chicks were recorded at the beginning of experiment (18-day old) and on a weekly basis thereafter. Weekly records of feed intake for each of the replicate groups of each treatment were also maintained. Feed conversion was calculated as the amount of feed consumed per unit of body weight gain.

B. Carcass yield and quality

Like the condition with all broiler chickens, birds destined for slaughter test had free access to feed and water until slaughter. Such unusual practice was intended in order to avoid a possible effect of feed withdrawal before slaughter (for a certain period of time) on the fat con-

tent of liver and/or other tissues of broilers, which may mask some of the effects of factors in question or confound the interpretation of the results obtained.

In order to see the effect of L-carnitine on fat deposition, we intended to delay the time of slaughter beyond the normal age used in common practice. The slaughter test was performed at the termination of experiment (53-day old) to determine some measurements of carcass yield and quality. These measurements included carcass yield and components (weights of eviscerated carcass, giblets, total edible parts, breast yield including bones, thighs + drumsticks yield including bones, breast meat yield and thighs + + drumsticks meat yield), abdominal fat content (as an absolute weight and as a percent of body weight) and composition of edible meat of broilers (in terms of dry matter, crude protein and ether extract contents of liver, breast meat and thigh plus drumstick meat). Six birds from each experimental group, with body weights around the mean value of the representative group, were selected and killed by decapitation. The carcasses were immediately scalded, feather picked and eviscerated, and then chilled overmight in a refrigerator at 4 ^{0}C in order to facilitate the removel of abdominal fat pad. The cutting up of carcasses was made according to the procedure described by Jensen /18/, and the abdominal fat pad was removed according to the method outlined by Fancher and Jensen /11/.

From one side of breast and one thigh + drumstick of each bird, meat (with skin) was excised and weighed separately. Total meat yields for each of breast and thighs (including drumsticks) were calculated by multiplying the amount of meat excised by two. Weights of cut-up parts of the carcasses and of breast and thigh meat were determined to the nearest gram, while those of abdominal fat pad and organs (liver, heart and gizzard) were measured to the nearest one-tenth gram, using a sensitive balance. Samples from liver, breast meat and thigh + drumstick meat were taken and frozen until analysis.

Laboratory analyses

The frozen samples of liver and breast and thigh meat were dried in a forced-draft oven at 65 ^{0}C, ground and used for chemical analysis. The proximate analyses for both the basal experimental diets and meat samples of breast, thigh and liver were carried out according to the official methods of analysis /1/. The gross energy contents of the basal experimental diets were also determined by an adiabatic oxygen bomb calorimeter.

Experimental design and statistical analysis

A completely randomized design in factorial arrangement of treatments, three levels of dietary crude protein (22, 20 or 18%) with or without L-carnitine supplementation (50 mg/kg diet), was used to separate effect of CP level from that of L-carnitine supplementation.

The statistical processing of data was performed using Statgraphics Program, STSC, software personal computer, MD, USA, Rockville /36/ based on multifactor analysis of variance, with P values of 0.05 or less considered significant. After analysis of variance, significantly different means for each parameter were separated using Duncan's multiple range test /10/.

Results

The analyzed CP values for the three basal diets used in this study were in good agreement with the calculated CP contents (Table 1).

As indicated in Table 2, neither dietary protein nor supplemental dietary L-carnitine affected feed intake of broilers throughout this study. However, feed conversion efficiency was significantly improved by supplemental dietary L-carnitine during the first two weeks of the experimental period (18 to 25 and 25 to 32 days of age) and during the entire experimental period from 18 to 53 days of age, irrespective of dietary CP level. Dietary CP level also had no effect on feed conversion efficiency (feed: gain ratio) of broilers, except during the period from 32 to 39 days of age where significant positive response was observed. Although birds fed the highest level of CP (22%) consumed slightly more feed than did birds fed on the lower CP levels (20 or 18%), the former achieved the best value of feed conversion during the 32 to 39-day period, regardless of dietary L-carnitine supplementation. But, the differences in feed conversion values between birds fed the 20% CP diets and those fed the 18% CP diets were not significant, irrespective of added L-carnitine. A significant interaction between dietary CP level and added dietary L-carnitine was noted on feed conversion efficiency of broilers during the same periods that gave significant responses to dietary L-carnitine.

The results presented in Table 3 indicate that, independently from dietary CP level, L-carnitine addition to diets of broilers resulted in significant increases in live body weight and body weight gain during the first two weeks of the experimental period. Apart from dietary L-carnitine supplementation, dietary CP level had no effect on body weight gain of broilers during the growing and finishing periods from 18 to 53 days of age. The same response was observed in live body weight of broilers, with the exception that a significant increase in 39-day live body weight occurred when dietary CP level increased from 18 to 22%. No significant differences were observed either between birds fed the diets contained 22% vs. 20% CP or between birds fed 20% vs. 18% CP diets. The interaction between dietary CP level and added dietary L-carnitine was not significant for live body weight throughout the course of this study. However, a significant interaction between dietary CP level and supplemental dietary L-carnitine was observed on body weight gain during the first two weeks of this study, irrespective of dietary L-carnitine supplementation.

Table 1

Percentage composition and proximate analysis of the basal diets

Ingredients, %	Dietary crude protein levels (%)		
	18	20	22
Yellow corn	50.73	47.76	45.15
Soybean meal, 47%	24.11	25.20	31.18
Animal-vegetable fat*	11.05	10.71	12.42
Fish meal, 64%	00.00	3.00	3.00
Wheat	10.00	10.00	5.00
Limestone	1.62	1.45	1.46
Monocalcium phosphate	1.27	0.91	0.87
DL-Methionine	0.27	0.17	0.12
L-Lysine HCL	0.15	0.00	0.00
Common salt	0.30	0.30	0.30
Vit. and Min. Premix+	0.50	0.50	0.50
Total	100.00	100.00	100.00
Calculated analyses:			
Crude protein, %	18.00	20.00	22.00
Crude fiber, %	3.10	3.12	3.36
Lysine, %	1.06	1.12	1.29
Methionine, %	0.53	0.50	0.48
Methionine + Cystine, %	0.82	0.82	0.82
Threonine, %	0.66	0.74	0.83
Tryptophan, %	0.21	0.23	0.26
Calcium, %	0.90	0.90	0.90
Phosphorus (total), %	0.60	0.60	0.60
Metabolizable energy, MJ/kg	13.50	13.50	13.55
Determined analyses:			
Dry matter (DM), %	94.05	94.50	94.07
Crude protein (CP), %	18.02	19.70	21.98
Ether extract (EE), %	3.79	3.48	3.65
Crude fiber (CF), %	3.17	3.20	3.67
Ash, %	5.95	5.85	5.95
Gross energy (GE), MJ/kg DM	16.31	16.26	16.65

*Made up of 40% lard and 60% corn flakes.

+Supplied per kilogram of diet: vitamin A, 7.870 IU; vitamin D_3, 2.009 IU; vitamin E, 9.81 IU; vitamin K_3, 3.8 mg; vitamin B_1, 3.52 mg; vitamin B_2, 9 mg; vitamin B_6, 5.28 mg; vitamin B_{12}, 0.015 mg; pantothenic acid, 25 mg; niacin, 35 mg; choline chloride, 1.750 mg; Ca, 8.89 g, P, 2.66 g; NaCl, 2.45 g; Mn, 60 mg; Fe, 45 mg; Cu, 8 mg; Zn, 50 mg; I, 3 mg; Se, 0.15 mg; ethoxyquin, 125 mg.

Table 2

Effects of dietary L-carnitine (CN) supplementation with different crude protein (CP) levels on feed consumption and feed conversion of broiler chickens during the growing and finishing periods from 18 up to 53 days of age

Dietary treatments		Feed intake, g/bird/period						Feed conversion, g feed:g gain					
CP level (%)	CN added, mg/kg	18-25[1]	25-32[2]	32-39[3]	39-46[4]	46-53[5]	18-53[6]	18-25	25-32	32-39	39-46	46-53	18-53
18	0	440	660	863	1040	1023	4027	1.37	1.94	2.18	2.28	3.14	2.19
18	50	450	647	873	1060	1030	4060	1.24	1.65	2.22	2.22	3.10	2.07
20	0	447	650	853	1060	1050	4067	1.34	1.81	2.17	2.18	3.11	2.12
20	50	453	640	820	1073	1057	4077	1.31	1.82	2.24	2.14	3.15	2.13
22	0	437	630	820	1087	1070	4043	1.29	1.76	2.02	2.26	3.16	2.10
22	50	447	637	833	1083	1067	4067	1.30	1.73	2.04	2.17	3.31	2.09
SEM[+]		12.3	19.2	20.3	25.7	22.4	33.2	0.026	0.033	0.036	0.042	0.090	0.019
Protein effect:													
18		445	653	868	1050	1027	4043	1.31	1.79	2.20[a]	2.25	3.12	2.13
20		450	645	837	1067	1053	4072	1.32	1.82	2.20[a]	2.16	3.13	2.12
22		442	633	827	1085	1068	4055	1.30	1.75	2.03[b]	2.22	3.24	2.10
SEM		8.71	13.6	14.4	18.2	15.8	23.5	0.018	0.023	0.025	0.029	0.063	0.013
L-Carnitine effect:													
0		441	647	846	1062	1048	4046	1.33[a]	1.84[a]	2.12	2.24	3.14	2.14[a]
50		450	641	842	1072	1051	4068	1.28[b]	1.73[b]	2.17	2.17	3.19	2.10[b]
SEM		7.11	11.1	11.7	14.8	12.9	19.2	0.015	0.019	0.021	0.024	0.052	0.011
Source of variation:		Probabilities											
Protein		0.7966	0.5935	0.1436	0.4223	0.2102	0.7000	0.6266	0.1372	0.0006	0.1355	0.3694	0.2732
L-Carnitine		0.4036	0.7335	0.8462	0.6474	0.8601	0.4372	0.0388	0.0026	0.1559	0.0695	0.5146	0.0244
Protein x L-carnitine		0.9879	0.8581	0.4634	0.8974	0.9673	0.9401	0.0452	0.0014	0.7600	0.8269	0.5820	0.0108

[1-6]Refer to age periods in days.

[+]Standard error of the means (n = 3).

[a-b]Means in the same column bearing different superscripts differ significantly ($P \leq 0.05$).

Table 3

Effects of dietary L-carnitine (CN) supplementation with different crude protein (CP) levels on live body weight and body weight gain of broiler chickens during the growing and finishing periods from 18 up to 53 days of age

Dietary treatments		Live body weights (g)						Body weight gains (g)					
CP level (%)	CN added, mg/kg	18[1]	25[2]	32[3]	39[4]	46[5]	53[6]	18-25	25-32	32-39	39-46	46-53	18-53
18	0	254	585	926	1322	1778	2104	321	341	396	456	327	1840
18	50	269	631	1024	1317	1845	2178	362	393	393	478	333	1959
20	0	258	593	952	1346	1834	2173	335	359	394	488	339	1914
20	50	277	625	983	1361	1861	2198	347	352	381	502	335	1921
22	0	271	610	971	1377	1854	2196	339	360	406	480	338	1925
22	50	272	616	984	1392	1892	2214	344	368	408	500	322	1940
SEM+		6.28	11.9	19.1	24.2	30.1	35.6	7.60	9.90	10.9	13.0	13.1	33.4
Protein effect:													
18		267	608	975	1320[bc]	1811	2141	342	367	395	467	330	1900
20		268	609	968	1353[ab]	1848	2186	341	356	387	495	337	1918
22		272	613	977	1385[a]	1873	2205	342	364	407	490	330	1933
SEM		4.44	8.40	13.5	17.1	21.9	25.2	5.37	7.00	7.72	9.18	9.26	23.6
L-Carnitine effect:													
0		265	596[b]	950[b]	1348	1822	2158	332[b]	353[b]	399	475	335	1893
50		273	624[a]	997[a]	1357	1866	2197	351[a]	371[a]	394	493	330	1940
SEM		3.63	6.86	11.0	13.9	17.9	20.6	4.39	5.72	6.30	7.50	7.56	19.3
Source of variation:		Probabilities											
Protein		0.7072	0.9008	0.8702	0.0287	0.1403	0.1907	0.9971	0.5013	0.1826	0.0731	0.8114	0.6041
L-Carnitine		0.1059	0.0047	0.0027	0.6655	0.0834	0.1804	0.0020	0.0303	0.6155	0.0838	0.6678	0.0833
Protein x L-carnitine		0.3295	0.2369	0.0687	0.8971	0.7996	0.6990	0.0415	0.0099	0.7861	0.9593	0.6877	0.1791

[1-6]Refer to age of birds in days.

+Standard error of the means (n = 30).

[a-c]Means in the same column bearing different superscripts differ significantly ($P \leq 0.05$).

As shown in Table 4, independently from dietary CP level, addition of L-carnitine in broiler diets from 18 to 53 days of age resulted in a significant reduction in abdominal fat content of broilers, either expressed as an absolute weight or as a percent of body weight. However, all other parameters measured for evaluating carcass yield and quality were not affected by dietary L-carnitine supplementation. Dietary CP level had a marked effect on some parameters of carcass yield and quality (Table 4), irrespective of dietary L-carnitine supplementation. Increasing dietary CP level from 18 to 22% in grower and finisher diets of broilers resulted in significant increases in breast yield (with bones), breast meat yield and thigh meat yield (meat excised from thighs + drumsticks). However, no significant differences were found in these parameters between birds fed 22% CP compared with 20% CP diets or between those fed 20% CP compared with 18% CP diets. Abdominal fat contents, either expressed as absolute weights or as percentages of live body weight, were significantly reduced (Table 4) in response to increasing dietary CP level, regardless of added dietary L-carnitine. All other parameters of carcass yield and quality were not significantly affected by dietary crude protein level. The interaction between dietary CP level and dietary supplemental L-carnitine was not significant for all parameters studied as criteria for evaluating carcass yield and quality, unsupplemented.

The dry matter (DM), crude protein (CP), and ether extract (EE) contents of breast meat, thigh meat and liver tissue of broilers, fed diets supplemented or unsupplemented with L-carnitine at different CP levels from 18 to 53 days of age, are summarized in Table 5. With respect to effect of dietary L-carnitine supplementation on the composition of liver, breast meat and thigh meat, analysis of variance revealed that EE content of the breast meat was significantly reduced in response to added dietary L-carnitine, irrespective of dietary CP level. Regardless of dietary supplementation of L-carnitine, increasing dietary CP level from 18 to 22% resulted in significant increases in CP contents of breast and thigh meat. The highest CP content of breast meat was achieved by birds fed the highest level of dietary CP (22%), but was not significantly different from that attained by the birds fed the medium level of dietary CP (20%). Although the highest CP content of thigh meat was observed for birds fed the medium level of dietary CP, it was not significantly different from that achieved by birds fed the highest level of dietary CP. Other proximate analyses determined for breast and thigh meat and for liver tissue were not affected by dietary CP level.

Table 4

Carcass yield and components of 53-days-old broiler chickens fed L-carnitine (CN)-supplemented diets with different levels of crude protein (CP) during the growing and finishing periods from 18 up to 53 days of age

Dietary treatments		Mean values (g)												
CP level, MJ/kg	CN added, mg/kg	LBW+	AFP[7]	EC‡	Liver	Heart	Gizzard	Giblets	BY[1]	BM[2]	TY[3]	TM[4]	TEP§	AF[5]
18	0	2112	2.88	1391	40.17	11.50	28.50	80.17	532	354	509	316	1471	60.83
18	50	2170	2.29	1465	39.33	11.83	28.00	79.17	566	378	536	339	1544	49.67
20	0	2177	2.31	1439	38.50	11.17	27.00	76.67	565	382	531	337	1516	50.67
20	50	2210	2.00	1454	38.83	12.00	28.67	77.83	581	395	534	349	1532	44.00
22	0	2207	1.96	1467	41.33	12.83	27.50	81.67	579	390	536	357	1549	43.00
22	50	2223	1.65	1483	37.00	11.50	31.83	80.33	589	406	538	364	1563	36.83
SEM[6]		51.9	0.15	36.0	1.66	0.52	2.20	2.69	12.5	12.5	13.3	11.5	38.0	3.48
Protein effect:														
18		2141	2.59^{a}	1428	39.75	11.67	28.25	79.67	549^{bc}	366^{c}	522	327^{bc}	1507	55.25^{a}
20		2193	2.16^{b}	1446	38.67	11.58	27.83	77.25	573^{ab}	389^{ab}	533	343^{ab}	1524	47.33^{b}
22		2215	1.81^{c}	1475	39.17	12.17	29.67	81.00	584^{a}	398^{a}	537	360^{a}	1556	39.92^{c}
SEM		36.7	0.00	25.5	1.18	0.37	1.56	1.90	8.82	8.84	9.42	8.10	26.9	2.46
L-Carnitine effect:														
0		2165	2.39^{a}	1432	40.00	11.83	27.67	79.50	558	375	525	337	1512	51.50^{a}
50		2201	1.98^{b}	1467	38.39	11.78	29.50	79.11	579	393	536	351	1456	43.50^{b}
SEM		30.0	0.09	20.8	0.96	0.30	1.27	1.55	7.21	7.22	7-69	6.61	21.9	2.01
Source of variation:		Probabilities												
Protein		0.3523	0.0001	0.4273	0.8097	0.4931	0.6860	0.3803	0.0268	0.0418	0.5321	0.0248	0.4378	0.0006
L-Carnitine		0.4098	0.0024	0.2503	0.2447	0.8990	0.3156	0.8625	0.0571	0.0861	0.3197	0.1464	0.2805	0.0085
Protein x L-carnitine		0.9218	0.5643	0.6463	0.3570	0.1137	0.5523	0.8809	0.6090	0.9138	0.5747	0.7704	0.6831	0.7336

+, ‡Refer to live body weight (LBW) and eviscerated carcass (EC), respectively, the latter is cooled carcass weight without neck and abdominal fat.

§Total edible parts (TEOÜ are eviscerated carcass plus giblets; the latter includes the edible offal of carcass (i.e. liver, heart and gizzard).

1-5Refer to breast yield (BY), breast meat (BM), thighs + drumsticks yield (TY), thighs + drumsticks meat (TM) and abdominal fat (AF), respectively.

6Standard error of the means (n = 6).

7AFP is abdominal fat content as a percent of LBW.

a-cMeans in the same column bearing different superscripts differ significantly ($P \leq 0.05$).

Table 5

Compositions of liver, breast meat and thigh meat of 53-days-old broiler chickens fed L-carnitine (CN)-supplemented diets with different levels of crude protein (CP) during the growing and finishing periods from 18 up to 53 days of age

Dietary treatments		Liver			Breast meat			Thigh meat		
CP level (%)	CN added, mg/kg	DM[1] (%)	CP[2] (%)	EE[3] (%)	DM (%)	CP (%)	EE (%)	DM (%)	CP (%)	EE (%)
18	0	26.31	19.81	5.22	26.05	22.95	2.42	26.36	19.47	5.96
18	50	26.25	19.77	5.17	26.56	23.12	2.07	26.95	19.94	5.72
20	0	26.22	19.88	4.93	27.09	23.90	2.28	27.48	20.35	5.83
20	50	26.10	19.82	4.77	26.85	24.06	1.93	27.49	20.56	5.71
22	0	26.33	19.81	4.92	27.33	24.15	2.02	26.92	20.42	5.65
22	50	25.98	19.84	4.58	26.47	24.27	1.45	26.98	20.36	5.45
SEM+		0.34	0.23	0.31	0.33	0.29	0.24	0.35	0.29	0.37
Protein effect:										
18		26.28	19.79	5.19	26.30	23.05[c]	2.24	26.65	19.71[c]	5.84
20		26.16	19.85	4.85	26.97	23.98[ab]	2.11	27.49	20.46[a]	5.77
22		26.15	19.83	4.75	26.90	24.21[a]	1.74	26.95	20.39[ab]	5.55
SEM		0.24	0.16	0.22	0.24	0.21	0.17	0.25	0.21	0.26
L-Carnitine effect:										
0		26.29	19.83	5.02	26.82	23.66	2.24[a]	26.92	20.08	5.82
50		26.11	19.81	4.84	26.63	23.82	1.82[b]	27.14	20.29	5.63
SEM		0.20	0.13	0.18	0.19	0.17	0.14	0.20	0.17	0.21
Source of variation:		Probabilities								
Protein		0.9126	0.9652	0.3434	0.1065	0.0009	0.1150	0.0727	0.0282	0.7211
L-Carnitine		0.5293	0.9215	0.4830	0.4816	0.5390	0.0415	0.4578	0.4003	0.5467
Protein x L-carnitine		0.9059	0.9781	0.8978	0.1331	0.9961	0.8721	0.6723	0.6650	0.9880

[1-3]Refer to dry matter (DM), crude protein (CP) and ether extract (EE), respectively.

+Standard error of the means (n = 6).

[a-c]Means in the same column bearing different superscripts differ significantly ($P \leq 0.05$).

For all analyses determined as parameters for evaluating composition of liver, breast meat and thigh meat, the interaction between dietary CP level and supplemental dietary L-carnitine was not significant.

Discussion

Effect of supplemental dietary L-carnitine

Apart from dietary CP level, the improvements observed in the present study in live body weight and body weight gain of broilers in response to added dietary L-carnitine may be attributable to an improved utilization of dietary nitrogen, achieved through more efficient fat oxidation by L-carnitine. We observed positive responses in body weight gain of broilers to dietary L-carnitine during the first two weeks of the experimental period. The increased rate of growth observed in this study during the period from 18 to 32 days of age in response to supplemental dietary L-carnitine may imply that the requirement of broilers for L-carnitine was higher during that period to support a maximal growth. However, it is logical to suppose that the rate of L-carnitine biosynthesis in the growing animals, including broilers, is lower than in adults. In human, it was concluded that the requirement for L-carnitine is increased by rapid growth, and that the biosynthetic capacity of L-carnitine is reduced by prematurity and infancy /12/. The higher body weight gains achieved by birds fed diets supplemented with L-carnitine compared with their control counterparts may explain the better feed conversion for the former than the latter, since feed intakes were approximately similar for the two groups, irrespective of dietary CP level.

The reduced content of carcass fat (as determined by abdominal fact content, both as an absolute weight and as a percent of body weight, and ether extract (EE) content of breast meat) observed in the current study in response to L-carnitine supplementation, may be due partly to a reduction in hepatic lipogenic capacity, since liver is the major site of lipogenesis in poultry, but other factors may also be responsible for the regulation of the rate of fat accumulation in adipose tissue and muscles. In this regard, research with Atlantic salmon (Salmo salar) has provided evidence to explain the mechanism by which dietary L-carnitine may alter some indices of intermediary metabolism through stimulating fatty acid oxidation /20/. The induc-

tion of pyruvate carboxylate (or a reduction of turnover) and enhanced protein synthesis was implicated in the mechanism for carnitine-induced changes in gluconeogenesis and nitrogen metabolism /20/.

The results of the present study are in agreement with those reported by other authors /15, 23, 39, 40/. When starter pigs were fed L-carnitine at levels of 0 or 1000 mg/kg of the diet from 0 to 2 weeks postweaning, followed by carnitine concentrations of 0, 250 or 500 mg/kg of diet for the next 3 weeks, an improvement in average daily gain was observed /40/. In another study on broilers, it has been shown that growth, slaughtering performance and carcass quality (meat and fat quality) tended to improve by increasing the level of added dietary L-carnitine from 20 to 60 mg/kg diet, the percent of C18:2 fatty acid was increased significantly by L-carnitine /23/. In addition, improvements in growth rate and feed conversion and a reduction in body fat have been reported for African catfish (Clarias gariepinus) fed diets supplemented with L-carnitine /39/. Other researchers reported that body weight gain and degree of protein conversion (efficiency of protein utilization) achieved by foals that received 10 g/d supplemental dietary L-carnitine for 78 days were significantly greater than those of the control group /15/.

In contrast, Cartwright /9/ reported that performance of broilers, in terms of body weight, feed consumption, carcass fat and abdominal fat content, was not affected by feeding diet supplemented with 0.05% L-carnitine of the diet from 5 to 7 weeks of age. Likewise, Barker and Sell /2/ observed no effect of added dietary L-carnitine, at levels of 0, 50 or 100 mg/kg diet, on performance or carcass composition of broiler chickens and young turkeys fed low- or high-fat diets. Leibetseder /22/ investigated the effectiveness of carnitine and its precursors (lysine and methionine) in reducing the formation of abdominal fat in broilers fed diets supplemented with 0 or 5% fat. He found that performance (body weight gain and feed conversion) and abdominal fat content of broilers were not influenced by dietary carnitine (L or DL form) at a level of 200 mg/kg diet. He also reported that carnitine concentrations in liver, kidney, heart and certain skeletal muscles significantly increased in response to dietary supplementation of L-carnitine. These studies may have been performed under managerial, housing or environmental conditions different from those applied in the present study.

Effect of dietary protein level

Regardless of dietary supplementation of L-carnitine, the improvement in feed conversion efficiency observed in the present study during the from 32 to 39 days of age coincided with insignificant and comparable feed intakes and body weight gains for broilers in response to increasing dietary CP level may imply that birds fed on the highest level of dietary CP had higher ability to utilize their diets than that of birds fed on the lower levels of dietary CP. This response agrees with the findings of other authors who reported improvements in feed efficiency of broilers when dietary CP level was increased, even in absence of a growth response /17, 27/. Dealing with male broiler chicks, it has been found that chicks fed ad libitum on a 18% CP diet were the least efficient at utilizing feed and the most efficient at utilizing protein for weight gain, when compared to those fed on 23% or 30% CP diets /32/. Research with growing broilers has also shown that CP level required for optimal feed efficiency was clearly higher than that required for optimal growth /11/.

However, the absence of growth responses of broilers to increasing CP level in most periods throughout this study may be due to the fact that protein and amino acid requirements of broilers are generally considered to decrease with increasing age.

The higher breast yield, breast meat and thigh yield, as well as high CP contents and lower abdominal fat contents for broilers fed high protein diets in the present study are consistent with the findings of other investigators. It has been reported that total meat and breast meat yields increased whereas carcass skin and abdominal fat yields decreased by increasing finisher protein levels /33/. Other researchers evaluated performance of male broilers in terms of yield and composition of various carcass parts by feeding diets of different protein contents (17, 20 or 23%) with or without of supplementation of methionine or metionine plus lysine /37/. They demonstrated that birds on the 17% diet had less carcass protein and more carcass fat than the higher protein diets. They also reported that, at similar weights, birds fed the higher levels of protein or birds fed diets supplemented with essential amino acids resulted in higher levels of carcass protein accretion. It has been reported in an earlier study employing diets varying in CP content from 16 to 25%, that feeding high protein diets for broilers produced relatively lean carcasses /4/. Those authors emphasized the importance of maining adequate protein levels in broiler diets up to the

time of marketing to prevent excessive fat deposition and to maintain feed efficiency.

However, the biochemical basis explaining the decrease in lipogenesis accompanying the feeding on high-protein diets is still unclear yet. One of the primary mechanisms involved in reducing carcass fat by feeding higher CP diets is the associated increased energy expenditure and associated increased heat increment involved in degrading excess amino nitrogen to uric acid /3/. In other words, it was proposed that excretion of excess amino acid N from diets containing high levels of CP would require energy. This requirement for energy would come at the expense of that used for fat synthesis /3/. Other authors found that increasing dietary protein for broilers resulted in an increase in GOT activity but decreased malic enzyme activity /32/. They hypothesized that higher activity of GOT in chicks fed high-protein diets would mirrored the rate of amino acid turnover and a requirement for the disposal of carbon fragments from these amino acids. Recently, it has been suggested that under conditions of feeding high protein deits, transamination of amino acids would deplete 2-oxoglutarate and force citrate from lipid synthetic pathways to 2-oxoglutarate production in order to support the transamination /31/.

Dietary L-carnitine x protein interaction

The perusal of data shown in Tables 2 and 3 indicates that the significant interactions between dietary supplementation of L-carnitine and dietary CP level on body weight gain and feed conversion of broilers during the period from 18 to 32 days of age may imply more effectiveness for supplemental L-carnitine with the lowest level (18%) than with the higher levels (20 or 22%) of dietary CP.

These results agree with the conclusion of other reports that carnitine seemed effective in improving body weight gain and feed conversion in groups of animals (fish, piglets and quail) fed diets marginally deficient in lysine and methionine plus cystine, respectively /35/.

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INFLUENCE OF DIETARY L-CARNITINE ON PERFORMANCE AND CARCASS QUALITY OF BROILER CHICKENS

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Effects of dietary L-carnitine supplementation on the performance, carcass yield and components, abdominal fat and composition of meat of broiler chickens were investigated. Ross broiler chicks, 18-day-old were used in this experiment. Four experimental diets were formulated, by adding three levels of supplemental L-carnitine (50, 100 or 150 mg/kg) to a basal diet and used from 18 to 46 days of age. Apart from the level of supplementation, L-carnitine (CN) addition resulted in significant increases ($P < 0.05$) in body weight gains (BWG) of broilers (means were 397 vs. 365 and 410 vs. 377 g, for the first and second weeks of the experimental period, respectively). Abdominal fat (AF) contents of 46-day-old broilers were significantly decreased, both as absolute weights ($P < 0.05$) and as percentages of body weight ($P < 0.01$), by added CN (means of AF were 36.8 vs. 44.8 g, equivalent to 1.87 vs. 2.32%). Level of added CN had no effect on BWG or AF of broilers. It was concluded that the effectiveness of supplemental L-carnitine for improving BWG and/or decreasing AF of broilers may depend on the age at which L-carnitine is added. Under the conditions of this study, a supplementation of 50 mg L-carnitine per kg diet proved to be effective.

Keywords: L-Carnitine — performance — carcass quality — broilers

Introduction

L-Carnitine (β-OH-γ-N-trimethylaminobutyric acid) serves two major functions. The well-established role of L-carnitine is facilitating the entry of long-chain fatty acids into mitochondria for energy-generating processes via β-oxidation. L-Carnitine also facilitates removal from mitochondria of short-chain and medium-chain fatty acids that accumulate as a result of normal and abnormal metabolism /21/. It has a widespread occurrence in nature; plants generally contain little carnitine comparing to animals /7/. In animals, its concentration varies widely in different species /30/ and

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among tissues /1, 7, 24/. Generally, L-carnitine is derived from the diet and by endogenous biosynthesis from the two essential amino acids lysine and methionine, in the presence of ferrous ions and a number of vitamins: ascorbate, niacin and pyridoxine /11, 22, 23, 26/. Most of cereal grains that constitute the major component of poultry diets are poor in their contents of both these two amino acids and L-carnitine /5, 6, 19/.

Information available in the literature on the response of avian species, particularly broiler chickens, to supplemental dietary carnitine is sporadic. The study carried out by Cartwright /9/ has indicated that no effect of L-carnitine fed at 0.5% of the diet from 5 to 7 weeks of age on abdominal fat weight, body fat, feed intake or growth of broiler chickens. Barker and Sell /4/ reported that supplemental dietary L-carnitine up to 100 mg/kg did not influence performance and carcass composition of broiler chickens fed low- or high-fat diets. Leibetseder /16/ has investigated if carnitine (L or D form) and its precursors (lysine, methionine) can reduce the formation of abdominal fat in broilers. He found that performance (body weight gain, feed conversion) and abdominal fat were influenced by dietary fat, lysine and methionine and were not altered by carnitine. In contrast, it has been reported that feeding diets supplemented with L-carnitine up to 60 mg/kg significantly affected the composition of abdominal fat and tended to improve fattening performance of broiler chickens /17/.

Research with other species (pigs, fish, foals) has demonstrated that growth performance improved by feeding diets supplemented with L-carnitine /14, 27, 28, 31/.

Excessive carcass fat accumulation, particularly in the abdominal and visceral areas, is one of the major concerns for broiler and turkey producers. This fat is generally undesirable for consumers and represents a waste product to the poultry processor. Numerous attempts have been made to minimize this fat accumulation, either genetically or by dietary manipulations, with different degrees of success.

Theoretically, dietary L-carnitine could play a role in reducing the undesirable fat in carcasses of broilers or turkeys. Pertaining this hypothesis, carnitine has a key role in facilitating the transport of long-chain fatty acids across the inner mitochondrial membrane prior to β-oxidation /6, 7/. Therefore, the transport of long-chain fatty acids under the conditions of L-carnitine insufficiency could be impaired. Thus, diets supplemented with L-carnitine should enhance the oxidation of these fatty acids, thereby

decreasing their availability for esterification to triglycerides and storage in the adipose tissues.

The aim of the present study was to investigate the effects of supplemental dietary L-carnitine on growth performance, carcass yield and components, abdominal fat pad and meat composition of broiler chickens.

Materials and Methods

One hundred and twenty, 1-day-old Ross broiler chicks obtained from a commercial hatchery were kept at conventional, wire-floored brooding batteries in an open-sided house provided with continuous lighting and equipped with central gas-heating system. The chicks were fed on a commercial starter diet up to 18 days of age. Thereafter, they were wing-banded and randomly divided into four experimental groups of three replicates each, and placed at 3-tier, wire-floored growing batteries equipped with nipple drinkers. A practical grower-finisher diet was formulated and served as basal (control) diet (Table 1). The experimental diets were prepared by adding three levels of L-carnitine (50, 100 or 150 mg/kg) in the form of Carniking· (LONZA Ltd.) to the basal diet and thoroughly mixing. Feed and water were provided ad libitum during the entire experimental period from 18 to 46 days of age.

Individual live body weights of the chicks were recorded at the beginning of experiment (18-day-old) and on a weekly basis thereafter. Weekly records of feed intake for each of the replicate groups of each treatment were also maintained. Feed conversion was calculated as the amount of feed consumed per unit of body weight gain. Growth performance was evaluated in terms of weekly live body weight, body weight gain, feed intake and feed conversion.

At 46 days of age, a slaughter test was performed to determine carcass quality parameters including carcass yield and components (weights and percentages of body parts and organs), abdominal fat pad and chemical composition of breast and thigh meat. Six birds from each experimental group, with body weights around the average value of the representative group, were selected and killed by decapitation. The carcasses were immediately scalded, feather-picked and eviscerated, and then chilled overnight into a refrigerator in order to facilitate the removal of abdominal fat pad. The cutting up of the carcasses was made according to the procedure described by Jensen /15/, and the abdominal fat pad was removed according to the method outlined by Fancher and Jensen /10/. Samples of breast and thigh muscles were taken and analyzed for dry matter, crude protein (N x 6.25), ether extract and ash contents. The proximate analyses for the basal diet and meat samples were made according to the standard procedures described by the Association of Official Analytical Chemists /2/.

Data were subjected to statistical analysis using Statgraphics Program, STSC, software computer /25/. Based on one-way analysis of variance, differences among means were considered to be significant at $P \leq 0.05$.

Table 1

Percentage composition and proximate analysis of the basal diet

Ingredients	%
Yellow corn, ground	56.00
Wheat, ground	15.00
Sunflower meal preparation[1]	15.00
Soybean meal, 47%	5.00
Fish meal, 64%	6.20
Limestone	1.50
Monocalcium phosphate	0.60
Vit. and Min. Premix[2]	0.50
Common salt	0.20
Total	100.00
Calculated analyses:	
Crude protein, %	19.30
Crude fiber, %	3.42
Lysine, %	1.14
Methionine, %	0.38
Methionine + Cystine, %	0.90
Calcium, %	1.02
Phosphorus, %	0.66
Metabolizable energy, MJ/kg	13.09
Determined analyses:	
Dry matter (DM), %	90.12
Crude protein (CP), %	19.22
Ether extract (EE), %	3.02
Crude fiber (CF), %	3.41
Ash, %	6.20
Gross energy (GE), MJ/kg DM	18.01

[1]Contained the following (g/kg): solvent-extracted sunflower meal, 858; sunflower oil, 120; L-lysine HCl, 15; DL-methionine, 7.

[2]Supplied per kilogram of diet: vitamin A, 7.840 IU; vitamin D_3, 2.009 IU; vitamin E, 9.81 IU; vitamin K_3, 3.8 mg; vitamin B_1, 3.52 mg; vitamin B_2, 9 mg; vitamin B_6, 5.28 mg; vitamin B_{12}, 0.015 mg; pantothenic acid, 25 mg; niacin, 35 mg; choline chloride, 1.750 mg; Ca, 8.89 g; P, 2.66 g; NaCl, 2.45 g; Mn, 60 mg; Fe, 45 mg; Cu, 8 mg; Zn, 50 mg; I, 3 mg; Se, 0.15 mg; ethoxyquin, 125 mg.

Results and Discussion

Growth performance

Regardless of the level of dietary L-carnitine added, its inclusion in broiler diets resulted in significant increases ($P < 0.05$) in body weight gain of broilers during the periods from 18 to 32 days of age (the first two weeks of the experimental period) and during the entire experimental period from 18 to 46 days of age (Table 2). Although birds fed L-carnitine-supplemented diets, independently from level of supplementation, consumed slightly higher feed (but not significant) compared with the control groups, they achieved numerical improvements (but also not significant) in feed conversion values (feed: gain ratio) during all periods investigated, as compared to the control. However, level of L-carnitine supplemented had no effect ($P > 0.05$) on all parameters studied for growth performance.

The improvements observed in the present study in body weight gain of broilers in response to added dietary L-carnitine may be attributable to an improved utilization of dietary nitrogen, achieved through more efficient fat oxidation.

Reports on the effects of dietary L-carnitine on growth performance are conflicting. Research with young pigs and fish has shown some favourable responses to added dietary L-carnitine. Studies on pigs have demonstrated that when starter pigs were fed L-carnitine at levels of 0 or 1000 mg/kg of the diet from 0 to 2 week postweaning, followed by carnitine concentrations of 0, 250 or 500 mg/kg of diet for the next 3 weeks, an improvement in average daily gain was noted /32/. The authors also found a linear improvement in feed efficiency of young pigs with increasing dietary carnitine concentration /32/. Other reports having dealt with fish indicated that supplemental dietary L-carnitine resulted in an increase in growth rate of sea bass (Dicentrarchus labrax L.) fry /27/. Recently, some investigators evaluated six levels of dietary L-carnitine, varying from the control level (about 125 mg/kg) to 3920 mg/kg, at two levels of dietary lipids in isonitrogenous diets for African catfish, Clarias gariepinus /31/. They demonstrated that growth rate and feed conversion improved significantly with increasing dietary levels of L-carnitine. Others dealing with fish, piglet and quail, found that L-carnitine seemed effective in improving body weight gain and feed conversion mainly in groups with diets marginally deficient in lysine and methionine plus cystine, respectively /28/. More recently, it has

Table 2

Performance of broiler chickens fed diets supplemented with L-carnitine during the growing and finishing periods from 18 to 46 days of age (Means ± SE+)

Criteria of performance‡	Levels of L-carnitine added (mg/kg)			
	0	50	100	150
Live body weights (g):				
18-day[1]	368 ± 12.2	364 ± 13.3	366 ± 12.4	356 ± 13.9
25-day[2]	733 ± 15.7	758 ± 20.4	759 ± 15.1	759 ± 17.8
32-day[3]	1110 ± 20.9	1173 ± 26.4	1166 ± 18.5	1167 ± 23.6
39-day[4]	1504 ± 25.2	1588 ± 29.4	1584 ± 22.3	1581 ± 33.2
46-day[5]	1908 ± 29.2	1994 ± 33.9	1990 ± 24.9	1989 ± 39.9
Body weight gains (g):				
18-25	365^{d} ± 7.85	394^{ab} ± 12.7	393^{abc} ± 10.2	403^{a} ± 7.09
25-32	377^{d} ± 8.17	414^{a} ± 11.2	407^{abc} ± 6.25	408^{ab} ± 9.40
32-39	395 ± 7.67	415 ± 8.16	418 ± 7.73	414 ± 11.7
39-46	404 ± 10.4	406 ± 11.7	405 ± 12.3	408 ± 12.0
18-46	1540^{d} ± 21	1630^{ab} ± 28	1623^{abc} ± 23	1633^{a} ± 30
Feed intake (g/bird/period):				
18-25	535 ± 11.5	559 ± 8.66	573 ± 12.7	571 ± 11.5
25-32	725 ± 10.4	751 ± 11.5	743 ± 14.4	748 ± 9.82
32-39	879 ± 11.5	898 ± 8.66	893 ± 11.5	891 ± 11.5
309-46	959 ± 8.66	1000 ± 9.81	993 ± 14.4	1000 ± 12.7
18-46	3098 ± 42.1	3208 ± 39.3	3202 ± 53.1	3210 ± 45.6
Feed conversion (g feed:g gain):				
18-25	1.47 ± 0.078	1.42 ± 0.043	1.46 ± 0.075	1.42 ± 0.059
25-32	1.93 ± 0.087	1.82 ± 0.066	1.83 ± 0.087	1.84 ± 0.068
32-39	2.23 ± 0.098	2.17 ± 0.075	2.14 ± 0.093	2.16 ± 0.079
39-46	2.38 ± 0.090	2.47 ± 0.105	2.46 ± 0.080	2.46 ± 0.104
18-46	2.02 ± 0.089	1.97 ± 0.070	1.96 ± 0.068	1.97 ± 0.077

+Standard error of the mean.

‡Values of body weight and body weight gain represent means for 30 birds per treatment, and those feed intake and feed conversion represent means for three replicate groups of 10 birds per treatment.

[1-5]Refer to age of birds in days.

[a-d]Means in the same row bearing different superscripts differ significantly ($P \leq 0.05$).

been reported that body weight gain and degree of protein conversion (efficiency of protein utilization) achieved by foals that received 10 g/d supplemental dietary L-carnitine for 78 days were significantly greater than those of the control group /14/.

The positive effect of supplemental L-carnitine observed in the present study on body weight gain agrees with the findings reported by the investigators mentioned above but disagrees with those reported by others /4, 9, 16/.

These inconsistent responses of animals to supplementary L-carnitine may be related mainly to inter-species differences, age, sex, plane of nutrition, feeding program, nutrient composition of the diet, levels of L-carnitine or its precursors in the diet and/or managerial or environmental conditions.

Carcass yield and quality

Table 3 shows parameters of carcass yield and components in terms of weights and percentages of body parts and organs, and abdominal fat of 46-day-old broiler chickens fed diets supplemented with L-carnitine. Analysis of variance proved that abdominal fat contents of 46-day-old broilers, either expressed as absolute weights ($P < 0.05$) or as percentages ($P < 0.01$) of body weight, were significantly reduced in response to added dietary L-carnitine, irrespective of the level of supplementation. However, level of added L-carnitine had no effect ($P > 0.05$) on the quantity or the percentage of abdominal fat deposited in broiler chickens at 46 days of age. All other parameters of carcass yield and quality were not significantly affected ($P > 0.05$) by either addition of L-carnitine or its level inclusion.

The fat-lowering effect of dietary L-carnitine obtained in the present study may be explained, at least partly, by a reduction in hepatic lipogenic capacity, since liver is the major site of lipogenesis in poultry but other factors may also be responsible for the regulation of the rate of fat accumulation in adipose tissue. This may imply that abdominal fat is the most susceptible component of a broiler carcass for alteration by dietary L-carnitine. This suggestion was supported by the findings of other investigators that supplemental dietary L-carnitine up to 60 ppm significantly affected the fatty acid composition of abdominal fat and tended to improve the fattening performance of broiler chickens /17/.

Limited comparative information could be found in the literature on the influence of dietary carnitine on carcass yield or quality for poultry or other species. The relative response of different species of poultry and other animals to supplemental L-carnitine is controversal. In the present study, the observed decrease in abdominal fat contents of broilers in re-

Table 3

Carcass yield and components of 46-day-old broiler chickens fed diets supplemented with L-carnitine during the growing and finishing periods from 18 to 46 days of age (Means ± SE+)

Measurements	Levels of L-carnitine added (mg/kg)			
	0	50	100	150
Live body weight, g	1923 ± 44.6	1965 ± 51.6	1967 ± 49.4	1973 ± 59.1
Eviscerated carcass[1], g	1241 ± 29.9	1269 ± 33.5	1270 ± 32.7	1277 ± 40.8
Eviscerated carcass, %[2]	64.63 ± 0.26	64.60 ± 0.11	64.58 ± 0.13	64.68 ± 0.20
Liver, g	33.33 ± 0.80	33.77 ± 1.15	33.23 ± 1.73	33.85 ± 1.47
Liver, %	1.74 ± 0.06	1.72 ± 0.06	1.70 ± 0.09	1.72 ± 0.05
Heart, g	9.57 ± 0.43	9.78 ± 0.31	9.58 ± 0.25	10.35 ± 0.58
Heart, %	0.497 ± 0.013	0.499 ± 0.015	0.490 ± 0.022	0.523 ± 0.018
Gizzard, g	30.83 ± 1.54	30.10 ± 1.28	30.92 ± 1.34	29.83 ± 0.87
Gizzard, %	1.61 ± 0.08	1.54 ± 0.10	1.57 ± 0.05	1.53 ± 0.08
Giblets[3], g	73.73 ± 2.02	73.65 ± 1.10	73.23 ± 2.41	64.03 ± 1.27
Giblets, %	3.84 ± 0.11	3.77 ± 0.13	3.73 ± 0.11	3.76 ± 0.07
Breast yield, g	443 ± 9.85	455 ± 12.8	456 ± 12.3	454 ± 13.9
Breast yield, %	23.03 ± 0.08	23.13 ± 0.17	23.18 ± 0.06	23.00 ± 0.06
Thighs + drumsticks, g	436 ± 9.61	449 ± 12.4	453 ± 12.8	453 ± 14.4
Thighs + drumsticks, %	22.67 ± 0.12	23.03 ± 0.08	23.03 ± 0.16	22.97 ± 0.17
Back, g	181 ± 5.72	179 ± 3.53	180 ± 5.35	182 ± 9.81
Back, %	9.42 ± 0.18	9.12 ± 0.19	9.16 ± 0.08	9.20 ± 0.27
Wings, g	156 ± 4.36	163 ± 6.41	161 ± 4.35	161 ± 4.64
Wings, %	8.13 ± 0.15	8.30 ± 0.21	8.19 ± 0.20	8.18 ± 0.20
Neck, g	131 ± 3.24	128 ± 6.47	131 ± 4.54	134 ± 4.79
Neck, %	6.85 ± 0.26	6.50 ± 0.19	6.65 ± 1.20	6.80 ± 0.10
Total edible parts[4], g	1315 ± 30.4	1343 ± 33.3	1343 ± 33.9	1350 ± 41.8
Total edible parts, %	68.35 ± 0.29	68.35 ± 0.18	68.32 ± 0.19	68.42 ± 0.18
Abdominal fat, g	44.8^{a} ± 1.99	35.8^{bc} ± 2.17	37.0^{b} ± 1.60	36.6^{bcd} ± 2.03
Abdominal fat, %	2.32^{a} ± 0.05	1.87^{bc} ± 0.08	1.88^{b} ± 0.06	1.85^{bcd} ± 0.07

+Standard error of the mean.

[1]Eviscerated carcass is cooled carcass weight without neck and abdominal fat (n = 6).

[2]All relative weights of carcass and its components, and organs were experessed as % of live body weight at slaughtering.

[3]Giblets refer to the edible offal of the carcass (i.e. liver, heart and gizzard).

[4]Total edible parts include eviscerated carcass plus giblets.

$^{a-d}$Means in the same row bearing different superscripts differ significantly ($P \leq 0.05$).

sponse to dietary L-carnitine agrees with the results obtained by other authors /8, 27, 32/ who reported that body fat and carcass fat (of fish and pigs) were significantly reduced by supplemental dietary L-carnitine. Conversely, other studies with poultry have been shown that abdominal fat did not affected by added dietary L-carnitine /4, 9, 16/. Likewise, Barker and Sell /4/ stated that breast meat yield of broilers was not affected by feediong diets containing L-carnitine up to 100 mg/kg.

Generally, the mean values for percentages of eviscerated carcass and total edible parts obtained in the present study were in a close consistency with those reported by Sherif /29/, but were considerably lower than those obtained by Raya /20/ who included the neck in determining eviscerated carcass and total edible parts. Other important factors that can affect dressing percentage and total edible parts of a chicken are sex, breed or strain, age at slaughter, plane of nutrition, and the nutrient composition of her diet.

Composition of meat

Data on the composition of breast and thigh meat are summarized in Table 4. Analysis of variance of these data proved that there were no significant differences ($P > 0.05$) in moisture contents or dry matter components for either breast or thigh meat could be attributable to dietary supplementation of L-carnitine. Also, level of supplemental L-carnitine had no effect on ($P > 0.05$) the proximate composition of meat. In spite of skeletal muscles constitute the major proportion of the total eidble parts of poultry carcasses, most of the nutritional studies have been focussed on gross

Table 4

Composition of breast and thigh meat of 46-day-old broiler chickens fed diets supplemented with L-carnitine during the growing and finishing periods from 18 to 46 days of age (Means ± SE⁺)

Component and analyses[1]	Levels of supplemental dietary L-carnitine (mg/kg)			
	0	50	100	150
Breast meat:				
Dry matter, %	25.43 ± 0.13	25.55 ± 0.08	25.52 ± 0.16	25.73 ± 0.16
Moisture, %	74.57 ± 0.13	74.45 ± 0.08	74.48 ± 0.16	74.27 ± 0.16
Crude protein, %	23.10 ± 0.13	23.25 ± 0.09	23.25 ± 0.20	23.43 ± 0.11
Ether extract, %	0.96 ± 0.01	0.87 ± 0.06	0.86 ± 0.07	0.85 ± 0.04
Ash, %	1.24 ± 0.03	1.30 ± 0.04	1.29 ± 0.04	1.26 ± 0.05
Thigh meat:				
Dry matter, %	25.90 ± 0.16	25.93 ± 0.26	26.02 ± 0.16	26.03 ± 0.15
Moisture, %	74.10 ± 0.16	74.07 ± 0.26	73.98 ± 0.16	73.97 ± 0.15
Crude protein, %	20.15 ± 0.16	20.20 ± 0.20	20.18 ± 0.21	20.02 ± 0.21
Ether extract, %	4.48 ± 0.06	4.46 ± 0.09	4.43 ± 0.11	4.45 ± 0.07
Ash, %	1.08 ± 0.05	1.06 ± 0.02	1.10 ± 0.03	1.09 ± 0.03

⁺Standard error of the mean.

[1]Means of six birds per treatment (% of fresh weight).

(total) carcass composition. Meanwhile, some reports presented comparable data on the proximate composition of breast and thigh meat of broiler chickens /12, 13, 20/.

In the present study we observed that the crude protein content of breast meat was higher than that of thigh meat, and the latter contained higher ether extract than the former. Also, the moisture content of breast meat was slightly higher than that of thigh meat. These findings agree with those reported by the investigators mentioned in the previous paragaph, regardless of the supplementation of dietary L-carnitine.

Dealing with total carcass composition, Barker and Sell /4/ stated that the proximate composition of 21-day-old poults and 45-day-old broilers was not changed by L-carnitine. However, a reduction in carcass fat of young pigs was reported when pigs were fed on a diet containing 1000 mg carnitine per kg, 0.1% of the diet /32/. This is not surprising since pigs, like most of mammalian species, differ from chickens in many aspects of carbohydrate and lipid metabolism, specifically in the site of lipid biosynthesis and the activity of lipid transfer protein which translocates the resynthesized lipids from the place of biosynthesis to the target tissues for storage /3, 18/.

In was concluded that the effectiveness of supplemental dietary L-carnitine for improving body weight gain and/or decreasing abdominal fat contents of broilers may depend on the age which L-carnitine is added. Under the conditions of this study, a supplementation of 50 mg L-carnitine per kg diet proved to be effective.

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SHORT COMMUNICATION

SIMULTANEOUS ISOLATION OF BOTH RNA AND DNA FROM MANY SMALL TISSUE SAMPLES

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There are several methods describing the simultaneous isolation of both DNA and RNA from different samples. However, most of them are not well suited for the simultaneous isolation of both RNA and DNA from many small tissue samples. Some of the published papers use a lengthy and costly ultracentrifugation step through a cesium chloride cushion /4, 6/. Other methods apply detergents for cell disruption, therefore these methods can be used only for tissue culture cells /5, 9, 11/. They are unsuitable to isolate RNA from a tissue with high RNase activity. Recently, Chomczynski and Sacchi's acidic RNA extraction method /3/ was modified to recover the DNA from the organic phase by adjusting the pH of the organic phase from acidic to alkaline /1/. However, the DNA yield of this procedure is rather low and the DNA recovered from the organic phase might be sheared. In two other published protocols either lithium chloride /7/ or a patended reagent /2/ is used to selectively isolate both RNA and DNA from tissue samples. With lithium chloride /7/, the samples should be incubated overnight at 4 ^{o}C to efficiently precipitate RNA and it is usually very laborious to dissolve the RNA pellet. Chomczynski's method /2/ might be too expensive for a small lab.

Recently, we have established a simple and economical protocol for the simultaneous isolation of both high molecular weight genomic DNA and total cellular RNA from many small tissue samples. We modified a published RNA isolation method /8/ to recover high molecular weight genomic DNA. In our suggested protocol the tissue is homogenized in a guanidium salt and the RNA and DNA are separated by selective precipitation of RNA by ethanol /8/. Then

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the RNA is further purified by acidic phenol-chloroform extraction and the DNA is isolated by conventional methods.

The protocol is briefly the following. Tissue samples are frozen in liquid nitrogen and stored at -70 °C. To isolate both RNA and DNA, samples are measured — they usually weigh 30-500 mg — and while they are still frozen, transferred into a Dounce type all glass homogenizer and homogenized in a guanidine-hydrochloride containing solution (7.5 M guanidine-hydrochloride, 0.1 M Tris-HCl, pH 7.5 and 0.1 M 2-mercaptoethanol) /8/ with pestle B. One ml solution is used for 100 mg tissue. The homogenization is the most critical step in this method. Overhomogenization will give higher RNA yield, but the DNA might be sheared. If the sample is not homogenized thoroughly, the RNA yield will be low. We usually homogenize until the tissue is just dissolved. The homogenate is transferred into a centrifuge tube, sodium-lauroyl-sarcosylate is added to 0.5% and the sample is mixed. Then 0.5 volume of absolute ethanol is added gradually and the sample is carefully mixed again. The mixture is left at -20 °C for one hour to precipitate the RNA. At this step it can be left overnight, too. The suspension is centrifuged at 27 000 g for 15 minutes at 4 °C in Sorwall RC5B centrifuge. Centrifugation with maximal speed in a minifuge might be also sufficient, but higher centrifugation force will yield a more compact RNA pellet. After centrifugation the DNA is present in the supernatant and the RNA is in the pellet. Next, we isolate the RNA, then the DNA. The DNA can be left in the guanidine-hydrochloride containing supernatant for several days at +4 °C.

For the additional purification of the RNA, the first step of the acidic phenol-chloroform extraction protocol was adopted /3/. Briefly, the RNA pellet is homogenized in solution D (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) in an all glass homogenizer with pestle B. Then 0.1 volume of 2 M sodium acetate, pH 4 is added and the mixture is treated with acidic phenol. Following centrifugation, the RNA is precipitated from the aqueous phase with isopropanol, washed in 70% ethanol and dissolved in distilled water as described /3/.

The DNA is isolated from the supernatant of the first centrifugation step. Briefly, sodium chloride is added to the supernatant to 0.5 M final concentration and the sample is extracted with an equal volume of Tris-HCl (pH 7.5) satured phenol and chloroform mixture (1:1). After centrifugation the supernatant is transferred into a fresh tube and the DNA is precipitated with 2 volumes of ethanol. The DNA is dissolved in TE (10 mM Tris-HCl, pH 7.5 and 10 mM EDTA) and purified by conventional RNase A and proteinase K treatments and organic extraction's /10/.

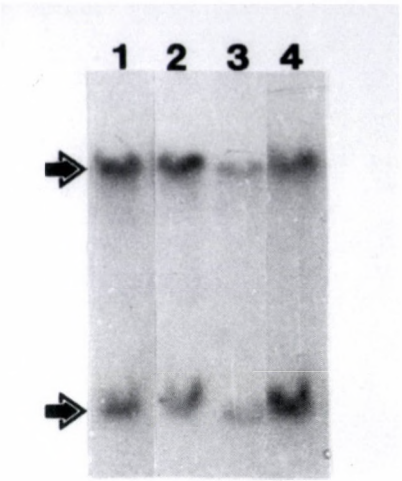


Fig. 1. Southern blot analysis of DNAs isolated from mouse lymphoreticular tumors. Lanes 1-4, 10-10 µg genomic DNA isolated from different tumors were digested with EcoRI and analyzed by electrophoresis on 0.7% agarose gel. Samples were capillary transferred onto a Hybond N+ membrane (Amersham) in 0.4 M sodium hydroxide. After overnight transfer the membrane was briefly rinsed in 2 x SSC and dried on air. Then the membrane was hybridized to a p53 specific probe labeled by primer extension with P^{32}-dCTP. The p53 probe was a 862 bp PstI-PstI fragment of pl76 /12/. The hybridization was performed overnight at 65 °C in a solution containing 5 x SSC, 2 x Denhardt's solution, 20 mM Tris-HCl, pH 7.5; 0.1% SDS and 100 µg/ml denatured salmon sperm DNA. Following hybridization the membrane was washed in 2 x SSC, 0.1% SDS at room temperature for 20 minutes, in 1 x SSC, 0.1% SDS at 65 °C for 20 minutes and in 0.2 x SSC, 0.1% SDS at 65 °C for 20 minutes. The autoradiography was done at -70 °C for 48 h with intensifying screen. The upper arrow points to the 16 kb long EcoRI fragment of the p53 gene and the lower arrow shows the 3.7 kb long EcoRI fragment of the p53 pseudogene

We have isolated both DNA and RNA using this protocol from a large number of different mouse tumors, present in liver, spleen, lymph nodes, lung, uterus, brain and thymus. Starting with 100 mg liver or lymphoreticular tumor the DNA yield is about 200-250 µg and the RNA yield is approximately 100-150 µg. Usually, the OD 260/280 ratios are 1.7-1.8 both for the RNA and DNA. The DNA preparation is free of contaminating RNA and vice versa.

The DNA was routinely used for Southern blot analysis of the ras, myc and p53 genes as well as for PCR amplification of the exons of ras and p53 genes. Figure 1 shows the Southern blot analysis of the p53 gene in mouse lymphoreticular tumors. The 16 kb long EcoRI fragment of the p53 gene can be easily detected. The RNA was used to study the expression of the H-, K- and N-ras and myc oncogenes as well as the expression of the p53 tumor suppressor gene in ionizing radiation induced tumors of mice. Figure 2 demonstrates the analysis of p53 expression in mouse lymphoreticular tumors.

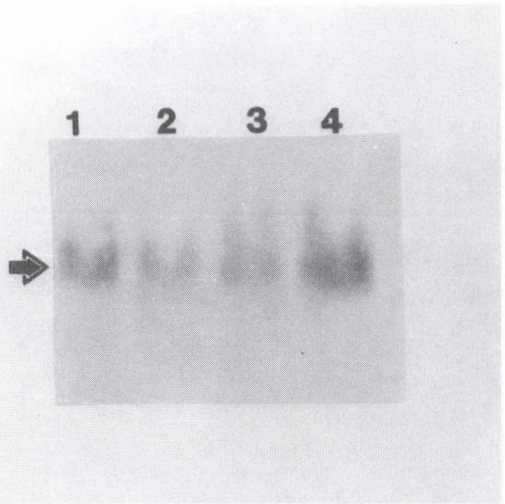


Fig. 2. Northern blot analysis of RNAs isolated from mouse lymphoreticular tumors. Lanes 1-4, 30-30 µg of total cellular RNA, isolated from the same tumors as in Fig. 1 were denatured at 75 °C for 5 min in 20 µl loading buffer (made by mixing 3.5 µl water, 12.5 µl formamide, 4 µl formaldehyde, 2.5 µl 50% glycerol and 5 µl 10 x MOPS buffer). The denatured RNA was analyzed by electrophoresis in 2 x MOPS buffer (40 mM MOPS, pH 7.0; 10 mM sodium acetate, 2 mM EDTA) on 1.2% agarose gel, containing 0.66 M formaldehyde. The electrophoresis buffer contained only 0.22 M formaldehyde. Following electrophoresis the RNA was capillary transferred onto HybondN membrane (Amersham) in 10 x SSC for 4 h. The membrane was briefly rinsed in 2 x SSC and dried for 2 h at 80 °C. Hybridization to p53 specific probe was performed as described in Fig. 1, except that the hybridization solution contained 10% dextrane sulfate. Autoradiography was done for 5 days as described in Fig. 1. The mouse p53 gene yields an about 2000 nucleotide long transcript (arrow)

In conclusion, the protocol is simple and useful for the simultaneous isolation of both DNA and RNA from small quantities of tissues. Many samples can be handled at the same time. The quality of the DNA and RNA satisfies the strict requirements of most molecular biological analyses, such as Northern and Southern blot, as well as polimerase chain reaction.

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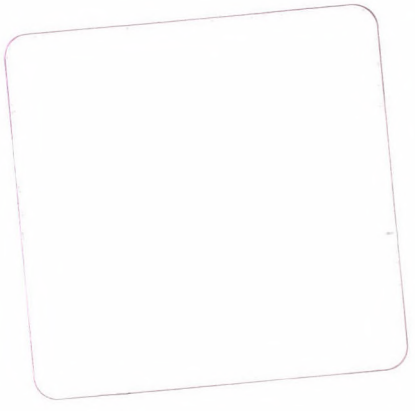
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MAGYAR
TUDOMÁNYOS AKADÉMIA
KÖNYVTÁRA

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EDITORIAL

With this issue Acta Biologica Hungarica intended to celebrate the 70th birthday of Professor Gábor Szabó, Ordinary Member of the Hungarian Academy of Sciences, member of the Editorial Board of this Journal. However, fate decided otherwise. While manuscripts of university colleagues, research associates and scholars were already under preparation to honour the jubilee, Prof. G. Szabó, founder and first head of the independent Department of Biology at the University Medical School, Debrecen, passed away on the 13th December 1996, three weeks before his birthday anniversary, 2nd January 1997. This way, the papers collected in this issue are dedicated to the memory and respect of the man with a wide intellectual horizon, to the researcher microbiologist and geneticist, university teacher, science politician, true friend and colleague.

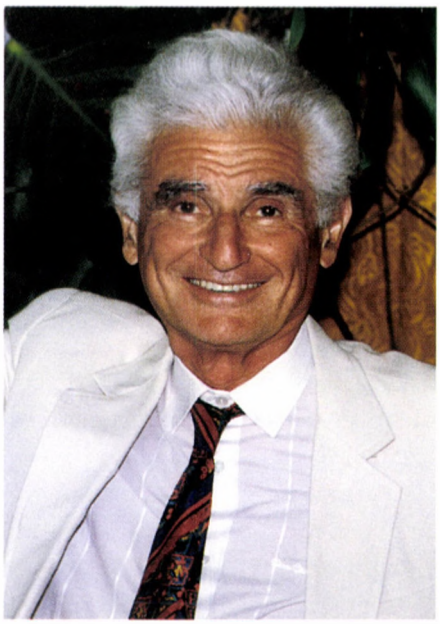
The educational, teaching and research carrier of G. Szabó is reviewed and praised in the following pages by one of his fellows in profession. As the Editor of this Journal and a close friend for nearly half a century I want to add and underline his great interest and enthusiasm about theoretical, methodological questions, his strict morality and exceptional devotion to duties he was entrusted at the University and at the Academy of Sciences. He was sensitive to social problems and paid great attention to the life of the research community. He served more than twenty years in our Editorial Board and contributed in many ways to its successful functioning with his initiatives, good proposals, criticism and by a high scientific demand.

The decease of Professor G. Szabó is a great loss for all of us. His name and achievements will be retained in history of Science and in our memory for ever.

János Salánki
Editor

Akadémiai Kiadó, Budapest

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GÁBOR SZABÓ

(1927—1996)

Even before it had begun, the Winter of early 1927 had relinquished its bitter hold. It was a Golden Bridge for the river, a mellow grape for vineyard and a warm summer night that the charming little town had ever so longed for. Gábor Szabó was born on the 2nd of January in the town of Sárospatak, probably fulfilling the quest for happiness in 1927. His personality and intellect were shaped by a loving family and the famous Reformed Alma Mater. His strong character was not spoiled but rather ennobled by the tragedies and miseries he suffered during the Second World War. He was born into the Jewish religion. His father, a multiply decorated and highly distinguished military officer of the First World War, was the doctor of and a legend for his town and fellow citizens.

His loving mother led the household and was an aid to medicine as a nurse. Gábor Szabó was brought up in the ideal of human education and the puritan morality of the Calvinist Collegium of Sárospatak. The spirit of Comenius educated him, and his youthful interest was guided toward literature by the inspiration of his educated teachers of Hungarian literature,

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Géza Képes and Sándor Maller. At this stage in his life, a career in the medical and natural sciences was only second on hist list.

The War, the ghetto and the labour service destroyed his parents and his elder brother. He survived. At the age of 18, after 13 months in a concentration camp and subsequently contracting tuberculosis, he set off for Debrecen in the footsteps of Petőfi (on foot) across the Plain. There, he registered as a student in the Medical School and in his third year he became a demonstrator of the Pharmacology Institute. Professors Kálmán Sántha, Tibor Vályi-Nagy and Endre Jenei were his mentors in science as well as in human dignity. His career started with research into antibiotics, but was soon redirected to the railways of genetics. Here, in the regnum of genetics, unfolded the scientist and later the teacher, educator, science-politician and scientist-publicist.

In the 50ties the Pharmacology Institute became an internationally renowned school of microbiological biochemistry, with strong ties to the University of Cambridge, England. First he studied the nature of the formation of mutations in the streptomycin producing microbe, Streptomyces griseus. This bacterium was the focal point of his research which was strongly supported by the national pharmacology industry at that time. His knowledge of antibiotics enabled him to visit and work in many eminent laboratories, such as those of Professors E. F. Gale, S. J. Alichanian and C. F. Gauze in Cambridge and Moscow. In 1959, a general biology research-education group was formed in the Pharmacology Institute, around Gábor Szabó, and the teaching of genetics also began. This step had an extraordinary significance as, let us not forget, the dark era of Lisenko had not yet ended! As a result of this research-education group, 1963 saw the formation of an independent Institute of Biology in the Medical University of Debrecen, of which Gábor Szabó had been the director for 30 years since its establishment.

Factor C

In 1960, an important discovery was published by Gábor Szabó and his colleagues in Nature, the holy journal of natural sciences. After systematic "hunt" for mutants he observed two types of cology morphology in the population of Streptomyces griseus, one of which produced much of the antibiotic streptomycin, while the other produced none. The latter type was able to further differentiate producing air mycelia. Long years of careful research showed that a regulatory protein, Factor C, was responsible for the differentiation pathway.

Pure Factor C was produced, and N-terminal amino acid sequence determined. Later, homologues of Factor C were found in a wide variety of species of the natural world, and since then, the protein has always been connected to regulation of developmental pathways. The isolation and cloning of the gene coding for Factor C proved to be a difficult task. However, the "first part" of the gene has been cloned and sequenced successfully. Today there is every hope that the "missing part" will give itself up to one of the newly developed, sophisticated PCR techniques. Once in the possession of the Factor C gene, we will have the opportunity to understand the mode of action of Factor C at the molecular and atomic level.

DUSF

In 1971, Gábor Szabó had the privilege to work with the Nobel laureate E. L. Tatum at the Rockefeller University, USA for one year. E. L. Tatum is one of the parents of molecular biology in the spirit of the aphorism: "one gene one enzyme". There, another significant result was born: they were the first (N. C. Mishra, G. Szabó and E. L. Tatum) to solve the genetic transformation of a eukaryotic organism, Neurospora crassa, the bread mould. In his Debrecen laboratory with many years of systematic and precise work, the substance stimulating transformability and the uptake of DNA: DNA Uptake Stimulatory Factor, DUSF for short, was identified. Similarly to Factor C, DUSF has been found in all sorts of species in the living world and its function has always been found to be the same, namely the introduction of DNA from the surroundings into the cell. The cloning of the gene coding for DUSF is currently in progress.

Gábor Szabó was a real scientist Professor, teacher, educator and an enthralling lecturer. Lack of preparation was unimaginable with him. Over 38 years, students have had the privilege to get a deep insight into genetics through his courses. They studied strict scientific logic, received model examples of classical education, culture, humanism, modesty and intellectual thinking. He is the sole or co-author of approximately 200 scientific publications, book chapters and public writings. Alongside molecular genetics, he was also involved in questions concerning human genetics. He worked a great deal on the harmonization of higher education, basic and applied research, modernization of university education and the postgraduate education of secondary school teachers in molecular biology and genetics. As a publicist, in noteworthy writings, he expressed his views on the relationship of reli-

gion and science, their harmonic cast and roles, the dialectics of scientific thinking and the relationship of science and society.

In 1963, he was appointed as a Professor at the Medical University of Debrecen. In 1973 he was elected to be corresponding member of the Hungarian Academy of Sciences, becoming a full member in 1982. He was the rector of the Medical University of Debrecen (1973--1979). He was the president of the Genetics Committee of the Hungarian Academy of Sciences (1980--1990), president of Debrecen Academy Branch (1982—1993), president of the Hungarian Human Genetics Society (1986) and advisor of the WHO (1987--1992). As follower of the prominent Hungarian geneticist Prof. Barna Győrffy, he was the founding member of the Society of Hungarian Genetical Society (1987). He was also an executive committee member of Microbiological, Biochemical and Biological Societies home and abroad.

In the August of 1996 he requested solitude from his colleagues, friends and disciples. He prepared for a long journey; on the 13th of December 1996 his ship set sail into the distance. He was our unanimously elected captain on the Ship of Genetics.

His elegant appearance -- because he always smart -- and his brilliant spirit will remain with(in) us forever.

The love towards him as the teacher of our bracket will always hold us together as an organizing power.

László Orosz

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AMINO ACID SEQUENCE HOMOLOGY OF FACTOR C PRODUCED BY STREPTOMYCES GRISEUS WITH REGULATORY PROTEINS OF ZINC FINGER TYPE*

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(Received: 1997-02-10)

Factor C is a regulatory protein produced by Streptomyces griseus 45H. Factor C-like antigen can be detected in the most diverse species examined. It is also present in human serum with an average of 219.4 U/ml of narrow dispersion. The level of factor C-like antigen shows an increase in the sera of patients with different hepatic disorders (341 U/ml), some values being 3-7 times higher than normal (600-1500 U/ml). Correlation was found between the elevated antigen levels and the amount of the bilirubin in the sera of patients with liver cirrhosis.

Amino acid sequence homology was found between factor C and zinc finger motifs of several known DNA binding proteins. In fact, factor C was successfully bound to and eluted from a Zn^{2+} affinity column. Our data show that factor C is probably a zinc finger type DNA binding protein.

Keywords: Factor C — Streptomyces griseus — regulatory protein — hepatic disorders — zinc finger motifs

Introduction

Streptomyces have complex life cycle and routes of morphological and metabolical differentiation. A great deal of observation reveals that this differentiation is a highly ordered and genetically determined process. Considering that most antibiotics are the products of streptomycetes and related organisms, the importance of the studies concerning their regulatory processes cannot be overestimated /4/. In this context, a promising field of research is the investigation of those endogenously produced substances (autoregulators) that were shown to influence the differentiation of Streptomyces (A-factor, B-factor, Pamamycin 607, and factor C), reviewed by Szabó et al. /6/.

*Dedicated to the memory of Professor Gábor Szabó.

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Factor C was detected in and later isolated from the cultivation medium of the "producer" S. griseus 45H strain /2, 7/ as a regulatory protein. It induces the formation of preconidia characteristic for the reproductive phase of the life cycle /13/ in submerged cultures of the "test" strain S. griseus 52-1 at concentrations as low as 0.5-1 U/ml (1U is about 1 ng of protein).

The effect of factor C was also detected by following the change of composition and synthesis of proteins in homogenates of mycelia grown after its addition /14/. Several other characteristics of the effect of factor C are reviewed in /9/.

Several experiments were aimed at clarifying the mode of action of factor C. Crude preparations of factor C elevated the T_m point of DNA, inhibited the overall in vitro RNA synthesis and the initiation of RNA transcription /5, 8/.

Purified factor C caused a release of intracellular K^+ when added to suspensions of washed Streptomyces mycelia of nine different strains — in concentration as low as 0.7 U/ml. The K^+ release followed a characteristic "slow kinetics" with about 3-10 min latency. From among many compounds tested only the effect of nicotinamide reminded for that of the factor C. It was assumed that NAD-glycohydrolase and possibly ADP-ribosylation of some transport proteins may be involved in the effects of factor C observed /9/.

Factor C was purified to electrophoretic homogeneity first by phosphocellulose and DNA-agarose chromatography and was found to have a molecular mass of 34.5 kDa /2/. Its pI was determined to be 9.75 by liquid phase isoelectric focusing /10/. Studies on the factor C got impetus from the introduction of new experimental methods such as elaborating ELISA and immunoblotting techniques with specific anti-factor C monoclonal antibodies /12/.

Materials and Methods

Streptomyces strains were generally maintained and cultivated as described earlier /11/.

Immunological methods. ELISA and immunoblotting techniques were applied as described by Szeszák et al. /12/.

29 N-terminal amino acid of factor C was sequenced according to the Edman's degradation method.

Zn^{2+} affinity chromatography was carried out as follows: Sepharose 6B Fast Flow-Iminodiacetic acid (SIGMA I-4510) was packed into 1.2 x 3 cm columns, washed thoroughly with distilled water, saturated with 25 mM $ZnCl_2$ and equilibrated with phosphate buffer containing 10 mM KPO_4, pH 7.1 and 5% w/v

glycerol. Crude preparations of factor C (second phosphocellulose step /2/) were dialysed against, put on the column at 10 ml/h and washed to baseline with the phosphate buffer. The bound protein was eluted with a 0-200 mM imidazole gradient dissolved in the phosphate buffer.

Results

Factor C is a protein conserved in evolution

At first, 23 Streptomyces strains belonging to 9 species were screened by ELISA and there was none found which, at least in some period of its cultivation, did not produce factor C-like antigen /9/. Moreover, as Table 1 shows factor C antigen was also detected with immunoblotting in a very broad range of different living organisms from archaebacteria to B. subtilis, Neurospora crassa and human tumour cell line. In all the specimens examined, 1 or 2 immunoreactive bands were found only either having the same molecular mass as determined in S. griseus /2/ or its double (Mr = 34.5 and/or 70).

Table 1

Molecular mass of factor C-like antigens in biological specimens of different origin

Strain	Cell homogenate	Culturing medium
	Molecular mass, kDa	
S. griseus 52-1	34	34
S. griseus 45H	34	34
S. flavofungini DV 147	34 and 70	34
S. coelicolor M-145	70	n.d.
S. antibioticus 77451	34	34
B. subtilis ATCC 6633	34 and 70	34
E. coli 3300	34	34
Halobacterium halobium NRL	34	34 and 70
Neurospora crassa RL-38 (wild)	34	34
Neurospora crassa FGSC-1118 (slime)	34	n.d.
Mouse erythroleukemia DS-19	34	34
Human leukemia HL-60	n.d.	34

Streptomyces strains were grown in liquid medium for 48-72 h, harvested, then samples were taken for SDS polyacrylamide gel electrophoresis and immunoblotting.

+ signs and Arg designations stand for the conservative replacement of amino acids along the polypeptide chains.

n.d. = not determined

Occurrence of factor C-like antigen in human sera

The ubiquitous presence of factor-like antigen in different species prompted us to examine its levels in human sera. From the blood samples of 282, randomly selected clinical cases a value of 218.19 U/ml ± 94.015 SD was obtained /10/. This value was substantiated by determining the level of antigen in 104 healthy individuals (Fig. 1, controlled blood donors). An average of 219.4 U/ml with narrow dispersion was obtained (the 2 highest values were around 500 U/ml). Then we started to determine factor C-like antigen levels in the sera of patients with different hepatic disorders. Such results obtained from the sera of patients with decompensated liver cirrhosis are shown in Fig. 2. Both the level and the dispersion of the values show a marked difference here. The mean of the samples is higher (341 U/ml) and its distribution is different, some values scattering upward (600-1500 U/ml) /3/.

In another sample group collected, correlation was found between the factor C-like antigen level and the quantity of different bilirubin frac-

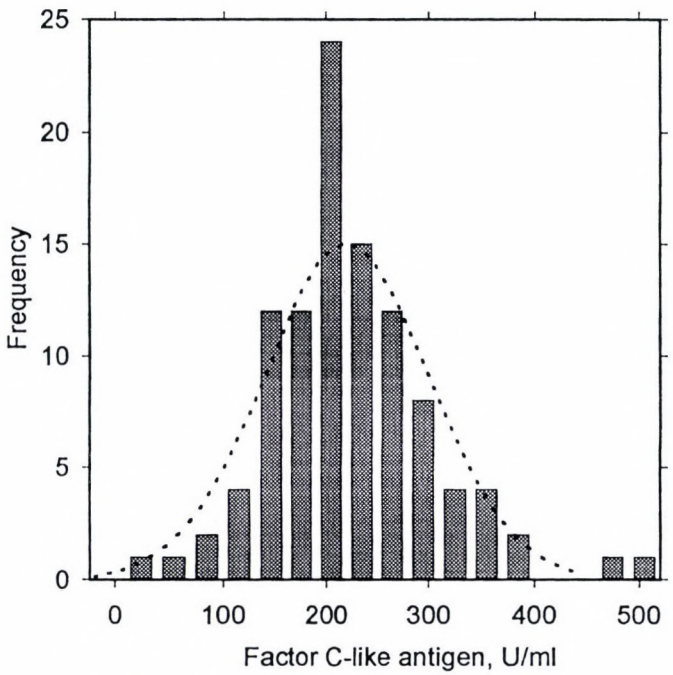


Fig. 1. Distribtuion of the factor C-like antigen levels in the sera of 104 healthy individuals. Columns: frequency of antigen levels; ..... Gaussian curve calculated

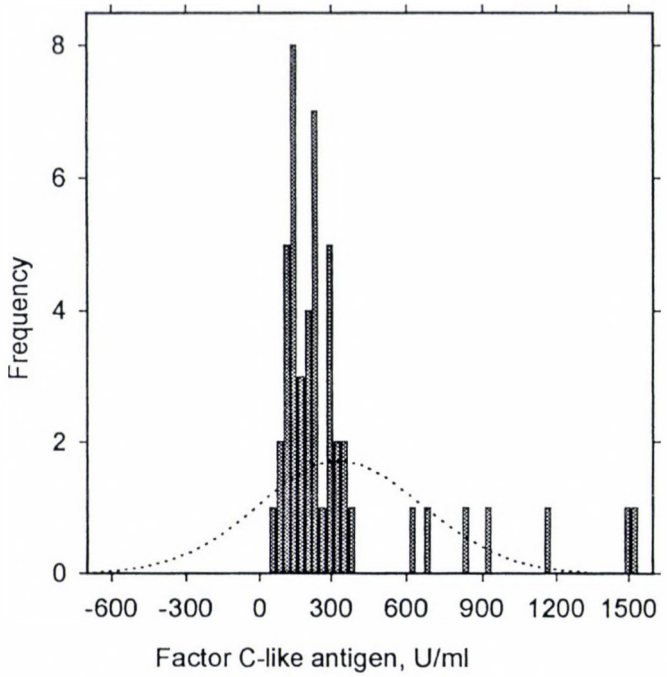


Fig. 2. Factor C-like antigen levels in the sera of patients with decompensated liver cirrhosis. Columns: frequency of antigen levels; ..... Gaussian curve calculated

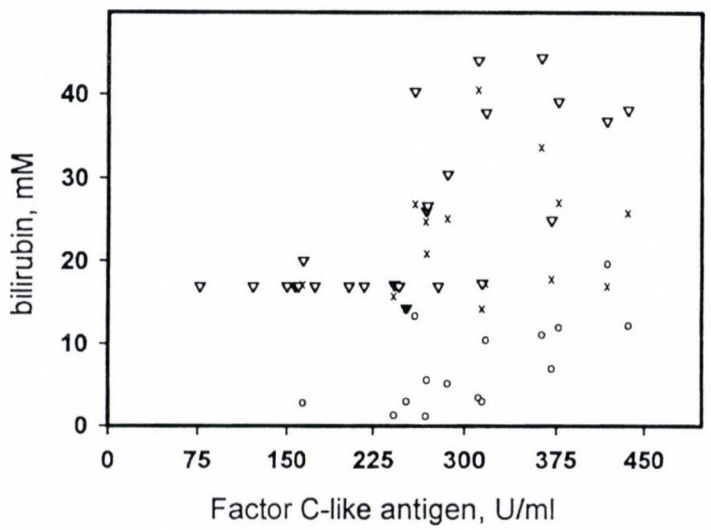


Fig. 3. Correlation between the factor C levels and the ones of different bilirubin fractions in the sera of patients with liver cirrhosis. ∇: total bilirubin; o: direct bilirubin; x: indirect bilirubin

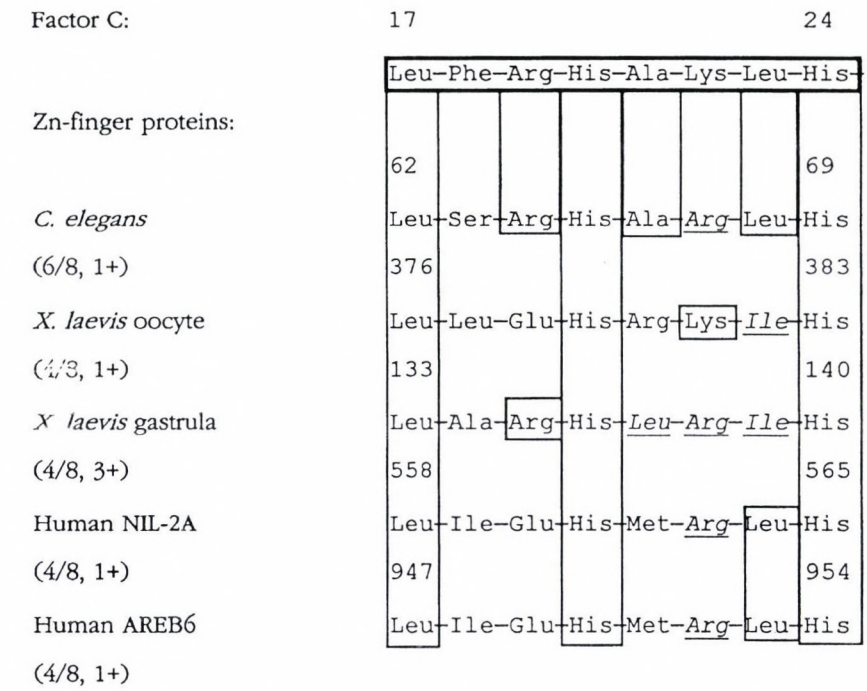


Fig. 4. Amino acid sequence homology of the 17-24 peptide stretch of factor C with different known Zn-finger proteins

tions in the sera of cirrhotic patients (Fig. 3). The correlation was particularly high between the antigen and the total bilirubin levels (r = 0.7093), the antigen and the direct bilirubin levels (r = 0.691). It was less pronounced between the antigen and the indirect bilirubin levels (r = 0.2325). By collecting larger sample sizes, we expect to find correlation between the elevated factor C-like antigen levels and some defined diagnostic group(s) of hepatic disorders.

Is factor C a Zinc-finger protein?

Searching for the gene of Factor C in S. griseus, the N-terminal 29 amino acids of the protein were sequenced. Amino acid sequence homology searches were performed using the BLAST network service and non-redundant protein sequence databases (Fig. 4) /1/. A marked homology was found between the amino acid sequences of several known Zn-finger proteins and the 17-24

peptide stretch of factor C. Taking also into consideration our earlier observations (increase of the cytomorphological effect by Zn^{2+} at concentrations of 10^{-5}-10^{-6} M, increase of T_m point, binding to DNA, and alteration of RNA polymerase activity in vitro) we have tried to bind factor C to Zn^{2+} affinity column successfully (Fig. 5). Factor C antigen was eluted as a narrow peak, well-separated from the non-bound proteins.

Discussion

Looking at the species examined, factor C would be among the most conserved proteins during evolution described until now. The presence of the same epitope in proteins with the same molecular mass makes it probable that all the species share a kind of protein closely related to factor C — an autoregulator of S. griseus. It probably is an inherent constituent of not only Streptomyces, but all kind of the cells and higher organisms.

On the basis of the data of Table 1 it was not unexpected that it is present in human serum. We do not know much of its role in the human body,

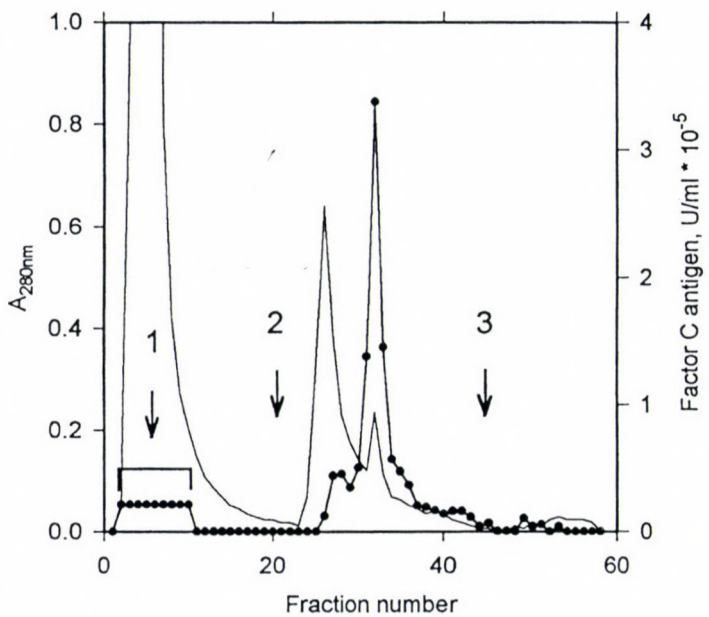


Fig. 5. Zn^{2+} affinity chromatography of factor C on a Sepharose 6B Fast Flow-Iminodiacetic acid column saturated with Zn^{2+} as described in Methods. ——: A_{280}; •: factor C antigen, $U/ml \cdot 10^{-5}$; 1: ELISA values measured from pooled fractions; 2: start of the imidazole gradient; 3: end of the imidazole gradient (replaced by 200 mM imidazole in phosphate buffer)

however, its definite increase in some hepatic diseases may lead to learn more on its function and can serve as a useful diagnostic tool.

Amino acid sequence homology of factor C with Zn-finger proteins supports our earlier assumptions /5/ that its primary site of action can be on the DNA itself. Our findings concerning the factor C induced potassium release and decrease of NAD-glycohydrolase activity /9/ do not rule out this possibility, because both effects have a latency period (3-7 min and 15-30 min, respectively), a time well enough in a prokaryotic cell for the synthesis of some protein(s) de novo. The data presented here support the assumption that factor C is a DNA binding protein of Zn-finger type. Experiments are under way to elucidate these findings.

Acknowledgement

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The technical assistance of Mrs. Andrea Kiss is kindly acknowledged.

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Acta Biologica Hungarica 48 (3), pp. 275–279 (1997)

A SHORT GC-RICH SEQUENCE INVOLVED IN DELETION FORMATION OF CLONED DNA IN *E. COLI*[*]

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(Received: 1997-02-10)

A spontaneous deletion probably caused by rec A independent recombination between short-direct repeat sequences was observed in pUC19 plasmid carrying a piece of *Streptomyces* genomic DNA after culturing in liquid medium. The deletion removed an unknown portion of the cloned DNA and the BamHI-EcoRI part of the multiple cloning site with an additional flanking 111 bp from the vector. At the junction a 13 bp GC-rich DNA sequence highly homologous to a known spontaneous deletion hotspot was found.

Keywords: Spontaneous deletion — recombination — direct repeat — GC-rich DNA — recA independent

Introduction

Spontaneous deletion of pieces of DNA through recA independent recombination between short directly repeated sequences in *E. coli* has been published by several authors /1, 2, 6-9/. The characteristics of this type of recombination and a replicational model explaining its mechanism has been discussed by Bi and Liu /2/. We are involved in the cloning of factor C, a protein which participates in the regulation of cytodifferentiation of *Streptomyces* strains /4/. In the course of that work we constructed a pUC19-based gene bank of *Streptomyces griseus* chromosomal DNA in *E. coli* JM109. Here we report a spontaneous deletion observed in one of the clones. In this deletion formation a short GC-rich sequence homologous to a known spontaneous deletion hotspot /1/ is inferred.

[*]Dedicated to the memory of Professor Gábor Szabó.

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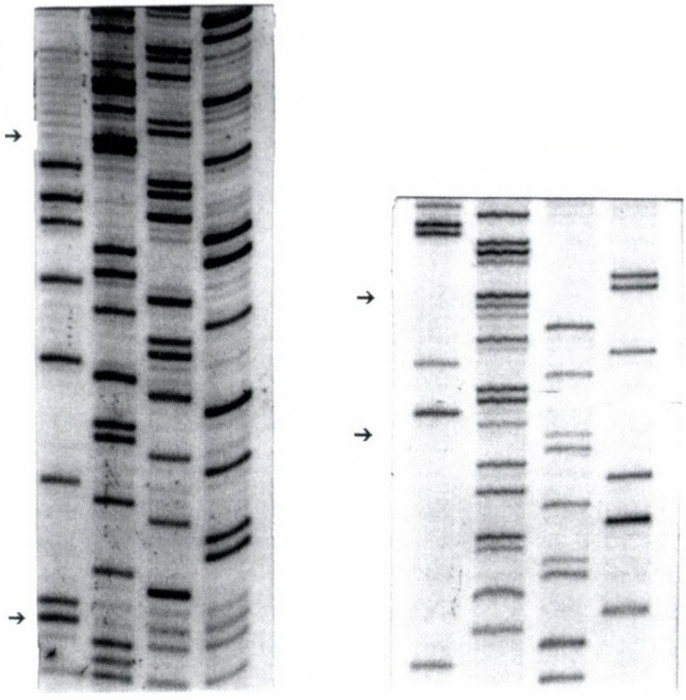
Materials and Methods

Bacterial strain and plasmid: E. coli JM109 (endA1, recA1, gyrA96, thi, hsdR17, relA1, supE44, Δ/lac-proAB/, /F' traD36, proAB, lacI^{q}ZΔM15/) and pUC19 were used throughout this work.

Recombinant DNA methods: Media and growth condtions, cloning, agarose and polyacrylamide gel electrophoresis, transformation, and selection of transformants were all performed according to standard procedures /10/. Plasmid DNA was extracted from the cells by the alkaline lysis procedure of Birnboim and Doly /3/ and further purified by the Promega Wizard DNA purification system. Sequencing was done according to Sanger /11/ using 7-deaza-dGTP in some of the reactions.

Results

We constructed a pUC19-based gene bank of Streptomyces griseus chromosomal DNA in E. coli. It consists of about 4500 clones with an average insert size of 5 kb as judged by restriction analysis of randomly chosen clones. It represents the genome of a Streptomyces strain with 95% probability. The first screening of our gene bank with a digoxigenin-labeled oligonucleotide (which is complementary to the 5' end of the gene coding for factor C) accomplished by transferring cells from the wells of the microtiter plates to Hybond N membrane yielded several hybridizing clones. After repeated cultivation in liquid medium we isolated plasmid DNA from these clones and analyzed them by restriction enzyme digestion. Since BamHI digested chromosomal DNA was cloned into the BamHI site of the plasmid we expected to recover the inserts with this enzyme. To our surprise, especially since we did not encounter difficulties in cutting out the inserts during our preliminary analysis of the gene bank, the plasmids were merely linearized by the BamHI. We further analyzed and sequenced one of the plasmids (designated 294a). This clone carries exactly 1000 bp Streptomyces DNA. The BamHI site and the polylinker region is intact at the HindIII side of the vector as shown in Fig. 1A. The other end of the insert, however, has a deletion. The BamHI-EcoRI region of the polylinker and an additional 111 bp are deleted together with an unknown portion of the cloned Streptomyces DNA. Figure 1B shows the sequence of the plasmid around the deletion. At the junction between the vector and the cloned Streptomyces DNA there is a 13 bp vector sequence (GCACCGATCGCCC) having surprisingly high GC content similar to the Streptomyces DNA which has about 73% G+C content. Since the complementary strand of this 13 bp sequence (GGGCGATCGGTGC) is very similar to a



A B

Fig. 1. Computer scanning of the sequencing gels showing the junctions between the vector and the cloned DNA. The order of the lanes is A, C, G, T.
(1A) The HindIII-BamHI intact portion of the multiple cloning site (between the two arrows in the figure and underlined below) and a short sequence of the cloned Streptomyces DNA. The sequence shown is: GCCCGAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCGGCTCCGCGGTCTC
(1B) The BamHI-EcoRI portion of the multiple cloning site and an additional 111 bp from the vector is deleted. The Streptomyces sequence is followed by the 13 bp vector sequence (between the two arrows in the figure and underlined below) which is probably responsible for the deletion formation. The sequence shown is GAGCTCGGCCTGCTCGGCACCGATCGCCCTTCCCAACA

deletion hotspot which causes large (700-1000 bp) deletions in the E. coli lacZ fusion system /1/, we believe that this sequence is responsible for the deletion found in our clone.

Discussion

Deletion of segments of cloned DNA in different host-vector systems is probably more frequent than one can estimate from the number of reports and systematic studies of this phenomenon. Ishiura et al. /6/ analyzed more than 1000 cosmid clones and showed that after prolonged propagation of the clones in liquid culture most clones suffered deletion irrespective of whether the cosmid was propagated in a recA or rec^+ host. This suggests that deletion is mediated by a recA independent recombination system. The most probable mechanism of this recombination is misalignment of direct repeat sequences during replication /2/.

Here we report a deletion probably caused by a short (13 bp) GC-rich (77% G+C) direct repeat sequence (GGGCGATCGGTGC). This sequence is very similar to the two conserved regions (underlined) of a short (17 bp) sequence (GGGCAAACAGTCGTTGC) which was reported to cause large deletions in the E. coli lacZ system /1/ and therefore considered to be a hotspot for spontaneous deletions. The sequence is expected to occur more frequently in GC-rich DNA such as the genome of Actinomycetes. This assumption is supported by the analysis of the sequences of the prokaryotic and bacteriophage division of the EMBL Nucleotide Sequence Data Library (release 16.0 October, 1995). The exact 13 bp sequence without mismatch was found only in one single gene besides pUC19 and its related plasmids. This was the trpC gene of the tryptophan pathway of Pseudomonas aeruginosa /5/. Interestingly this gene was cloned and sequenced in pUC19. If our assumption about the role of this 13 bp sequence is correct, the authors had probably also observed deletion at this position and had been able to determine the sequence in a subclone where the vector and insert 13 bp sequences were in opposite orientation or widely spaced. Although not mentioned in their paper, this is indicated by the sequencing strategy of the relevant portion of that operon (around nt. 2300 shown in Fig. 1). The 13 bp sequence with one mismatch was found in 41 other entries. These are mainly sequences of microorganisms with high GC DNA (Mycobacterium, Pseudomonas, Streptomyces, Xanthomonas, etc.) or the aminoglycoside nucleotydil transferase gene in low GC species. The latter species probably acquired the aminoglycoside resistance gene from Actinomycetales. Therefore the 13 bp sequence described in this paper may cause problems when DNA from species having GC-rich genome is cloned in pUC19 or in related plasmids.

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A POSSIBLE ROLE OF THE EFFECT OF METHIONINE ON THE ACTIVITY OF ASPARTOKINASE IN SPORULATION OF A STREPTOMYCES FRADIAE MUTANT*

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In addition to methionine, aspartate may also induce sporulation in the Streptomyces fradiae St3110 mutant that requires methionine for sporulation but not for growth. Partially purified aspartokinase (EC 2.7.2.4.) of the mutant was tested for feed-back control by methionine, threonine, and lysine. Methionine or threonine alone did not have any significant effect but they had a concerted inhibitory effect together that was further increased by lysine. The threonine-lysine combination also caused inhibition of the enzyme activity, while lysine alone activated the enzyme. Methionine was also found to partially repress the aspartokinase activity to about one third of the control. The sporulating aerial mycelia induced by aspartate were lacking the characteristic sporulation pigmentation present in the wild type or after induction of spores by methionine. No significant difference in heat resistance between the two types of the spores was detected. The number of spores produced by aspartate was only about one fourth of that after induction by methionine. The data may indicate a role of aspartokinase control by methionine in restoring the normal sporulation, although in addition to this methionine may act by a different mechanism, as well.

Keywords: Streptomyces — sporulation — methionine — aspartate — aspartokinase

Introduction

The multicellular, mycelial Streptomyces have complex developmental processes including aerial mycelia formation and sporulation. The regulation of these processes are interesting not only from a theoretical but also a practical point of view, since these organisms produce significant secondary metabolites of commercial interest. Developmental mutants have successfully been used to explore several aspects of the regulatory mechanisms /2, 3/. The quality of the N-source and some amino acids have been known to have an effect on the sporulation of some Streptomyces species. Glycine inhibits the

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formation of aerial mycelia and spores, while aspartate, glutamate, and histidine allow the growth of aerial hyphae but suppress sporulation in S. viridochromogenes /4/. In S. clavuligerus, NH_4Cl alone does not support sporulation unless combined with certain amino acids /1/. A related species requires methionine for its sporulation /14/. The precise explanations for these effects are still missing. Still little has been done in bringing the genetic approach and metabolic studies together.

One of the developmental mutants is the S. fradiae St3110 strain that has a rather specific methionine requirement for sporulation but not for growth. Revertants to normal differentiation can be selected by norleucin, a methionine analogue. This case, because of the definable mutation and metabolic block with the simultaneous developmental defect, provides a promising system for exploration of links between metabolism and regulation of development. Methionine is required only during sporulation, i.e. after 48 h of culture age. At that age Co^{++} and vitamin B_{12} were also effective in inducing sporulation /13/. In the biosynthesis of methionine, the first enzyme of the pathway, aspartokinase, is regulated by different compounds. The enzyme activity can be increased by lysine, not affected significantly by threonine alone, while lysine in combination with threonine is strongly inhibitory /6, 8, 9/.

Materials and Methods

Bacterial strain used

Streptomyces fradiae St3110 mutant ($ArgF^-$, Str^r), described earlier /12/. Storage of spores was maintained in 25% (v/v) glycerol at -20 °C.

Culture media

For surface cultures, sucrose-nitrate agar (SNA) medium was used /11/ containing ($g \times l^{-1}$): sucrose, 30; $NaNO_3$, 2; K_2HPO_4, 1; $MgSO_4 \times 7\ H_2O$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; Agar, 15; (pH 7.2). For liquid cultures, agar was omitted resulting the sucrose-nitrate (SN) medium.

Culture conditions

The liquid media (20 ml in 100 ml flasks) were inoculated by 10^7 spores and shaken at 250 min^{-1} frequency at 34 °C in an incubator (New Brunswick Scientific). For inoculation, spores were scraped off the surface of one-week-old cultures on SNA media, suspended in 0.9% NaCl, and vortexed for 10 min. The density of the spore suspensions were verified by determining

the number of colony forming units. In critical cases, when it was important to have individual spores without contaminating hyphae, 0.1% Tween 80 was added /10/, hyphae were removed by filtering through sterile cotton wool, and the still remaining mycelial fragments were eliminated by a 3 x 20 s sonic treatment that did not change the viability of spores (they were resistant up to 5 min of sonic treatment) but killed 98-99% of the vegetative hyphae. The surface cultures were incubated at 30 °C.

Sonic treatment

Twenty kHz waves produced by a Braunsonic 300S instrument at the highest power were used. The treatment was performed in 20 s periods keeping regular 20 s breaks between them, under continuous cooling.

Preparation of cell homogenates

After growth in liquid SN media mycelia were sedimented by centrifugation, washed twice with 1/4 volume of 0.9% NaCl solution, then suspended in the appropriate buffer for enzyme activity measurements. Cells were homogenized by 2 min of sonic treatment, the debris was pelleted by a 2500 g centrifugation, and the supernatant was used for further determinations.

Determination of aspartokinase activity

Aspartokinase (AK, EC 2.7.2.4.) was partially purified from the crude homogenates according to Mendelowitz and Aharonowitz /8/. The enzyme activity was then measured by the method of Hänel et al. /6/.

Determination of the protein content

Proteins were precipitated by cold 5% TCA for 1 h, sedimented by centrifugation, and extracted using 1 N NaOH at room temperature for 24 h. After a 10-fold dilution the protein content was determined by the method of Lowry et al. /7/. Always at least two determinations were averaged. Bovine serum albumin (Serva) was used as standard.

Determination of the heat resistance of the spores

Spore suspensions were sonicated for 3 min effective time (see above), washed and suspended in 0.9% NaCl solution. Heat treatment was performed in two kinds of experiments: aliquots of equal volume and density of spore suspensions were (a) treated at various temperatures for 10 min, or (b) kept at 60 °C for various length of time. Then the viability of the spores was determined by plating and counting the number of colony forming units.

Determination of the number of spores per unit of area produced in surface cultures

To agar plate cultures that were standardized for the volume and thickness of media, number of inoculated spores, etc., 200 µl 200 mM aspartate or 200 µl 20 mM methionine solution was added in a centrally located

hole of 1 cm of diameter at 48 h of cultivation. The concentrations given were determined as optimal in preliminary experiments. After sporulation completed, from the sporulating zone around the hole 5-5 agar discs of 1 cm of diameter were cut out. The spore layer was thoroughly collected from each disc, and suspended in 0.1% Tween 80, 0.9% NaCl solution. In order not to loose spores, the suspensions were not filtered. The hyphae were removed by sonic treatment for 3 x 20 s. Then the number of colony forming units was determined.

Microscopy

The simple microscopic methods used were described in detail earlier /11, 13/.

Results

The finding that methionine was able to restore sporulation in the S. fradiae 3110 mutant, when added at the age of 48 h, led us to retest intermediates of the methionine pathway at this age of the cultures. In addition to the already published Co^{++} and B_{12} /13/, aspartate provided at 48 h to the cultures in a gradient test /11/ was also found to support sporulation. Aspartate is the amino acid from which synthesis of methionine starts. Threonine and lysine, whose biosynthesis also starts from aspartate, did not induce sporulation. This raised the possibility that the developmental defect in this mutant was caused by a relative aspartate deficiency. This could be resulted from an inefficient methionine biosynthesis leading to a missing feed back control and derepression of the methionine biosynthetic pathway, and, consequently, using up much of the aspartate synthesized. By adding exogenous methionine the feed back inhibition of the pathway by methionine could save aspartate for the cell.

The expected point for the feed back control of this pathway was the first enzyme, aspartokinase. Therefore, we partially purified the enzyme, and tested the effects of relevant pathway end products on its activity (Table 1). Methionine and threonine alone were without any significant effect, while lysine had a ca. 30% activation. Methionine was inhibitory only in combination with threonine or threonine and lysine. Without threonine, methionine did not change the activatory effect of lysine.

Another possibility for regulation of AK activity in the cells was repression of its synthesis by methionine. So, we grew the mutant cultures in the presence or in the absence of 1 mM exogenous methionine, made the enzyme extracts and determined the specific enzyme activity (i.e. activity

Table 1

Effect of pathway end products on the activity of partially purified AK from S. fradiae St3110 mycelia

Compound[a]	AK activity (%)
None	100
Met	105
Lys	131
Thr	106
Met + Lys	127
Met + Thr	52
Lys + Thr	18
Met + Lys + Thr	16

Average of two independent experiments with a difference of ca. 10%. For the enzyme source see Materials and Methods.

[a]Each compound was present at a concentration of 5 mM.

per mg protein) in each. The specific activity of AK of the cultures supplied with methionine was significantly lower. The specific activity in the enzyme preparation from cells grown without methionine supplementation was regarded as 100%. When the activity was related to the protein content of the enzyme preparation, the specific activity of AK from cultures supplemented with methionine was only about 46%. When the AK activity was related to the total cellular protein of the cultures, the difference was even bigger: only 35% specific activity remained in the presence of methionine compared to the cultures not supplemented with this amino acid (data not shown). These results indicate a partial repression of the AK synthesis by methionine.

If methionine acted simply through aspartate on restoring normal development, then the spores produced by the two amino acids should be indistinguishable from each other. To test this point we compared some of the characteristics of the spores produced by the presence of exogenous aspartate and methionine, respectively.

It was clear even by simple inspection of the surface cultures that the spore layer formed on the addition of methionine had a pink pigmentation characteristic of the wild type strain, while sporulating mycelia produced

Table 2

Comparison of the number of spores induced in *S. fradiae* St3110 by methionine or aspartate at 48 h age of the cultures

Inducing compound	No. of spores/cm^2 (x 10^6)	%
Methionine	140 + 38	100
Aspartate	37 + 13	26

[a]See the details of the experiment at Materials and Methods.

by aspartate induction had a white or slightly yellowish color, although the shape and refractive properties of the spores under phase contrast microscope looked the same. This difference in pigmentation could have reflected an incomplete maturation of the spores in the presence of aspartate. In order to test the maturity of the spores, we determined the heat resistance of the spores produced in the two different ways. Both kinds of spores were resistant to heat treatment for 10 min up to 60 ^{o}C, then their resistance rapidly decreased with further elevation of the temperature between 60 to 65 ^{o}C, with slight, not significantly higher resistance of the methionine-induced spores. Similarly, no significant difference in the time courses of heat inactivation at 60 ^{o}C of spores induced by methionine or aspartate, respectively, was observed in another type of experiment. Investigation of resistance against acetone, chloroform, and NaOH also gave similar results (data not shown).

Because attempts to establish a significant difference in maturation of spores induced by methionine or aspartate failed, we decided to get some data on the extent of sporulation in the aerial mycelia, i.e. to count the number of spores produced on a defined area of the surface culture. The number of spores collected from standard agar discs of the cultures (see Materials and Methods) revealed that only 26% spores per cm^2 were induced by aspartate in comparison to the number found after methionine induction -- representing 100% (Table 2). It is important to note that cells needed ten times higher concentration of aspartate than methionine for this result. While 20 mM methionine was enough to produce abundant sporulation, the very rare spores induced by aspartate applied at the same concentration could hardly be found under phase contrast microscope.

Discussion

The fact that in addition to methionine and the cofactors of its biosynthesis, Co^{++} and B_{12}, aspartate can also induce sporulation when added at 48 h of culture age may suggest that instead of methionine, aspartate is the really missing amino acid for normal development in the mutant. An impaired methionine biosynthesis and a consequently low intracellular methionine pool in the mutant that could release the feed-back control of the enzyme would lead to a constantly high rate of flux of aspartate into the derepressed pathway. Exogenously added methionine may act by decreasing the activity and/or synthesis of AK, the first enzyme of its biosynthetic pathway. This would save aspartate for the cells. This explanation is supported by the finding that methionine can at least partially repress AK, and — together with threonine -- also inhibits its activity. Although both effects are partial only, their concerted action may decrease the AK activity to a very low level in the cells. Methionine alone, i.e. without threonine, does not inhibit AK but it is likely that with derepressed AK there is enough threonine for the concerted action. The properties of AK found are similar to those reported for other Streptomyces species /6, 8, 9/, but in contrast to S. clavuligerus the enzyme from S. fradiae is inhibited by the combination of methionine and threonine. Some data contradict the above hypothesis. Lysine and threonine together are very potent inhibitors of AK but neither of them nor their combination induced sporulation in the mutant. This may reflect that AK activity is not really relevant for sporulation, or there is some permeability barrier for threonine and/or lysine, or the inhibition is not effective without methionine in vivo.

There have been differences observed between the effects of methionine and aspartate. These included that (i) the characteristic spore color was not seen on the aerial mycelia when sporulation had been induced by aspartate, (ii) methionine was required at a much lower concentration that aspartate. The former is not very likely to be explained by rough differences in spore maturation, since heat resistance of the spores was similar in both cases to that reported for S. fradiae spores /5/, although resistance to heat does not necessarily indicates the same maturation status as pigmentation does. It seems more likely that the characteristic pink color of the sporulating S. fradiae mycelia was missing when spore formation was induced by aspartate because of the less abundant sporulation of the white aerial mycelia causing a 'dilution' of the color. For the latter difference men-

tioned above, it is possible that the transport of aspartate is not efficient enough, or methionine acts also by an additional mechanism to be explored. In order to find such other possible mechanism(s) investigations are planned on post-translational modification of macromolecules by methylation and on regulation of N-metabolism in S. fradiae.

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PURIFICATION AND CHARACTERIZATION OF THE CATALYTIC SUBUNIT OF PROTEIN PHOSPHATASE 1 FROM NEUROSPORA CRASSA*

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Protein phosphorylation is a universal regulatory mechanism in eukaryotic cells. The phosphorylation state of proteins is affected by the antagonistic activities of protein kinases and phosphatases. Protein phosphatases (PPs) can be classified as serine/threonine and tyrosine specific phosphatases. Ser/Thr phosphatases are divided into four subclasses (PP1, PP2A, PP2B, PP2C) on the basis of their substrate specificity, metal ion dependence and inhibitor sensitivity. We were able to detect the activities of all four Ser/Thr protein phosphatases in the mycelial extract of Neurospora crassa. The catalytic subunit of PP1 was purified 1500-fold with a yield of 1.3% using ammonium sulfate-ethanol precipitation, DEAE-Sephacel, heparin-Sepharose and MonoQ FPLC chromatography. The protein product was nearly homogenous, as judged by SDS-polyacrylamide gel electrophoresis. The most important properties of the enzyme were the following: /1/ its molecular mass proved to be 35 kD, /2/ it was completely inhibited by inhibitor-2, microcystin and okadaic acid, /3/ it was bound to heparin-Sepharose, and /4/ its specific activity was 2000 mU/mg. These biochemical properties are very similar to those of the homologous enzyme from rabbit muscle and indicate a high level of conservation of PP1 structure during evolution.

Keywords: Protein phosphorylation — Ser/Thr protein phosphateses — protein purification — okadaic acid — Neurospora crassa

Introduction

Protein phosphorylation is a mechanism widely utilized by eukaryotic organisms to regulate their cellular functions. Changes in the phosphorylation state of proteins on their serine, threonine and tyrosine residues may result in conformational and functional changes. The phosphorylation state of a given protein depends on the opposing activities of protein kinases and protein phosphatases.

*Dedicated to the memory of Professor Gábor Szabó.

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Protein phosphatases (PPs) can be classified as serine/threonine and tyrosine specific phosphatases. Ser/Thr phosphatases are divided into four subclasses (PP1, PP2A, PP2B, PP2C) on the basis of their substrate specificity, metal ion dependence and inhibitor sensitivity /4/. According to a recent classification PP1, PP2A and PP2B belong to the PPP gene family, whereas PP2C and the mitochondrial pyruvate dehydrogenase phosphatase are members of the PPM family /3/. Regulatory or targeting subunits that bind to the phosphatase catalytic subunit also play important role in the enzyme's action: either by altering the selectivity of the enzyme towards a particular substrate or by targeting the phosphatase to the subcellular location of its substrates.

PP1 is widely distributed among diverse species and the primary structure of its catalytic subunit has been extremely well conserved during evolution. Yeast and filamentous fungi are versatile model organisms to study the biological function of this enzyme. A PP1 catalytic subunit is encoded by the GLC7 gene in budding yeast. It is involved in regulation of glycogen metabolism and cell cycle progression /12, 17/. The protein product of the GAC1 gene was recognized as a regulatory (targeting) subunit responsible for glycogen binding /14/. In fission yeast there are two genes encoding PP1 catalytic subunit isoforms: dis2^{+} - the homologue of GLC7 - and sds21^{+}. These phosphatases negatively regulate entry into mitosis and play and important role in inactivation of p34^{cdc2}/cyclin complex during mitosis /29/. The sds22 protein, which is considered to be a regulatory subunit, controls the substrate specificity and is necessary for the progression from metaphase to anaphase /29/.

A mutation in an Aspergillus gene encoding a PP1 enzyme (bimG) leads to defects in mitosis and cell growth /8/. In Neurospora crassa the first known gene encoding a well-defined phosphatase (the Ca^{++}-dependent PP2B) activity was cloned by Higuchi et al. /16/. Later it was shown that this activity was essential for hyphal elongation /23/. Inactivation of a single PP2A gene (pph-1) proved to be lethal in N. crassa /30/. The activities of PP1, PP2A and PP2B in N. crassa cell extract were first detected by Szöőr et al. /28/. The purification and biochemical characterization of the PP2A catalytic subunit was also described /27/. Recently Zapella et al. /31/ successfully resolved and partially characterized PP1, PP2A and PP2C activities from a mycelial extract of N. crassa. In this work we describe the purification and biochemical characterization of the PP1 catalytic subunit ($PP1_C$) from N. crassa.

Materials and Methods

Materials

Okadaic acid and microcystin-LR were purchased from GIBCO BRL. /γ-^{32}P/ ATP was obtained from ICN. DEAE-Sephacel, heparin-Sepharose, MonoQ HR 5/5 and Superdex 200HR were the products of Pharmacia, and FPLC of the same company was used. Casein was from the Sigma Chemical Company. Other chemicals were obtained from Reanal Fine Chemicals (Budapest, Hungary).

Strain and cultivation

Two agar slants of wild type Neurospora crassa strain RL3-8 (FGSC stock number 2218) were used to inoculate 400 ml Vogel's minimal medium /7/. One liter flasks were incubated for 48 h at 27 °C in a temperature controlled laboratory shaker. The mycelial mat was collected by filtration through cheesecloth, weighed, frozen in liquid nitrogen and stored at -70 °C.

Protein preparations

Phosphorylase b was purified from rabbit skeletal muscle /13/ and was converted to phosphorylase a with rabbit muscle phosphorylase kinase using /γ-^{32}P/ATP /18/. Inhibitor-1 (I-1) and inhibitor-2 (I-2) were purified from rabbit skeletal muscle /5/. The dephosphorylated form of I-1 was phosphorylated with bovine cAMP-dependent protein kinase using /γ-^{32}P/ATP /21/. ^{32}P-labelled casein was prepared according to /21/. ^{32}P-labelled myosin light chain was prepared using the procedure of Erdődi et al. /11/. The catalytic subunit of protein phosphatase 1 isolated from rabbit skeletal muscle /15/ was used in control experiments.

Protein phosphatase assays

Protein phosphatase assays were carried out with four different substrates.

(i) ^{32}P-labelled phosphorylase a substrate was used as described /6/. The assay mixture contained 5 μM ^{32}P-phosphorylase a dimer and 5 mM caffeine in the extraction buffer (see Results section) in a total volume of 30 μl. The reaction was initiated by the addition of the substrate and was terminated after 10 min incubation at 30 °C by the addition of 100 μl 10% trichloroacetic acid (TCA). TCA soluble radioactivity was counted by Cherenkov radiation.

(ii) Measurements using ^{32}P-labelled I-1 as a substrate were performed according to /16/. The reaction mixture contained 3 μM ^{32}P-labelled I-1, 40 mM Tris-HCl (pH 7.0), 0.4 mM dithiothreitol (DTT), 0.2 mM $CaCl_2$, 1 mM $MnCl_2$ and 12 μM calmodulin in a total volume of 30 μl. The reaction was started by the addition of substrate and after 10 min incubation at 30 °C it was terminated by the addition of 100 μl 20% TCA and 100 μl 6% bovine serum albumin (BSA). The released radioactivity was counted as above.

(iii) Reactions with ^{32}P-labelled casein were carried out according to /21/. The assay mixture contained 60 mM magnesium acetate, 50 mM Tris-HCl (pH 7.0), 0.1 mM /ethylene glycol-bis(oxyethylenenitrilo)/tetraacetic acid (EGTA), 0.1% (v/v) 2-mercapthoethanol in a total volume of 30 μl. The reaction was initiated with 10 μl ^{32}P-labelled casein. After 10 min at 30 °C,

the reaction was terminated by addition of 100 µl of 20% TCA. The suspension was allowed to stand for 2 min at room temperature, then centrifuged for 2 min at 13 000 x g. The released radioactivity was counted in 120 µl of the supernatant.

(iv) Assays with ^{32}P-labelled myosin light chain were performed as described /11/. The 100 µl reaction mixture contained 25 mM Tris-HCl (pH 7.5), 1 mM DTT and ^{32}P-labelled myosin light chain (0.5 mg/ml). The reaction was initiated by the addition of the phosphosubstrate and terminated after 10 min at 30 °C by addition of an equal volume of 20% TCA. After centrifugation at 15 000 x g for 5 min 100 µl of the supernatant was counted for radioactivity.

Using an extraction method /2/, we proved that in all phosphatase assays more than 95% of radioactivity was due to the presence of $^{32}P_i$. One unit (U) of protein phosphatase activity was defined as the amount of enzyme which catalyzed the liberation of 1 µmol inorganic phosphate from the substrate in 1 min.

Protein analysis

Protein concentration was measured according to Read and Northcote /23/ using BSA as standard. SDS-polyacrylamide gel-electrophoresis was carried out as described /19/. For the visualization of the proteins after gel-electrophoresis we used the silver staining method /22/.

Chromatographic procedures are described in the figure legends. NaCl concentration of fractions was determined by measuring their conductivity.

Results

Distribution of four Ser/Thr protein phosphatase activities in the mycelial extract of N. crassa

There is no "universal" substrate which would be suitable to detect all four Ser/Thr protein phosphatase activities under the same reaction conditions. For this reason we used four different substrates in our activity measurements. Rabbit muscle phosphorylase a and myosin light chain were used to determine the activity ratio of PP1 and PP2A (Table 1). Since subnanomolar concentrations of okadaic acid (OA) specifically inhibit PP2A, the difference between activities measured with and without 1 nm OA was considered PP2A activity, whereas the residual activity was attributed to the tpye 1 enzyme. This activity was fully abolished by 10 000 U/ml inhibitor-2. Reciprocal experiments using 10 000 U/ml inhibitor-2 to block PP1 activity gave essentially the same results (data not shown). The activity ratios of PP1 and PP2A show very similar values with phosphorylase a and myosin light chain (60:40 and 55:45, respectively). PP2B activity was determined in the

Table 1

Detection of protein phosphatases from N. crassa mycelial extract

Protein phosphatases[a]	Distribution of specific protein phosphatase activities as determined with various substrates (%)			
	Phosphorylase a	Myosin light chain	Casein	Inhibitor-1
PP1	60 (3.1)[b]	55 (12.0)	11 (2.6)	—
PP2A	40 (2.0)	45 (10.0)	16 (3.9)	39 (0.028)
PP2B	—	—	—	61 (0.045)
PP2C	—	—	73 (17.4)	—

[a]For exact description of each assay see text and Materials and Methods.

[b]Absolute specific activity values (mU/mg) are given in parentheses. Average of 3-5 experiments is shown. The standard deviation of the assays was ± 20-30% of the total activity.

presence of Ca^{++}, calmodulin and 3 μM OA, with rabbit muscle inhibitor-1 as substrate. Since 3 μM OA inhibits PP1 and PP2A, and Ca^{++}-calmodulin activates PP2B, the activity corresponds to that of PP2B, while the activity measured in the presence of 0.5 mM EDTA corresponds to PP2A. (PP1 activity cannot be detected with this substrate.) The Mg^{++}-dependent PP2C was measured with casein substrate. The difference between values obtained with and without Mg^{++} (i.e. in the presence of 0.5 mM EDTA) was considered PP2C activity. The ratio of PP1 and PP2A activities with this substrate was established as above. The PP1 activity was rather low with ^{32}P-casein (Table 1).

Purification of the catalytic subunit of PP1 from N. crassa

The first three steps of the purification were identical with those described for PP2A /27/. In these steps no separation of PP1 and PP2A activities could be observed (see Table 2). Protein phosphatase activities were measured with ^{32}P-phosphorylase a substrate at the different purification steps.

N. crassa mycelia from a 48 h culture were disrupted in mortar with pestle in liquid nitrogen. The mycelial powder was mixed with 2 volumes/weight extraction buffer (50 mM Tris-HCl /pH 7.4/, 1 mM EDTA, 0.1 mM EGTA, 0.6 mM Mn^{++}, 0.1% 2-mercaptoethanol) containing a mixture of freshly diluted

Table 2

Purification of protein phosphatase 1 catalytic subunits from N. crassa

Purification step	Volume (ml)	Protein (mg/ml)	Activity (mU/mg)	Specific activity (mU/mg)	Residual activity (%)		Purification	Yield (%)
					OA[a]	I-2[b]		
Crude extract	72	1880	2.5	1.3	70	38	1	100
$(NH_4)_2SO_4$ + ethanol treatment	12	589	6.4	10.9	72	37	8.4	42.7
DEAE-Sephacel chromatography	6	339	6.8	20.1	68	40	15.5	22.7
Heparin-Sepharose chromatography	8	8	1.5	188	96	7	144.6	6.7
MonoQ FPLC	1.2	0.8	1.6	2000	94	7	1538	1.3

Starting material: 30 g.

[a]Activity was measured in the presence of 1 nM okadaic acid.

[b]Activity was measured in the presence of 3000 U/ml inhibitor-2.

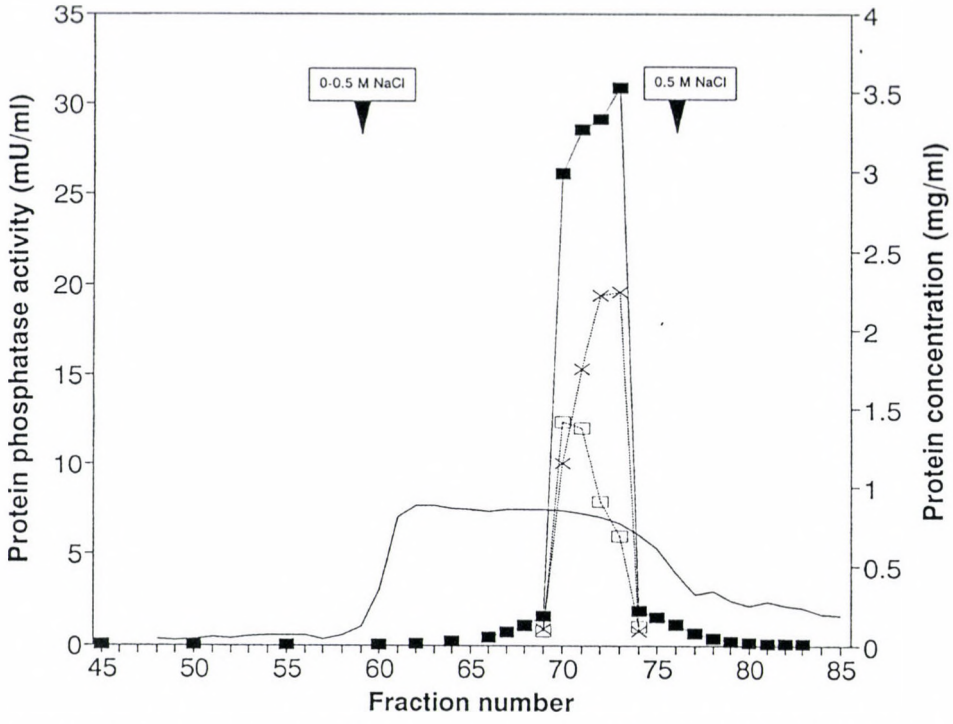


Fig. 1. Ion exchange chromatography of N. crassa protein phosphatases on DEAE-Sephacel. Fractions were assayed for phosphorylase phosphatase activity without additions (■), and in the presence of 1 nM okadaic acid (□), or 3000 U/ml inhibitor-2 (X), as well as for protein concentration (x). Column size: 2 cm x 21 cm, column buffer: 50 mM imidazole, 5 mM EDTA, 10 mM 2-mercaptoethanol, 10% v/v glycerol (pH 7.2), flow rate: 35 ml/h, fraction volume: 2.8 ml. A 50-50 ml 0-0.5 M NaCl gradient was started at the arrowhead, fractions 65-75 were pooled and concentrated by dialysis against IEM buffer containing 60% v/v glycerol for further purification

protease inhibitors (5 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM o-phenantroline). The extraction was completed by mixing the ice cold homogenate at a full speed for 1 min in an MSE blender. Then the mixture was filtered through glass wool and was centrifuged at 4 ^{o}C and 15 000 x g for 15 min. The supernatant was used as mycelial extract in the phosphatase assays. Addition of Mn^{++} ions slightly increased specific activity of the extract and it had especially beneficial effect on the stability of PP1 during purification. The presence of Mn^{++} did not change the ratio of PP1 and PP2A, significantly.

Then ammonium sulfate precipitation was carried out at 70% saturation, and the resuspended pellet was precipitated again, this time with 5 vol. of 96% ethanol at room temperature. The ammonium sulfate-ethanol treatment li-

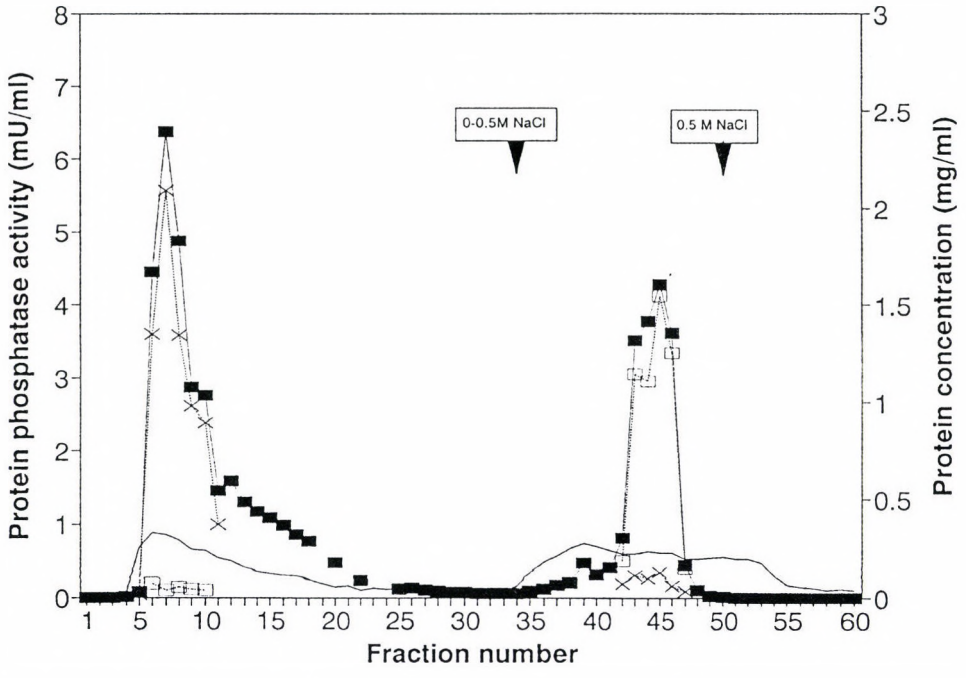


Fig. 2. Separation of N. crassa protein phosphatase catalytic subunits on heparin-Sepharose. Symbols and buffer composition are the same as in Fig. 1. Concentrated peak fractions from the previous step were diluted twofold with IEM buffer to reduce glycerol content, and were loaded on a column of 2.5 cm x 9 cm. Flow rate: 35 ml/h, fraction volume: 2.8 ml. A 35-35 ml 0-0.5 M NaCl gradient was started at the first arrowhead, fractions 48-53 containing PP1 activity were pooled and concentrated as in Fig. 1. The second arrowhead indicates the start of washing the column with 0.5 M NaCl

berated the catalytic subunits of protein phosphatases ($PP1_C$ and $PP2A_C$) from their holoenzymes /20/. The pellet was extensively dialyzed against 50 mM imidazole, 5 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.4 (IEM buffer). This and all the subsequent steps were carried out at 4 ^{o}C.

Protein phosphatase catalytic subunits were separated from the bulk of the proteins by DEAE-Sephacel chromatography (Fig. 1). Activities of $PP1_C$ and $PP2A_C$ were only partially resolved on this column; $PP1_C$ was eluted somewhat earlier than $PP2A_C$ based on assays with OA and I-2.

$PP1_C$ was separated from $PP2A_C$ by heparin-sepharose affinity chromatography /15/. The phosphatase activity of the flowthrough fractions proved to be $PP2A_C$ (Fig. 2). Since the second peak (eluted at 0.32 M NaCl concentration) was abolished by I-2 and only slightly affected by 1 nM OA, it was considered to be $PP1_C$.

The final purification step was carried out on MonoQ FPLC. The $PP1_C$ activity was eluted as a single peak at 0.34 M NaCl concentration (Fig. 3). The addition of heat stable I-2 nearly completely inhibited PP activity, while 1 nM OA caused only slight (10%) reduction in the specific activity of the peak fraction.

Our protocol resulted in about 1500-fold overall purification and 1.3% yield (Table 2). The specific activity of N. crassa $PP1_C$ was in the range of 800-2000 mU/mg protein in 3 independent purification experiments. These values are similar to the ones described for rabbit skeletal muscle (1750 mU/mg /25/) and for bovine platelet (2371 mU/mg /10/), suggesting that the preparation was nearly homogeneous.

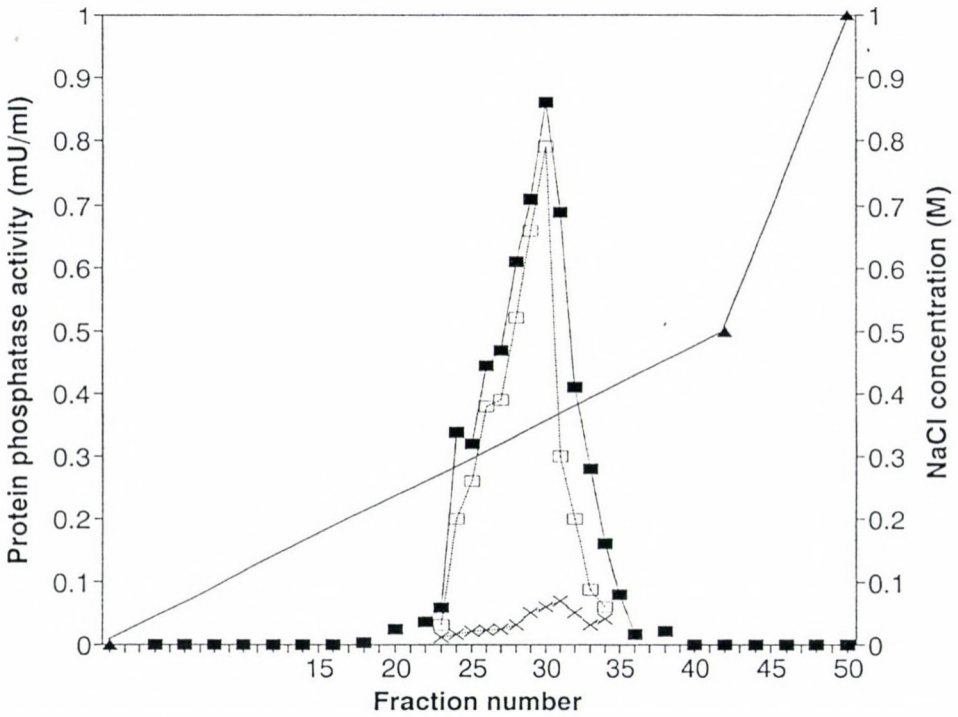


Fig. 3. MonoQ FPLC of N. crassa protein phosphatase 1 catalytic subunit. Fractions were assayed for phosphatase activity (■), a two step NaCl gradient elution was performed as shown (▲). Column buffer was the same as in Fig. 1. The sample was loaded after twofold dilution with IEM buffer. Flow rate: 10 ml/h, fraction volume: 0.8 ml

Characterization of N. crassa PP1 catalytic subunit

The purified $PP1_C$ gave a single band of 35 kD on an SDS polyacrylamide gel (data not shown). The same molecular mass was obtained in a gel filtration experiment on FPLC (Fig. 4).

The purified $PP1_C$ preparation was compared with its homologue originating from rabbit muscle (Table 3). The two enzymes exhibit considerable similarity with regard to their specific activity, sensitivity to OA and microcystin LR, as well as their molecular mass.

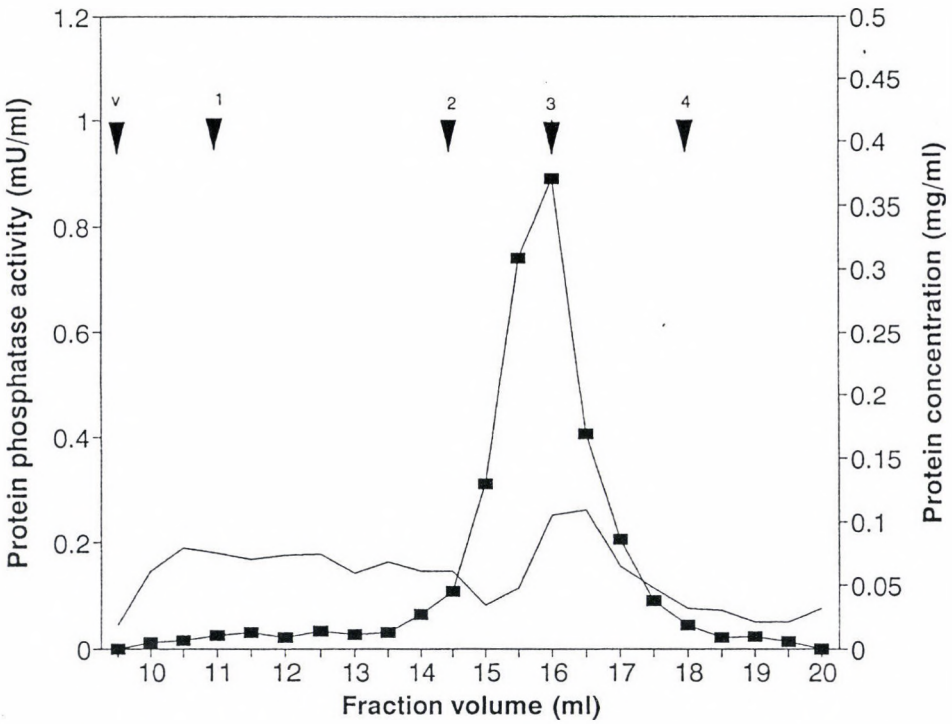


Fig. 4. Gel filtration of N. crassa protein phosphatase 1 catalytic subunit on Superdex 200HR. The elution profile obtained with partially purified $PP1_C$ (■) is shown. The elution position of proteins used for the calibration of the column are indicated by arrowheads: (1) ferritin, 480 kDa; (2) BSA, 68 kDA; (3) rabbit skeletal muscle $PP1_C$, 36 kDa; (4) myoglobin, 17.8 kDa; VO denotes void volume. Column buffer: 50 mM imidazole, 5 mM EDTA, 100 mM NaCl, 10 mM 2-mercaptoethanol, 10% glycerol, pH 7.2, flow rate: 0.5 ml/min, fraction volume: 0.5 ml. 0.2 ml samples were loaded on a prepacked column connected to an FPLC system

Table 3

Comparison of protein phosphatase 1 catalytic subunits from N. crassa and rabbit skeletal muscle

	N. crassa $PP1_C$	Rabbit $PP1_C$
Specific activity (mU/mg)	2000	1750
Okadaic acid inhibition (IC_{50})	30 nM	20 nM
Microcystin LR inhibition (IC_{50})	0.12 nM	0.1 nM
Molecular mass (kD)	35	35

Discussion

Protein phosphatases play an essential role in various regulatory processes within the eukaryotic cell. For a more complete understanding of their biological significance and function, as a first step, it is necessary to study these proteins from a biochemical point of view. All four different classes of Ser/Thr protein phosphatase activities can be found in an N. crassa mycelial extract. However, quantitative ratios of the different activities should be evaluated with caution, because they might show significant differences depending on the applied substrate. Our result for the relative activity of PP1 (60%) measured with phosphorylase a substrate is in good agreement with the data of Zapella et al. /31/. In addition we determined the activities of PP1 and PP2A with other substrates, as well (Table 1), indicating a broad substrate specificity of these enzymes.

We attempted and successfully carried out purification and biochemical characterization of the catalytic subunit of a type 1 protein phosphatase from N. crassa. Starting out from 30 g mycelium, after five purification steps we obtained about 1 mg purified catalytic subunit of PP1 enzyme with a specific activity of 2000 mU/mg. The protocol was repeated several times and it proved to be reliable and reproducible. The protein product was nearly homogeneous, as judged by SDS polyacrylamide gel electrophoresis. The most important properties of the enzyme were the following: /1/ its molecular mass proved to be 35 kD, /2/ it was inhibited by inhibitor-2, microcystin LR and OA, /3/ it bound to heparin-Sepharose. All these properties are very similar to those of the rabbit muscle $PP1_C$ and indicate a high level of conservation of its structure during evolution (Table 3). To our knowledge this is the first report on the purification of a type 1 protein phosphatase from a filamentous fungus.

The catalytic subunit of PP1 exerts its biological function in association with regulatory (targeting) subunits, which bind it to its targets. It was shown, that the retinoblastoma protein was one of the proteins that associated with $PP1_C$ in human cells /9/. It was also suggested that PP1 acted in the dephosphorylation (activation) of the retinoblastoma protein /1/, thus playing an important role in the braking mechanism regulating cell division. In lower eukaryotes the GAC1 and sds22$^+$ genes encode regulatory subunits in budding yeast and fission yeast, respectively /14, 26/. To date there is no information on regulatory subunit of PP1 in N. crassa. However, preliminary gel filtration experiments in our laboratory have indicated the existence of a high molecular mass (~ 200 kD) holoenzyme form of PP1, strongly suggesting the presence of noncatalytic subunits associated with $PP1_C$ (Yatzkan et al. unpublished results). Inhibitor proteins that can also bind to $PP1_C$ are thought to modulate protein phosphatase activity in vivo. A thermostable protein phosphatase inhibitor was identified in N. crassa which effectively inhibits both N. crassa and mammalian PP1 /31/.

In order to approach the biological functions of PP1 in filamentous fungi it would be necessary to clone the genes encoding the subunits of this enzyme, then to disrupt them and analyze phenotypic effects that correspond to the inactivated gene. The cloning of the catalytic subunit could be facilitated by our results demonstrating the similarity between the biochemical properties of the fungal and mammalian enzyme. Based on these observation a screening for the PP1 gene can be initiated with a heterologous DNA probe from animal sources.

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LESSONS TO LEARN FROM THE CELL DEATH AND HEAT SHOCK GENES OF CAENORHABDITIS ELEGANS*

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The nematode Caenorrhabditis elegans is an applicable experimental system for simulation of complex biochemical processes of mammalian cells and tissues. The genetic pathway of programmed cell death (PCD) has been partially clarified in the nematode. Analysis of cell death genes of C. elegans led to the conclusion that PCD is conserved in the animal kingdom. Our intention is to study the role of tissue transglutaminase and heat shock proteins in the process of PCD. Tissue transglutaminase is often observed to be induced and activated during the molecular mechanism of PCD. The connection between the heat shock proteins and PCD is not well understood, but it is clear that many apoptosis inducers lead to increased synthesis of heat shock proteins and production of heat shock proteins is coupled with the appearance of apoptosis in numerous experimental systems. Our preliminary observations made by studying cell death mutants of C. elegans we suggest that transglutaminase and heat shock proteins are involved in the death program of the nematode.

Keywords: Caenorhabditis elegans — programmed cell death (PCD) — transglutaminase — heat shock proteins

Introduction

The well-known model organism of developmental genetics, the nematode Caenorhabditis elegans is a small free-living soil worm. It feeds on bacteria and reproduces with a life cycle of about three days under optimal conditions. Eggs are layed after the fertilization and embryogenesis take place within these eggs. After hatching it develops through four larval stages and finally mature adults are formed. C. elegans is easily maintained in laboratory, where it can be grown on agar plates or in liquid culture with Escherichia coli as a food source. It has two sexes, hermaphrodites and

*Dedicated to the memory of Professor Gábor Szabó.

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males. Hermaphrodites produce both oocytes and sperm and can reproduce by self-fertilization but cannot fertilize each other. Males, which are produced spontaneously at low frequency, can fertilize hermaphrodites. Regarding its short-life cycle and hermaphrodite mode of reproduction, C. elegans is applicable for genetic analysis. Chemical mutagens (EMS) or X-ray mutagenesis can induce mutations at a high frequency /95/. Several hundreds of mutants were isolated and analysed until now. The expression pattern database and the genome project of the animal are highly progressed; more than the half of its genome bas been sequenced /91/. Its genetic map has been aligned with the physical map of the genome, so each chromosome is now nearly completely represented by overlapping collections of cosmid or YAC clones. In addition, it is possible to construct transgenic animals and the activities of individual genes can be assayed in vivo /10/. Complex mammalian cell phenomena, such as cell death and stress process can be studied in the nematode modell system.

Cell death in C. elegans

Cell death occurs as a normal aspect of animal development as well as in tissue homeostasis and aging. Programmed cell death (PCD) can act to regulate cell number, to facilitate morphogenesis, to remove abnormal cells or to eliminate cells that have already performed their functions /12/. Gene expression within dying cells is thought to be required for these cells to die, since the process can be blocked by inhibitors of RNA and protein synthesis /52/.

The nematode C. elegans seems well suited to investigation of genetically specified cell death. The complete cell lineage of this nematode has been recorded /83, 84/. During the ontogenesis of hermaphrodite nematodes 1090 somatic cells (their position and life history are exactly known) are generated, from which 131 cells undergo PCD. Because the cuticle of the animal is transparent and individual cell nuclei are easily identified by Nomarski optics, cell death can be observed while it occurs in the living animal /10/. PCD in C. elegans can be divided into four distinct stages (Fig. 1 /12/). These include the decision of whether a given cell will die, the death of the cell, the engulfment of the dead cell by phagocytes, and the degradation of the engulfed corpse. Dying cells disintegrate within an hour or so and are quickly engulfed by neighbouring cells /12/.

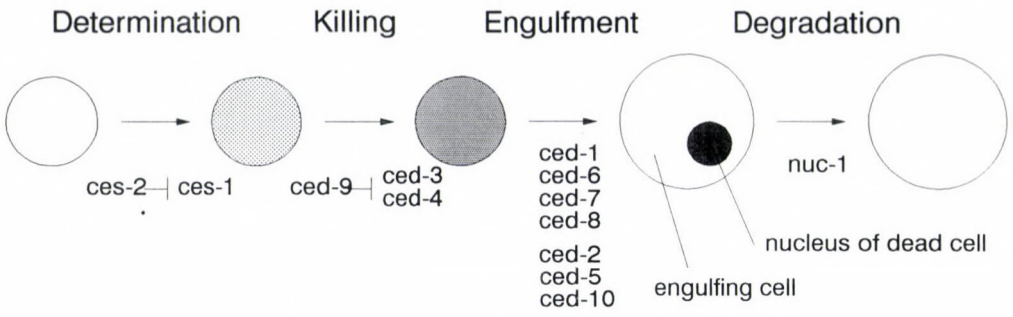


Fig. 1. Mutations of known cell death genes block PCD at various steps

The morphology of cells undergoing death in C. elegans has been described /68, 83/. It shares a lot of features with apoptosis, the mode of cell death observed in vertebrates /41/. During C. elegans cell's death its cytoplasm and nucleus condense, and dying cells fragment into several pieces. These features of the animal has allowed mutations to be identified that disrupt the normal pattern of PCD.

Numerous genes that are involved at various steps of this process were described, defining a genetic pathway for PCD in the nematode (Fig. 1 /12/). Recessive loss-of-function mutations of ced-3 or ced-4 /15/ and the dominant gain-of-function mutation of the negative regulator ced-9 /34/ genes cause all cells that normally die instead survive, differentiate and function /2, 15/. Mutations of ced-3 or ced-4 genes can block the excess cell death and lethality in ced-9 loss-of-function mutants indicating that ced-3 and ced-4 are genetically downstream from ced-9 /99/. Genetic mosaic analysis indicates that ced-3 and ced-4 most likely function within cells that die /101/, and they are expressed primarily during embryonic development, when most cell deaths occur /98, 100/. Seven known genes (ced-1, ced-2, ced-5, ced-6, ced-7, ced-8, ced-10) are required for the engulfment of dead cells, in mutants defective in one of these genes cells die as a result of PCD, but their corpses are not phagocyted by the surrounding cells and they can persist for many hours. There are several double phagocytosis mutants, for example the ced-5;ced-7 double mutatnt, which has the most expressive phenotype. Genetic analysis of double mutants' genes that may be involved in the control of engulfment can be divided into two groups (set 1: ced-1, ced-6, ced-7, ced-8; set 2: ced-2, ced-5, ced-10). Animals with mutations in both sets of genes have the highest number of unengulfed cell corpses /13, 31/. Moreover the nuc-1 gene encodes a DNAse that is used to digest the DNA of

dead cells, but mutations in it do not affect neither the death of cells nor the engulfment process /12/. All of these genes are involved in the death of all cells in the nematode, but the entry into the process is determined by cell specific genes /14, 86/.

Homologues of C. elegans cell death genes in mammalian cells

The molecular mechanism that control PCD seems to be conserved in the animal kingdom /79, 99/. Cloning and molecular characterization of the ced-9 gene showed that it encodes a protein containing sequence and structural similarities to the mammalian protooncogene bcl-2 /32, 33/. The translocation of bcl-2 coding region results in an abnormal activation of bcl-2 expression, that promotes oncogenesis because of the increased life span of cells caused by the inhibition of the PCD, which could normally eliminate the cells after the performance of their functions /87/. Overexpression of bcl-2 can block PCD in the nematode and the ectopic cell deaths that occur in ced-9 loss-of-function mutants /32, 89/.

The ced-3 gene encodes a protein that is similar to a cysteine protease, interleukin-1β-converting enzyme (ICE) /98/, and it has been shown that the CED-3 has a protease activity /96/. CED-3, ICE /59/, Nedd2 /43/, Ich-1 /90/ and CPP3225 /17/ are members of the ICE-like protease family and can induce apoptosis when they are expressed in mammalian cells. Expression of baculovirus antiapoptoic protein p35 inhibits PCD in C. elegans, thus extra cells were observed in the anterior part of the pharynx in transgenic animals /82/. Baculovirus p35 inhibited CED-3 of the worm and also blocked ICE family proteases in mammalian cells, which resulted in the inhibition of PCD in experimental systems /63, 96/. In addition, the product of cowpox virus gene crmA, a well-known ICE inhibitor, prevented the vertebrate neuronal death /24/.

Ced-4 does not show similarity to any known proteins. Two regions of CED-4 have some similarity to calcium-binding domain known as the EF-hand, suggesting that PCD in C. elegans may be regulated by calcium /100/.

The sequence of ced-7 showed similarity to mammalian ABC (ATP-binding casette) protein, but its possible role in the engulfment process has remained unclear /50/. Moreover, there is a new evolutionary conserved cell death supressor gene family called dad-1. C. elegans dad-1 can substitute for the mammalian gene and the expression of the human gene is able to in-

Table 1

Mammalian homologues of C. elegans cell death proteins

C. elegans protein	Mammalian protein
CED-9	BCL-2
CED-3	ICE, NEDD2, ICH-1, CPP32
CED-7	ABC transporter
DAD-1	DAD-1 in a temperature sensitive hamster cell line

hibit the cell death in the nematode /81/. It is clear that members of ced-9/bcl-2 and dad-1 families and ICE like proteases have a very important role in the general PCD process which has evolutionary conserved steps (Table 1).

The transglutaminase connection

It has been observed in our laboratory that tissue transglutaminase is often induced and activated during the molecular process of PCD /19/. The calcium-dependent protein crosslinker cytosolic tissue transglutaminase acts in an acyl transfer reaction where the γ-carboxamide group of a peptide bound glutamine serves as acyl donor and the ε-amino group of peptide bound lysine residue acts as acyl acceptor. The result of this reaction is a covalent ε(γ-glutamyl)lysine isopeptide bond between the proteins; this linkage is resistant to proteases /22, 49/. The enzyme accumulates to high level in some terminally differentiated cells /62, 64/ as well as in number of cell types undergoing cell death both in vivo and in vitro /19, 20/. Biochemical analysis of apoptotic bodies formed during the cell death has shown that they contain high level of ε(γ-glutamyl)lysine isopeptide bond in the strongly cross-linked protein envelopes /18/. It is likely that cross-linking of proteins stabilizes the apoptotic bodies and limits the leakage of intracellular constituents into the extracellular space /20/.

Numerous transglutaminases were described in mammals and their existance has also been established in microorganisms /26, 42/, in grasshopers (Schistocera americana /75/), in fish (Pagrus major /97/), in crabs (Tachypleus tridentatus /85/), in birds (Gallus gallus /94/), and in nematode (Brugia malayi /76/). Since the role of transglutaminases has not been

completely clarified so far, we turned to the nematode system to get more information using the ced mutants.

This enzyme was unknown in C. elegans, so our first aim was to detect it both in wild and cell death mutant strains. We demonstrated that there is a transglutaminase protein in C. elegans /19/. The enzyme is expressed constitutively in some cells of the nematode and induced in those programmed to die. Some ced mutant organisms show characteristic changes in the activity of the enzyme, and wide variety of crosslink level was observed in those where the cells die and are not eliminated because of mutations in genes required for the engulfment process. Our data suggest that the transglutaminase is part of the basic cell death program in the worm, but further experiments, including cloning and modifying the C. elegans transglutaminase gene, are needed to reveal its exact role in the death process.

Heat shock proteins and cell death

Heat shock (stress) proteins are highly conserved throughout the evolution and were detected in cells from bacteria till human beings. They are produced under changed environmental conditions such as sudden change of temperature, presence of environmental pollutants, ionophores, inhibitors of energy metabolism and amino acid analogues; also under certain pathophysiological states as fever and inflammation, ischemia, oxidant injury, aging, etc. /47, 60/. Certain stress proteins are present in the cells under non-stressful conditions, and play roles in wide spectrum of physiological processes /6, 54, 93/. The level of phosphorylation state of stress proteins is often regulated by growth factors, oncogenes, cytokines /1, 23, 55/ and changes at various stages of cell cycle /58, 74/. Most of these proteins are necessary to normal growth and proliferation of cells /39/.

The most intensively studied role of the stress proteins is their chaperone function, i.e. taking care of newly synthesized proteins, accelerating the proper folding and preventing abnormal associations in the crowded environment of cytoplasm, and also to guide protein movements throughout membranes /27, 60, 72/. Under stress conditions when there are more damaged proteins in the cells, stress proteins serve as tags to mark and destine the damages ones to degrading systems, i.e. the lysosomes or proteosomes /8, 66/. However, if the level of stress is further increased, and even the higher amounts of stress proteins are unable to protect crucial proteins and

Table 2

Comparison of inductions of hsp production and apoptosis

Hsp production is induced	Apoptosis is induced
heat shock	heat shock
serum restriction, glucose restriction	serum restriction
fever, inflammation	fever, inflammation (cytokines)
free radicals	free radicals, UV light
oxidative stress, H_2O_2	oxidative stress, H_2O_2
calcium ionophores	calcium ionophores
growth, differentiation	growth, differentiation

organelles of the cell, the apoptotic program is activated, and the cell commits suicide, eliminating the damaged cell /71/. This way, both processes — production of the stress proteins and programmed cell death — are responsible for the general "well-being" of the organism.

In many situations however, the apoptotic program acts as the regulator of the proper cell number in development of C. elegans /5, 79, 99/. It has been supposed recently that stress proteins act as regulators of the apoptotic program /56/. Although there is surprising analogy between stressors inducing the heat shock response and inducers of apoptosis, results obtained so far using different cell cultures are contradictory (Table 2).

In experimental studies the apoptotic program is often turned on by growth factor withdrawals or treating the cells with ionophores, topoisomerase II or protein kinase C inhibitors, etc. which act on very different cellular processes. It has been detected on many cell lines that inducers of the cell death program same time change the level or the phosphorylation state of certain stress proteins (Table 3).

In cells which stably or transiently overexpressing heat shoch proteins the programmed cell death is very often delayed or decreased indicating role of heat shock proteins in these systems (Table 4).

It has been our intention to study the possible connection between the programmed cell death and production of heat shock proteins using C. elegans as a physiological apoptosis model. There is no experimental data considering the role of heat shock proteins in the programmed cell death of the nematode. It is our intention to find a connection between hsp production (level) and programmed cell death using C. elegans cell death mutants, comparing heat shock protein pattern of the wild type worm to the cell death mutants (ced-3, ced-4, ced-9) /34, 98, 100/.

Table 3

Effect of apoptosis inducers on hsp production

Inducer of apoptosis	hsp
TNF	shsp becomes phosphorylated /55/
t-retinoic acid	shsp becomes phosphorylated /78/
menadion, H_2O_2	shsp becomes phosphorylated /74/
phorbolester	shsp becomes phosphorylated /16/
quercetin	hsp 70 level decreases /11, 92/
topoisomerase II inhibitor	hsp 60, 90 level decreases, collogin level increases /9/
butyrate, propionate, acetate	hsp 70 mRNA level decreases /21/
hsp 70 antisense oligo	hsp 70 level decreases /92/
dexamethasone	hsp 60 and 70 are expressed on the surface of $CD3^{low/-}$ cells /67/

Table 4

Overproduction of hsps may inhibit apoptosis

Change in heat shock protein level	Apoptosis
heat shock (high level of hsps)	butyrate induced apoptosis is delayed /21/
heat shock (high level of hsps)	protects against apoptosis caused by glucocorticoid /57/
brief heat shock	resistance to apoptosis in human leukemic T cells /61/
heat shock (high level of hsp 27, 70)	decreases apoptosis caused by etoposide, actinomycin D, camptothecin on U937 and Wehi-s cells /71/
transient overexpression of hsp 70 mRNA	decreased apoptosis (caused by butyrate) in HL-60 cells /21/
hsp27 stabil transfection	decreased apoptosis mediated by FAS/Apo in murine L929 cells /56/
hsp 70 and shp 72 stabil transfection	decreases the level of cell death caused by etoposide, actinomycin D, camptothecin in U937 and Wehi-s cells /71/
transgene mice expressing hsp 70	T cell lymphoma develops due to the blocked apoptosis in the T cell selection /73/
expression of human, Drosophila shp 27 human alfa-B chrystallin	decreases the level of apoptosis caused by staurosporin in murine L929 cells /56/
hsp 90 overexpression	increased apoptosis caused by UV light or serum restriction /25/
hsp 90 low level	decreased apoptosis /25/

The major groups of heat shock genes of C. elegans are already known and prove the conservative nature of these proteins. During the normal development of the nematode and in the dauer larva, eight different heat shock protein families can be detected (16-81 kDa) increasing the normal growth temperature from 20 ^{o}C to 29-35 ^{o}C /77/. However, heat shock response of C. elegans shows important differences comparing to the bacterial or human heat shock system. The heat shock genes in C. elegans include introns, and the mature mRNA-s are both cis- and trans-spliced, which is unusual in this protein family /3, 29/.

The most important heat shock proteins in C. elegans are the hsp 70-s, which are coded by a multigene family. At least 9 different hsp 70 genes coding three subfamilies of the protein, and being located in the cytosol, transported to the mitochondria or endoplasmic reticulum have been detected /35/. Both constitutive and inducible forms exist which are differentially expressed under heat shock and cadmium treatment /30/. These proteins are produced during the whole life cycle of the worms.

Hsp 90 in higher eukariotes plays role in regulation of hormone sensitivity of steroid hormone receptors. In C. elegans high level of hsp90 (15 times more) was detected at the mRNA level in the non-aging dauer larval form of the nematode comparing to other developmental stages /7/.

In C. elegans UbiA, the major polyubiquitin locus, and the ubc-1 and ubc-2 genes coding ubiquitin conjugating enzymes were already detected /28, 37/. None of these genes were induced by heat shock and are expressed during the whole life but ubc-2 (corresponds to let-70) show developmental and tissue specific expression and essential to normal development /102/. Enhanced expression of poliubiquitin gene was found in apoptosis of certain cell types /51/.

Small heat shock proteins (shsps) in Caenorhabditis elegans are strongly inducible, and from the two hsp16 genes at least five different proteins are produced /38, 69/. These shsp-s have lower molecular weight than the only human hsp27, and also show high homology with α-chrystallin /36, 70/. Transgene worms that carry fusion of hsp16 genes to the E. coli lacZ reporter gene were produced recently, and were suggested to use as rapid, cheap and simple biological monitors of environmental stress /4, 80/. Small heat shock proteins in C. elegans probably are involved in the thermotholerance and extended lifespan of the worms beside the age and daf genes /48/. C. elegans is often used as model of aging /44, 53/. These long-lived strains are also resistant to oxidative environmental stresses /44, 88/.

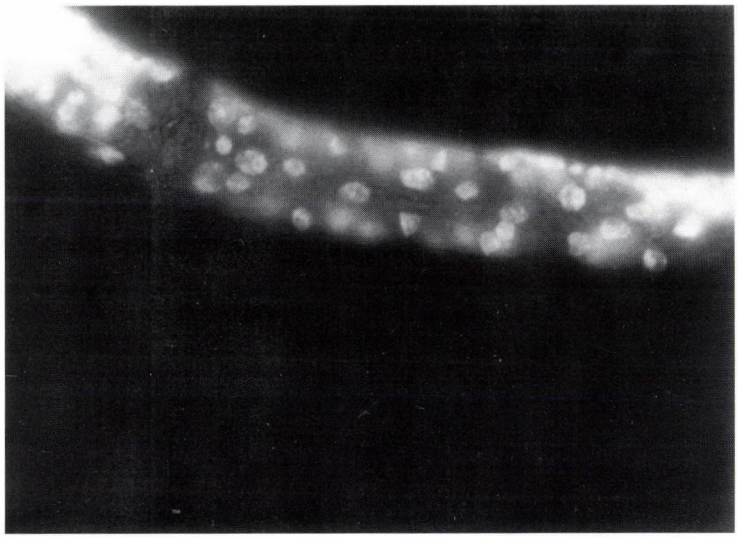


Fig. 2. Localization of different proteins in C. elegans can be visulaized with double immunofluorescence after fixation and permeabilization of the cuticule. Cell nuclei serve stained with propidium iodide (shown here) serve as reference for localization of the cells

Cyclophilin (peptidylprolyl cis-trans isomerase) genes were cloned in C. elegans only recently /65/, and ten different isoforms were detected. Certain cyclophylins are heat shock proteins in other species, but it has not yet been proven in C. elegans.

Chaperonins are located in mitochondria (hsp 60) and in the cytosol of eukariotic cells (TCP1 complex), this latter plays role in tubulin and actin biogenesis /46/. The single copy of tcp-1 gene in C. elegans was mapped, isolated and sequenced only in 1996 /45/. Unlike the evolutionary related hsp60 chaperonin genes, tcp-1 is not upregulated at elevated temperature, but instead appears to be down-regulated. It is 57 kDa protein subunit of high-molecular mass complex.

We found in our experiments that the pattern of heat shock proteins are different in the wild type and PCD mutant worms: hsp 70 is not produced under normal temperature in the wild type and ced-9 mutants but induced under stress conditions. Both ced-4 and ced-3 mutants produced hsp 70 even at normal temperature, which was further increased in heat shock.

Hsp 90(81) is present in the wild type in very small amount and it is strongly induced after heat shock, same way as in ced-3 mutant.

Small heat shock proteins (hsp 16-18) — which are related to the alfa-chrystallin family -- are a bunch of totally inducible proteins in the wild

type and ced-3 mutants, but some of them are present in the ced-4 and ced-9 mutants already at normal temperature. The different proteins could be different due to phosphorylation state.

Using different monoclonal hsp 70 antibodies we have tried to detect hsp 70 in C. elegans not only in homogenized protein samples after Western blots but in intact worms as well (Fig. 2).

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RAPID DETECTION OF STAPHYLOCOCCUS SAPROPHYTICUS USING PRIMER SPECIFIC PCR*

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Staphylococcus saprophyticus is one of the most frequently encountered clinically significant members of the coagulase-negative staphylococci. A set of species-specific PCR primers was defined for the detection of Staphylococcus saprophyticus. These primers target variable regions (V3 and V6) of the 16S rRNA gene. Primer-specific PCR has potential applications in epidemiological studies and diagnosis of Staphylococcus saprophyticus.

Keywords: Coagulase-negative staphylococci — Staphylococcus saprophyticus — 16S rRNA

Introduction

Coagulase-negative staphylococci (CNS) are abundant in the normal flora of skin and mucous membranes /8, 9/. CNS represent one of the most common contaminants in hospitals /7/. The ability of CNS to cause clinically significant infections is becoming increasingly apparent. Staphylococcus saprophyticus is one of the most frequently encountered clinically significant coagulase-negative organism /2, 3, 5, 6, 11, 12/.

Currently, ribosomal RNA (rRNA) analyses are the most powerful methods available for determining phylogenetic relationships as well as for identification of microorganisms /4, 10/. In recent years, there has been great interest in PCR-based tests which use the small subunit 16S rRNA gene (16S rDNA) to detect bacterial pathogens. Some regions of the 16S rRNA molecules are highly conserved. However, other regions of the 16S rRNA's are hyper-

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variable. Oligonucleotides complementary to the highly variable regions of the 16S rDNA can be used as a specific PCR primers for unique detection of bacterial species.

The present study was undertaken to develop, optimize, and evaluate a primer-specific PCR for detection of S. saprophyticus.

Results and Discussion

In order to design specific PCR primers for detection of S. saprophyticus, the complete sequence of the S. saprophyticus 16S rDNA was determined (GenBank, L20250). Comparison of the S. saprophyticus sequence with those of other CNS 16S rDNA's revealed small but consistent sequence differences within the variable regions, V3 and V6, to design a set of specific primers for detection of S. saprophyticus (Table 1). Thus, two oligonucleotides complementary to the selected sequences were synthesized and named pSV3 and pSV6. To determine whether the primer set pSV3-pSV6 can be used for unique detection of S. saprophyticus, PCR was performed with DNA isolated from 32 CNS species. DNA was isolated directly from a single colony grown on an agar plate. A bacterial colony was suspended in PCR reaction mixture and after 15 min of incubation at 100 ^{o}C, 2.5 U of Taq DNA polymerase (Boehringer) were added. DNA amplification was carried out as described in Fig. 1. Amplification product (605 bp) was observed following PCR of S. saprophyticus and five other CNS staphylococci: S. cohnii, S. equorum, S. haemolyticus, S. simulans and S. xylosus. According to Kloos et al. /9/, S. cohnii, S. equorum and S. xylosus belong to the same S. saprophyticus species group. The sequence of the pSV6 primer was identical to the homologous region of 16S rRNA of S. equorum and S. xylosus. However, the sequence of the second primer, pSV3, differed by two bases from the homologous region of 16S rRNA of these two species. In order to improve the specificity of the PCR, the temperature of primer annealing was increased (Fig. 1). Thus, under a stringent temperature (65 ^{o}C) of primer annealing, the amplification product was only observed following PCR of S. saprophyticus (Fig. 1). The results obtained by primer-specific PCR under the stringent conditions were reproducible (data not shown). The applicability of the primer-specific PCR for the identification of clinical isolates of S. saprophyticus is currently under investigation.

Table 1

Alignment of the V3 and V6 variable regions of staphylococcal 16S rDNA sequences

Species	V3 region	V6 region	Source*
S. saprophyticus	5' GAGTG**ATGAAGGGTTTCGGCTCG**TAAAACT 3'	5' TTGA**CATCCTTTGAAAACTCTAGAG**ATAG 3'	L20250
S. cohnii	----------TC-----A----------	----------C----------	D83361
S. equorum	----------T------A----------	----------	Z26895
S. haemolyticus	----------TC-----A----------	----------C----------	X66100
S. simulans	----------TC-----A----------	----------C----------	D83373
S. xylosus	----------TC-----A----------	----------	D83374
S. auricularis	----------TC-----A----------	----------CCG----------	D83358
S. caseolyticus	------A-----T------A----------	----------C----------	D83359
S. chromogenes	----------TC-----A--------G-	--------T-----CC----------	D83360
S. epidermidis	----------TC-----A----------	----------C---CCC----------	L37605
S. felis	----------TC-----A--------G-	----------CCG----------	D83364
S. hominis	----------TC-----A----------	----------CCCT----------	X66101
S. hyicus	----------TC-----A--------G-	--------T-----TCG----------	D83368
S. lentus	----------TC--A--A----------	----------TCG----------	D83370
S. pulvereri	----------TC--A-A----------	----------TCG----------	U12764
S. schleiferi	----------TC-----A--------G-	----------CCG----------	D83372
S. sciuri	----------TC-----A----------	----------CCG----------	D83569
S. simulans	----------TC-----A----------	----------CAA----------	D83373
S. warneri	----------TC-----A----------	----------CCG----------	L37603

The sequences of the specific primers are boxed.

*accession number (GenBank)

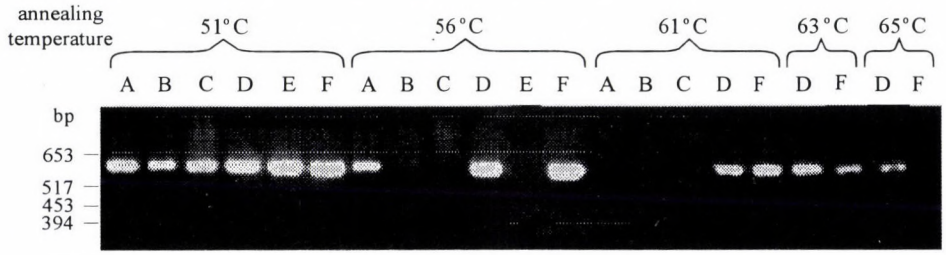


Fig. 1. Primer-specific PCR. The PCR was performed using the pSV3 and pSV6 primers. The reaction mixture (100 µl) contained 1 µM primers, 100 ng of DNA template, all four deoxynucleotide triphosphates at 0.2 mM each, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin and 2.5 U Taq polymerase (Boehringer). Lines: A: S. cohnii, B: S. equorum, C: S. haemolyticus, D: S. saprophyticus, E: S. simulans, F: S. xylosus

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GENETIC AND DEVELOPMENTAL ANALYSIS OF MUTANT *KETEL* ALLELES THAT IDENTIFY THE *DROSOPHILA* IMPORTIN-β HOMOLOGUE*

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The *Ketel* gene of *Drosophila melanogaster* was identified by four $Ketel^D$ dominant female-sterile mutations and their 27 revertants. The X-ray and the P-induced $Ketel^R$ alleles delineated the *Ketel* locus to the 38E1.2-3 cytological position. Although oogenesis proceeds normally in the $Ketel^D/+$ females, embryogenesis comes to a deadlock shortly after fertilization inside the normal-looking eggs of the $Ketel^D/+$ females. The $Ketel^D$ alleles are dominant negative mutations of antimorph type. Cytoplasm of the $Ketel^D/+$-derived eggs induce lesions when injected into wild-type eggs and the $Ketel^D$ alleles can be reverted. Zygotes homozygous for loss-of-function (revertant) $Ketel^R$ alleles die in second larval instar. Analysis of the cold-sensitive *Ketel* alleles and the genetic interactions between importin-α and $Ketel^R$ mutant alleles indicate an involvement of the *Ketel* gene product in oo-, embryogenesis and larval life and show interaction of the KETEL protein with different components of nuclear processes. Molecular analysis (to be published elsewhere) confirmed the genetic data and revealed that the *Ketel* gene encodes the *Drosophila* homologue of importin-β, an essential component of nuclear protein import.

Keywords: Nuclear protein import — maternal effect — female sterility — zygotic lethality — *Drosophila*

Introduction

Genetic dissection of the initiation of embryogenesis implies first identification and understanding the function of single components and the subsequent reconstruction of the process. Since there is little if any transcription form the embryonic genome during early cleavage divisions in *Drosophila*, most components required for the initiation and early steps of embryogenesis are maternally provided /28/. It is to be expected that genes

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encoding the maternal components can be identified by maternal-effect lethal mutations, a special type of female sterile (Fs) mutations that allow egg production but interfere with embryogenesis. The true maternal effect genes are rare. It is rather common that early embryogenesis is governed by genes that have both maternal and zygotic functions and, in fact about 90% of the fs mutations are weak (hypomorph) alleles of genes with essential zygotic functions /18, 24/. Loss of function mutations in genes with both maternal and zygotic functions result in lethality and hence do not allow readily analysis of the maternal function.

Genes with both maternal and zygotic functions may be identified by dominant female sterile (Fs) mutations that disrupt maternal but permit zygotic functions. We have identified 50 genes of Drosophila by 75 Fs mutations /5, 25/. Thirty-three of the Fs mutations, that identify altogether 23 genes, bring about termination of embryogenesis soon after fertilization. The Ketel gene, one of the 23, appears to be a key component in the initiation of the embryogenesis: development is arrested in eggs of the $Ketel^{D}/+$ females at the first zygotic division due to lack of nuclear membrane formation. It may be suggested, based on the $Ketel^{D}$ mutant phenotype, that the normal Ketel gene product is required for a nuclear membrane related function at the onset of embryogenesis. Revertants of the $Ketel^{D}$ alleles are zygotic (larval) lethal mutations. The $Ketel^{R}$ homozygotes perish without any characteristic morphological defects providing no clues about function of the normal Ketel gene product. We describe in this paper a combined genetic and developmental analysis of the Ketel mutant alleles. We conclude that the Ketel gene product maternally provided and is required during both embryogenesis and larval life. We propose that the Ketel gene product is required to a nucleus related function and confirm the molecular data: the Ketel gene product is involved in nuclear protein import.

Materials and Methods

The mutant Ketel alleles

Three Fs(2)Ketel (=$Ketel^{D}$) alleles were isolated in the screen for dominant female-sterile mutations /5/. Fs(2)Hont is allelic with Ketel and will be labelled $Ketel^{D4}$ henceforth. For isolation of recessive $Ketel^{R}$ alleles, either the dominant $Ketel^{D}$ alleles or $Ketel^{RS1}$ were reverted by EMS, X-rays or the P-element, a type of Drosophila transposons. $Ketel^{RS1}$ is a spontaneous allele possessing dominant cold sensitive lethality. Revertants induced by P-element were generated by crossing dysgenic $Ketel^{RS1}$/SM5

Table 1

List of the Ketel mutant alleles

Dominant $Ketel^D$ alleles	Marker mutations on the founder chromosome	Revertant $Ketel^R$ alleles induced by Spont. (RS)	EMS (RE)	X-rays (RX)	P-element (RP)
$Ketel^{D1}$	1t bw	RS1	RE11	RX1 - RX3 RX10 - RX19	RP11 - RP13
$Ketel^{D2}$	none			RX21, RX22	
$Ketel^{D3}$	1t bw		RE31-RE34	RX31, RX32	
$Ketel^{D4}$	none			RX41	

Notes:

- $Ketel^{RE34}$ is a cold sensitive revertant allele possessing dominant female sterility on 29 °C.
- $Ketel^{RS1}$ is a dominant cold-sensitive lethal.
- The $Ketel^{RX10-RX19}$ and the $Ketel^{RP11-RP13}$ alleles were generated through reversion of the $Ketel^{RS1}$ — associated dominant cold-sensitive lethality.
- $Ketel^{RE31}$ is a recessive cold-sensitive lethal allele.
- Four of the $Ketel^{RX}$ revertant alleles are deficiencies: $Ketel^{RX10}$: 38E1.2-3; 38F1-39A1.2. $Ketel^{RX21}$: 38C5.6-10; 39A1.2-3. $Ketel^{RX22}$: 38B6-C1.2; 38F6-39A1.2. $Ketel^{RX32}$: 38D1.2-3; 38E4-5.6.
- Three of the $Ketel^{RX}$ revertant alleles are (2;3) translocations in which a third chromosome segment become juxtaposed to a second chromosome segment with a common break point in 38E1.2-3, delineating the Ketel locus. The corresponding third chromosome break points are as follows. $Ketel^{RX2}$: 89C. $Ketel^{RX17}$: 75D. $Ketel^{RX19}$: 62B.

males with CyO/Pm females on 18 °C. The $Ketel^{RS1}$-carrying as well as the SM5/CyO and the SM5/Pm zygotes die and thus only the $Ketel^R$/SM5 and the $Ketel^R$/Pm flies survive. The $Ketel^R$ recessive loss-of-function alleles are kept as zygotic lethals (see Table 1). If otherwise stated, the experiments were conducted on 25 °C. For explanation of the genetic symbols throughout the paper (see /14/).

The dominant mutant phenotypes

The $Ketel^D$ dominant phenotype was established by immunostaining and confocal microscopy. The spindle apparatus was made visible by a monoclonal mouse anti-tubulin primary (Amersham) and an anti-mouse fluorescein-labelled secondary antibody (Jackson Labs. Inc.). DNA was stained by propidium iodine. Optical sections were made by a Zeiss confocal microscope in freshly deposited eggs of both wild-type and $Ketel^D$/+ females.

The recessive mutant phenotypes

To establish the loss-of-function mutant phenotypes, y/y; $Ketel^R$/y+CyO females were mated with y/Y; $Ketel^R$/y+CyO males. The y+CyO balancer chromosome carries a y^+ transgene (A. Shearn, personal communication). The descending y/y (or y/Y); $Ketel^R/Ketel^R$ zygotes are yellow and can be separeted from the heterozygous non-yellow (y+CyO-carrying) siblings. Eggs and embryos were prepared for microscopic analysis following standard procedures /29/. The $Ketel^D$ alleles were EMS induced and thus the founder chromosomes are likely to carry second site lethal mutations /25/. Additional second site lethal mutations might have originated during the second mutagenesis to revert the $Ketel^D$ or the $Ketel^{RS1}$ alleles. Since presence of common second site lethal mutation on different founder chromosomes is very unlikely, revertants of different $Ketel^D$ alleles were used to determine the loss-of-function phenotype.

The $Ketel^D$/+/+ females

To determine whether the $Ketel^D$ alleles are (a) antimorph and disturb the same process in which the wild-type allele functions, or (b) neomorph and interfere with a different pathway, we constructed $Ketel^D$/+/+ females by crossing XX/Tp(2;Y)G; Df(2L)Sd68/CyO females with X/Y; $Ketel^D$/ScO males. The Tp(2;Y)G transposition spans from 36C1 to 40F and covers both the Ketel and the purple loci (in 38E0.2-3 and 38B1-B2, respectively /14/). The Df(2L)Sd68 deficiency spans from 37B3-7 to 38E3-5 and uncovers the Ketel as well as the purple loci. As determined from a cross between XX/Tp(2;Y)G; Df(2L)Sd68 pr/CyO, pr females and X/Y; pr/pr males, 39% of the CyO, pr/pr females are XX/Tp(2;Y)G and thus 39% of the $Ketel^D$/CyO females is expected to be XX/Tp(2;Y)G; $Ketel^D$/CyO, i.e. $Ketel^D$/+/+ from the above cross.

Genetic interaction Ketel and importin-α mutants

To elucidate the possible function of the Ketel gene product in nuclear protein import we tested genetic interaction between the Ketel and the importin-α alleles. Importin-β has been known to interact physically and form active receptor for nuclear localisation signal /6, 17, 21/. The Drosophila importin-α (also called pendulin) mutant alleles are available /13, 26/. Analysis of dominant genetic interaction was performed with all Ketel and mutant alleles of the importin-α gene (see Table 2). Viability, female and male fertility of the Ketel+/+ importin-α flies were studied.

Results

The $Ketel^D$ dominant phenotype

The $Ketel^D$/+ females are fully viable. They deposit normal numbers of normal-looking eggs. The development of egg primordia (as followed by anti-tubulin antibody, DNA staining and confocal microscopy) appears normal in the $Ketel^D$/+ females. Cytological analysis clearly showed that both the

Table 2

The Ketel ↔ importin-α interaction

Ketel alleles	Importin 60 alleles															
	144/1		D1		D3		D10		D14		D18		D19		D21	
	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
$Ketel^{D1}$	St		St		St		St		St		St		St		St	
RE11						↓										
RX1										↓↓↓				↓		
RX10		↓										↓				
RX11		↓										↓				
RX13										↓						
RX16		↓		↓												
RX17														↓		
RX18				↓												
RP11		↓														↓
RP12		↓↓														
RP13				↓↓		↓↓								↓		↓
$Ketel^{D2}$	St		St		St		St		St		St		St		St	
RX21		↓						↓								
RX22		↓↓↓		↓						↓		↓				↓↓↓
$Ketel^{D3}$	St		St		St		St		LETH.		St		St		St	
RE31						↓						↓				
RE32								↓								
RE34					St		St		St							
RX31				↓↓↓								↓		↓		
RX32						↓		↓↓		↓						
$Ketel^{D4}$	St	↓	St	↓	St		St	↓	St		St		St		St	

Notes:

— F = female, M = male.

— Only the interacting and the female sterile (St) combinations are shown.

— The $Ketel^{RE34}$ +/+ D3, $Ketel^{RE34}$ +/+ D10 and $Ketel^{RE34}$ +/+ D10 females deposit normal numbers of normal looking eggs without any sign of embryogenesis inside.

— ↓, ↓↓ and ↓↓↓ = reduced fertility. No quantitative evaluation was made. Of the tested (>10) males several were sterile, and only a variable proportion of the males gave rise to a few offspring each.

first and the second meiotic divisions are indistinguishable from wild type in the $Ketel^{D}$/+ females. Essentially every egg of the $Ketel^{D}$/+ females is fertilized as revealed by the presence of sperm tyil in the egg cytoplasm. The first visible deviation from wild type embryogenesis becomes apparent at the time of gonomeric division (Fig. 1): the failure of nuclear membrane formation in the $Ketel^{D}$-derived eggs leads to lack of daughter centrosome separation, abnormal microtubule organization and chromosome segregation

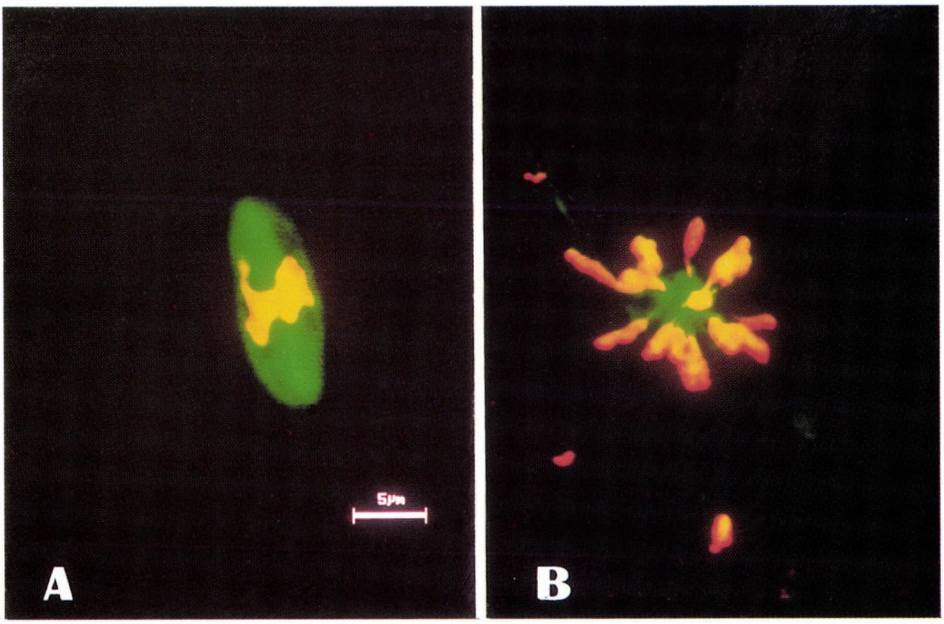


Fig. 1. **A:** A wild type first mitotic (gonomeric) metaphase spindle. **B:** Abnormal arrangement of the first mitotic apparatus in an egg of a Ketel^{D1}/+ female. Merged confocal micrographs in which tubulin and the chromosomes are labelled with green and red, respectively. Overlaying green and red signals appear yellowish-orange

that is followed by chromatin degradation, destructed embryogenesis and an eventual decay of the egg content (Fig. 1 /6/).

The Ketel^{D} alleles are of gain-of-function nature

Three lines of evidence verify the involvement of mutant KETEL gene product in origin of the Ketel^{D} mutant phenotype.

(a) Cytoplasm of the Ketel^{D1} females is toxic. Following injection of 0.2 pl cytoplasm from eggs of Ketel^{D1}/+ females into wild type eggs, the developing 59 larvae did not have the appropriate head structures around the site of injection. /The cytoplasm was collected from the 75% egg length region and injected into identical location of wild type eggs that were less than 30 min "old". The 75% egg length region gives rise to characteristic landmark head sense organs /11/./ Of the 72 control larvae, that developed following injections of wild type cytoplasm into wild type embryos, only 5 showed head defects. The entire anterior parts of the embryos were missing following injection of about 1 pl mutant cytoplasm/wily type egg (44 eggs).

(b) The Ketel^{D2} and the Ketel^{D4} alleles are antimorph: the mutant phenotype may be "cured" by increasing the normal gene product ratio to the mutant. We generated and analyzed Ketel^{D}/+/+ /in fact XX/Tp(Y;2)G; Ketel^{D}/CyO) females/. As described in Materials and Methods, 39/ of the descending Ketel^{D} females are XX/Tp(Y;2)G; Ketel^{D}/CyO, i.e. Ketel^{D}/+/+. There were 38, 56, 19 and 37 females individually tested, respectively in case of the four Ketel^{D} alleles. Although embryos died, visible cuticular structures developed in about 1% of the eggs deposited by the Ketel^{D2}/+/+ females, a phenomenon never observed in eggs of the Ketel^{D2}/+ females. Only fragments of the larval cuticle were present in the dead embryos. Every of the cuticle landmark structures could be identified, albeit in different embryos indicating an unspecific and widespread cell death /10, 11/. A few (1-8 throughout their life) offspring derived from 13 (23%) of the 56 Ketel^{D2}/CyO females. Outcrosses clearly showed the presence of the Tp(Y;2)G chromosome in 7 of the 13 offspring-yielding females. A few dead embryos ($<<$ 1%) developed in eggs of the Ketel^{D4}/+/+ females, showing the antimorph nature of the Ketel^{D4} allele. Embryos did not develop to the stage of cuticle differentiation in eggs of the Ketel^{D1}/+/+ and the Ketel^{D3}/+/+ females.

Experiments with the Ketel transgene (labelled +) clearly show that the Ketel^{D1} and Ketel^{D3} are also antimorph: the Ketel^{D1}/+/+/+ and the Ketel^{D3}/+/+/+ females produce a few offspring (Szabad, J. unpublished results). The antimorph nature of the Ketel^{D} alleles clearly show that the (a) mutant and normal KETEL proteins participate in the same process and (b) the KETEL protein molecules interact with either KETEL and/or other proteins.

(c) Every of the four Ketel^{D} alleles can be reverted during second mutagenesis. Altogether 11 Ketel revertant alleles were isolated following mutagenesis of the Ketel^{D} alleles by EMS or X-rays (Table 1, /5/). The revesion frequency of the Ketel^{D} alleles is about 10^{-3} upon 25 mM EMS or 4000 R of X-rays. A spontaneous dominant cold-sensitive lethal revertant allele (Ketel^{RS1}) was also recovered (Table 1). The dominant cold sensitive lethal nature of Ketel^{RS1} allowed convenient recovery of further Ketel^{R} revertant alleles by first mutagenizing the Ketel^{RS1} allele and screening subsequently for revertants on 18 ^{o}C, the restrictive temperature. The Ketel^{RX10} - Ketel^{RX19} and Ketel^{RP11} - Ketel^{RP13} alleles are revenants of the dominant cold-sensitive lethal phenotype (Table 1).

The Ketel^{RX} revertants delineated the Ketel locus into the cytological position 38E1.2-3

Salivary gland chromosomes were prepared and analyzed from all of the Ketel alleles. Seven (all X-ray induced) of the 27 Ketel^{RX} alleles are associated with visible chromosomal rearrangements (Table 1). Four alleles (Ketel^{RX10}, Ketel^{RX21}, Ketel^{RX22} and Ketel^{RX32}) are deficiencies and eliminate the Ketel as well as a few adjacent loci. Three in the Ketel^{RX} alleles (Ketel^{RX2}, Ketel^{RX17} and Ketel^{RX19}) are associated with a common break point in the 38E1.2-3 cytological region. In situ hybridizations detected P-element insertion in 38E1.2-3 in every of the three P-induced Ketel^{RP} alleles. Four Ketel^{RX} and the three Ketel^{RP} alleles delineated the Ketel locus into the 38E1.2-3 cytological position and were of great help in molecular cloning of the Ketel gene /16/.

Zygotic requirement of the Ketel gene function

Zygotes trans-heterozygous for Ketel^{R} alleles and the Ketel^{R}/- hemizygotes were generated by crossing y/y; Ketel^{R}/y^{+}CyO or y/y; Ketel^{RX32}/y^{+}CyO females with y/Y; Ketel^{R}/y^{+}CyO males. (Ketel^{RX32} is a small deficiency removing the Ketel and few adjacent loci.) Most of the Ketel^{R}/Ketel^{R} as well as the Ketel^{R}/- combinations are second larval instar lethals, suggesting that second larval instar death is the complete loss-of-function (amorph) phenotype. The Ketel^{R}/- larvae reach second instar with minor delay, live for about one additional day, become sluggish and die without any characteristic morphological defects. Death of the mutant loss-of-function Ketel^{R} zygotes clearly shows the requirement of the normal Ketel gene product during zygotic life.

The -/Ketel^{D} larvae, from crosses between y/y; Ketel^{RX32}/y^{+}CyO females and y/Y; Ketel^{D}/y^{+}CyO males, also die in second larval instar and look alike the Ketel^{R}/- larvae. The identical phenotypes of the Ketel^{R}/- and Ketel^{D} zygotes show that the Ketel^{D} encoded mutant gene product (a) cannot support zygotic development and that (b) it is, once again, involved in the same process as the normal Ketel gene product.

The temperature-sensitive Ketel alleles reveal requirement of the Ketel gene product during oogenesis and pupariation

Three of the Ketel alleles process cold sensitivity (Table 1). The Ketel^{RS1} allele possesses dominant cold-sensitive lethality. The Ketel^{RE34} allele is a recessive cold-sensitive revertant. The Ketel^{RE31} allele shows recessive cold-sensitive lethality. Temperature shift experiments with the above alleles may reveal stages of development when the Ketel gene product is required.

Dominant cold-sensitive lethality of Ketel^{RS1}

To determine the cold-sensitive period of the Ketel^{RS1}/+ zygotes, wild-type (+/+, Oregon-R) females were mated with Ketel^{RS1}/CyO males. In the shift up experiment the descendants first spent 2, 4, 6 etc. days on the restrictive (18 ^{o}C) and continued development on the permissive temperature (25 ^{o}C). In the shift down experiment the progeny first spent 1, 2, 3 etc. days under permissive (25 ^{o}C) and were subsequently subjected to restrictive conditions (18 ^{o}C). (Note that egg to adult development takes 9 and 24 days on 25 and 18 ^{o}C, respectively; Fig. 2). Viability of the +/Ketel^{RS1} progeny, as compared to the sibling +/CyO zygotes, is shown on Fig. 2A. It is apparent that the cold-sensitive period of the Ketel^{RS1}/+ zygotes is during the larva-pupa transition period, indicating requirement for Ketel gene function during pupariation. When exposed to restrictive conditions, the Ketel^{RS1}/+ zygotes accomplish both pupal and adult moults and perish without any apparent visible defect.

Recessive cold-sensitive lethality of Ketel^{RE31}

The Ketel^{RE31}/- (where - stands for a deficiency) zygotes are fully viable on 25 ^{o}C, indicating that Ketel^{RE31} can support zygotic development in hemizygous condition and is apparently a partial revertant. However the Ketel^{RE31}/- zygotes die as pupae on 18 ^{o}C: the Ketel^{RE31} allele possesses recessive cold sensitive lethality. To determine the cold-sensitive period of Ketel^{RE31} allele, we crossed Ketel^{RX13}/CyO females with Ketel^{RE31}/CyO males and determined the survival rate to adulthood of the Ketel^{RE31}/- zygotes (Ketel^{RX13} is null allele). In the shift down experiment, eggs were collected on 25 ^{o}C in one day periods, eggs and the enclosed larvae were transferred to 18 ^{o}C after 1, 2, 3 etc. days. The relative survival rate of the Ketel^{RE31}/- zygotes was determined as compared to the 25 ^{o}C values.

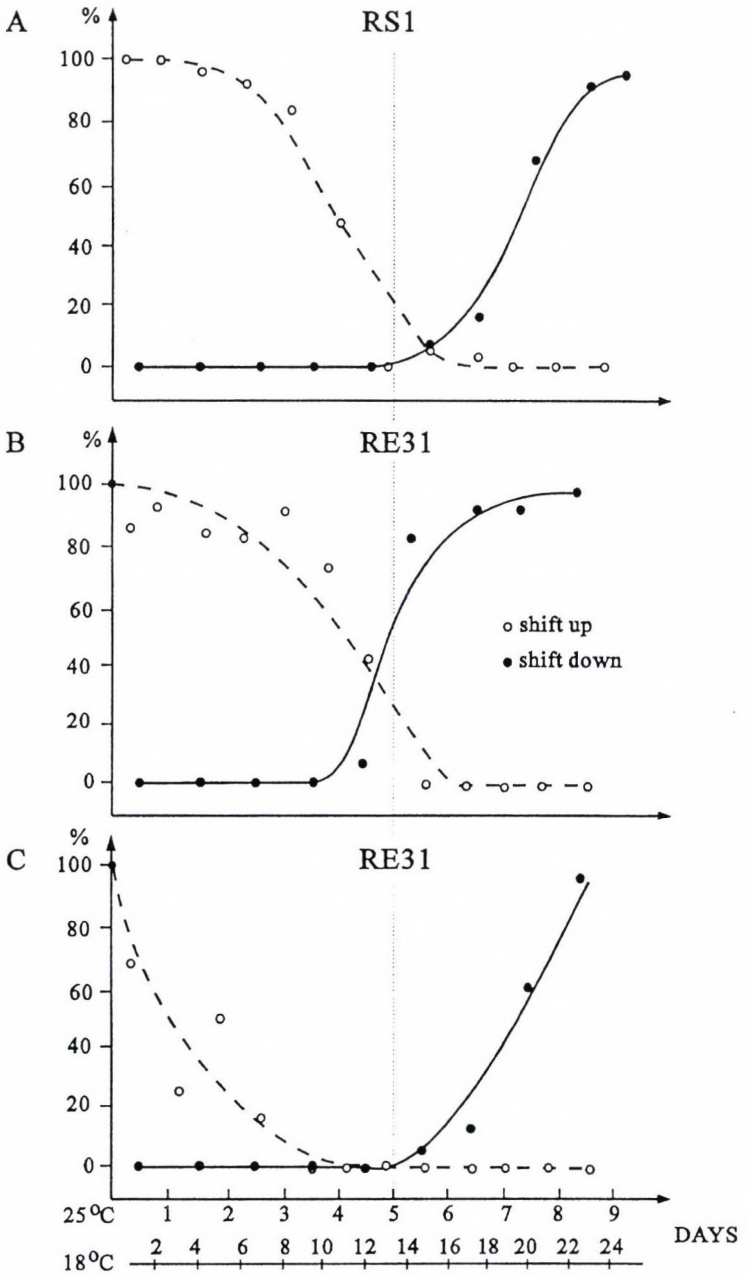


Fig. 2. Results of the temperature shift experiments. The relative survival rate, as compared to survival rate on the permissive temperature, in shift down (open circles) and shift up (dots) experiments. **A:** Ketel^{RS1}/+; **B** and **C:** Ketel^{RE31}/-. In **B** and **C** the Ketel^{RE31} allele was of paternal and of maternal origin, respectively. (See text for details.) The vertical dotted line shows the time of pupariation

As shown in Fig. 2B, the cold-sensitive period is confined to the time of pupariation: the Ketel^{RE31}/- zygotes that pupariate on 18 °C die as pupae. In the shift up experiment, eggs were collected on 18 °C in two day intervals and shifted to 25 °C after, 2, 4, 6 etc. days. The shift up experiment also defined the time of pupariation as the cold sensitive period (Fig. 2B).

In the previous experiment the Ketel^{RE31} allele was of paternal origin. In the reciproc cross, where the Ketel^{RE31} allele derived from the females and Ketel^{RX13} from the males, the shift down and shift up experiments clearly showed a maternal effect associated with the Ketel^{RE31} allele (see Fig. 2C). In the shift down experiment, the maternally-supplied mutant Ketel^{RE31} gene product extended the cold sensitivity.

Although the Ketel^{RE31}/- females are viable on 25 °C, they are sterile. They deposit normal-looking eggs that never hatch. (Cuticle analysis of the dead embryos revealed widespread and random cell death as seen in eggs of the Ketel^{D2}/+/+ females.) About 7% of the eggs deposited by the Ketel^{RE31}/- females on 25 °C possess weak ventralization: the dorsal chorionic appendages are shorter than normal and fused at the base /22/. After shifting the Ketel^{RE31}/- females to 18 °C, the restrictive temperature, an increasing fraction of the eggs were ventralized. Beyond day 18, almost 100% of the eggs were strongly ventralized, showing that the Ketel^{RE31} enconded mutant gene product interferes with establishment of the dorso-ventral egg polarity.

Ketel^{RE34} possess heat-sensitive female sterility

Under permissive conditions (25 °C), larvae hatch from about 67% of the eggs of the Ketel^{RE34}/+ females, indicating that Ketel^{RE34} is a partial revertant. (Cuticles of the dead embryos were very similar to those descended from the Ketel^{D2}/+/+ and the Ketel^{RE31}/+ females and, once again, revealed extensive unspecific cell death.) To determine the temperature-sensitive period of the Ketel^{RE34}-related Fs phenotype, we raised Ketel^{RE34}/+ females on 25 °C and mated them with wild-type males. The parents were shifted to 29 °C. Eggs were collected in 8 hours intervals and the ratio of the non-hatching eggs was determined. As illustrated in Fig. 3A, larvae hatched only from a few percent of the eggs already after one day on 29 °C. Cuticle was present in a decreasing fraction of the eggs (Fig. 3B). The original Ketel^{D} mutant phenotype (lack of embryogenesis) developed in essentially all the eggs beyond the second day on 29 °C, showing that the mutant Ketel gene product forms already during the last stages of oogenesis and persists in the egg cytoplasm.

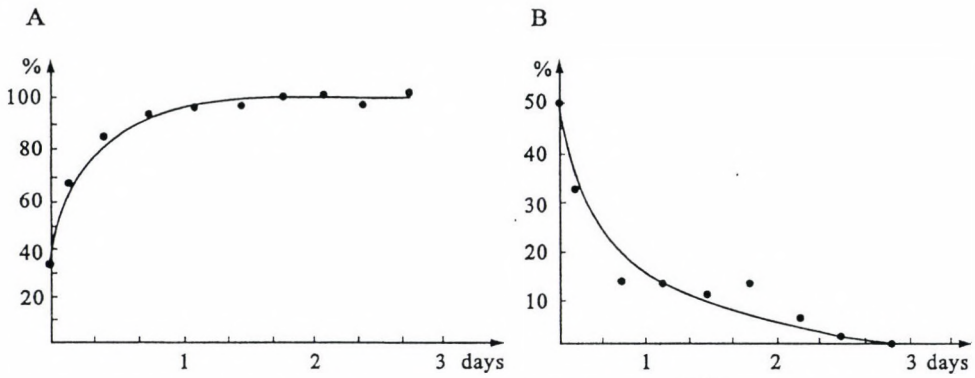


Fig. 3. A: Death rate (%) of the embryos descending from Ketel ^{RE34}/+ females after transferring the females to 29 °C, the restrictive temperature. B: Fraction of the cuticle-producing embryos among those shown in Fig. 3A

Genetic interactions suggest that Ketel gene functions in the nuclear protein import

The Ketel mutant phenotypes suggested the involvement of the Ketel gene in nuclear membrane related process(es). There are 8 importin-α mutants available, mutant alleles that identify the importin-α gene, an essential component of nuclear protein import /26/. We constructed trans-heterozygous conditions including the 8 importin-α as well as the 4 Ketel^{D} and the 27 Ketel^{R} alleles and studied viability and fertility of the offspring females and males. Results of the analysis are summarized in Table 2. All the 162 Ketel^{R}+/+ importin-α double heterozygous combinations are fully viable. The Ketel^{RE34} allele shows dominant interaction with three of the importin-α alleles D3, D10 and D14: the double heterozygous females are sterile. They deposit as many eggs as the wild-type females. Although the eggs look normal, embryogenesis never commences inside the eggs. In 32 of the 162 Ketel+/+ importin-α double heterozygous combinations strong reduction of male fertility was apparent: most of the otherwise vigorous males were sterile and only few of them yielded a few offspring over the two week test period (Table 2). The Ketel^{R} ↔ importin-α dominant interactions substantiate the involvement of the Ketel gene product in nuclear protein import.

Discussion

The Ketel gene product is involved in nucleus related process(es)

Products encoded by the $Ketel^{D}$ mutant alleles hinder the initiation of embryogenesis by disrupting assembly of the nuclear membrane, suggesting function of the normal Ketel gene product in a nuclear membrane related process. Similar phenotype, i.e. failure of nuclear membrane assembly was also reported in two systems. 1. The piml-dlts mutation of the fission yeast Schizosaccharomyces pombe prohibits reestablishment of the nuclear membrane following mitosis /4, 12/. The piml gene encodes RCC1, regulator of chromatin condensation, the guanine nucleotide exchange factor for Ran /19/. 2. T24N-Ran, a dominant negative mutant allele of Ran, encodes a protein that profoundly disrupts nuclear membrane assembly in the Xenopus laevis egg extract system /3/. Ran, a Ras-related protein, is a GTP-ase that interact with RCC1 and is required in nuclear processes (for recent reviews see /6, 17, 21/).

Additional support for the nuclear-related function of the Ketel gene product comes from the observation that the $Ketel^{RE31}/-$ hemizygous females produce ventralized eggs under restrictive conditions. Ventralized eggs form (a) following the elimination of oocyte nuclei, through UV-extirpation, during stages 6-9 of oogenesis /16/ and also following the elimination, by mutations, the gurken-torpedo signalling system /1, 9, 22/.

That the KETEL protein may be involved in nuclear protein import, a nucleus-related process, was indicated by the dominant genetic interaction between the Ketel and the importin-α mutant alleles: 35/216 of the $Ketel^{R}+/+$ Importin-α double heterozygous combinations possess female sterility and/or reduced male fertility. /Dominant genetic interaction has been considered as an indicator of the involvement of the interacting genes in the same genetic pathway /9/./ Importin-α is the receptor for the nuclear localization signal /8, 19, 20, 26/ and the Ketel gene encodes the Drosophila homologue of importin-β that, together with importin-α, assist nuclear protein import /6, 17, 21/. Indeed, molecular analysis of the Ketel gene showed a 61% amino acid identity and an 81% similarity between human importin-β and the KETEL protein /15/.

The Ketel gene is expressed during oogenesis, is required for the commencement of embryogenesis and during pupariation

The $Ketel^{R}/Ketel^{R}$ (as well as the $Ketel^{R}/-$) die as second instar larvae, demonstrating zygotic requirement of the Ketel gene during larval life. In addition, shift up and down experiments with the temperature-sensitive $Ketel^{RS1}$ and $Ketel^{RE34}$ alleles clearly show requirement of the Ketel gene product around the larval-pupal transition period. The $-/Ketel^{D}$ zygotes, where $Ketel^{D}$ is of paternal origin, also die during second larval instar, indicating that (a) the $Ketel^{D}$ alleles behave as recessive zygotic lethal mutations and that (b) the $Ketel^{D}$ encoded gene product cannot support zygotic development. It is thus apparent that the $Ketel^{D}$ mutations identify maternal function of an otherwise zygotically required gene. We propose that alternatively splied Ketel mRNAs function during oo-, embryogenesis and larval life, and by eliminating — through mutations — different Ketel sub-functions, different mutant phenotypes originate.

The temperature sensitive Ketel alleles indicate conformational changes and/or protein-protein interactions

Three of the mutant Ketel alleles ($Ketel^{RE11}$, $Ketel^{RE31}$ and $Ketel^{RS1}$) are cold sensitive, suggesting conformational changes and/or interactions of the Ketel protein with other proteins. (It is generally accepted that cold-sensitive mutant phenotypes develop in the case of proteins that go through conformational changes; /2/). Importin-β, the Ketel homologue, has been shown to interact with the following components of the nuclear protein import machinery: importin-α, some components of the nuclear pore complex, Ran, p10 (a nuclear translocation factor) /6, 17, 21/. $Ketel^{RE34}$ possesses female sterility on 29 ^{o}C, when the conformational change is not prohibited. On 18 ^{o}C, the mutant protein cannot accomplish the conformational change and hence the females are fertile (dominant female sterility cannot be expressed). The non-temperature sensitive nature of reversion of dominant female sterility and simultaneous acquisition of dominant cold-sensitive lethality of the $Ketel^{RS1}$ allele reveal that the normal Ketel protein interacts with at least two different types of molecules. That the Ketel protein may indeed interact with different types of molecules is supported by two genetic interactions: one with the importin-α mutant alleles and a dominant suppressor mutation (on the third chromosome) of the $Ketel^{RS1}$-associated do-

minant cold sensitive lethality (Erdélyi, M., unpublished). The cold-sensitive Ketel alleles are likely to alter function of the interacting sites of the encoded protein, providing tools to identify molecularly essential functional sites in the Ketel protein.

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THE PHYSIOLOGY AND BIOSYNTHESIS OF SECONDARY METABOLITES*

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Recently, several excellent reviews appeared /9, 12, 15, 29/ summarizing new findings of the last years and describing the topic in the new interconnections. The signalling systems through which changes in environmental conditions affecting the growth of microorganism are sensed, transmitted and converted into mechanisms controlling the production of antibiotic can now be investigated with the techniques of molecular genetics. Evidence has been accumulated that demonstrated the key roles played by diffusible molecules in regulating cellular differentiation even among prokaryotic microorganisms. This is exemplified by A-factor and its analogues, which act as autoregulators for morphological differentiation and secondary metabolism in Streptomyces.

In our article we have concentrated on the physiological changes, which can occur during the growth in natural environment or during cultivation under laboratory conditions. Recent evidence for the presence of molecular signalling systems in Streptomyces is reviewed, along with the inherent implications. The constitution of the metabolic type during the first hours of cultivation has been previously reviewed /36/.

Keywords: Growth in natural environment — regulatory mechanisms — autoregulatory factors

Working hypothesis

1. Streptomycetes in natural environment probably never start to grow under optimal conditions.

2. The low rate of growth is accompanied by a very early synthesis of small amount of antibiotics which is however very important during the first hours of growth to protect the limited amounts of nutrients.

3. During transition from the exponential to the stationary phase of growth overlapping of different regulatory mechanisms occurs some of them are

*Dedicated to the memory of Professor Gábor Szabó.

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extending the growth and escaping the culture from the beginning of starvation. The others regulate the transition of cells into the stationary phase and sporulation.

4. Regulatory mechanisms characteristic for the end of exponential growth and responsible for overlapping the beginning of starvation are similar to mechanisms regulating the nutritional deficiency during germination and during first hours of growth when metabolic type is being constituted.

Introduction

Streptomycetes have characteristic biological aspects such as a mycelial form of growth that culminates in sporulation and the ability to form a wide variety of secondary metabolites including most of the antibiotics. Complex morphological development in these genera is phenotypically related to secondary metabolism. Both differentiation and secondary metabolism are probably responses to environmental conditions that must be performed so that a signal is transferred in succession to many genes in the complex regulatory relay. However the life cycle in natural environment was not studied in any detail and most of the claims of relation between the growth rate and production of secondary metabolites come from the laboratory experiments in submerged cultures on shakers.

The majority of papers focus the attention to metabolic events which are performed during transition from the exponential growth phase to the stationary phase when the exhaustion of some nutrition factors takes place and the induction of the synthesis of secondary metabolite starts.

Growth in natural environment

In nature microorganisms must have the ability to regulate metabolic activity in response to changing environment and this is a prerequisite for their survival. As the temperature, pH, oxygen level, nutrients vary the organism must optimize and integrate its function to suite new circumstances.

In general it can be assumed than bacteria have envolved complex interlocking regulatory networks that respond to nearly all possible environment conditions. Bacteria react to their environment by measuring extracellular

mitting that information to regulatory proteins revealing that most genes whose products provide fuel or precursors for cell growth are regulated in more than one way /19/.

Streptomycetes in their natural environment never start to grow under so-called "optimal conditions" which under laboratory conditions lead to the excessive growth. Restrictive growth in natural environment caused by unbalanced accessibility of primary metabolites for building new cells leads to a very early synthesis of antibiotics which have fundamental relevance in the competition with other soil comensales for limited amount of nutrients. There is nothing known about the amount of antibiotic compound produced, however, even small amount may have protective effect during the outgrowth of mycelia.

Growing streptomycetes form on the solid media colonies in which some of the short-term changes in the environment can be modulated or eliminated. Nevertheless it is assumed that soil bacteria during the growth elaborate continuously up to fifty signals.

Temperature

Soil microorganisms are well adapted to temperature changes which occur during alternation of day and night. Proteins which are formed during cultivation under laboratory conditions as a result of temperature changes are called heat-shock proteins (hsps). In their review Yura et al. /61/ show that the primary structure of the hsps appears to be highly conserved during evolution, suggesting that they serve similar function in all organisms. Transient induction of hsps represents an important protective and homeostatic mechanism to cope with physiological and environmental stress at the cellular level. The synthesis of a set of hsps is rapidly induced. The induction of hsps in E. coli occurs almost immediately after temperature shift, reaches its maximum at about 5 min and declines a new steady-state level in 20-30 min. The other very important observation was that most hsps are synthesized all the time, even under nonstress conditions, albeit at reduced rates and research indicated that hsps play fundamental roles in normal cell physiology.

The heat shock regulon is under σ^{32} control and includes genes for about 20 proteins. Most of major E. coli (the same holds for hsps of streptomycetes) have homologous eukaryotic counterparts (Dna K, Dna J, Grp E,

Gro EL, Gro ES and Htp G). Many of them are chaperons that assist correct folding and assembly of proteins and consequently affect diverse cellular processes including DNA replication. Recent proposal is that free pool of Dna K, Dna J, Grp E etc. (and its eukaryotic homolog) serves as a cellular thermometer that monitors changes in cellular concentration of unfolded or denaturated proteins and regulates the expression of all hsps in prokaryotes and eukaryotes /13/.

Guglielmi et al. /23/ in a set of 15 streptomycetes observed the induction of a small Hsp of 18 kDa and Servant and Mazodier /54/ reported the cloning and nucleotide sequence of one of the hsp 18 genes in Streptomyces albus. Disruption mutagenesis of hsp 18 showed that Hsp 18 is not essential for growth in the 30 to 42 ^{o}C temperature range. However, Hsp 18 is involved in thermotolerance at extreme temperatures.

In a work following the developmental control of the heat-shock stress regulation in liquid batch cultures in Streptomyces coelicolor in rapid growth 1, transition, rapid growth 2 and stationary phases Puglia et al. /50/ have shown that all heat shock proteins were highly dependent upon the growth phase.

Some hsps can be induced by other agent, including DNA-damaging agent, amino acid analogues, stringent response, starvation for carbon source, lambda phage infection, unfolded proteins, alkaline shift and ethanol, suggesting that hsps may protect cells against a variety of environmental stress.

Bacterial sigma factors

It is obvious that most of our concepts of a role of sigma factors have come from studies in E. coli and B. subtilis. In the eubacteria, transcription initiation requires specific proteins, known as sigma factors that bind reversibly to catalytically active core RNA polymerase to form a holoenzyme with the minimal composition $\beta\beta'\alpha_2\sigma$ (Table 1). Only holoenzyme can accurately initiate transcription at promoter sequences /35/.

In streptomycetes, RNA polymerase heterogeneity was first discovered by Westpheling et al. /58/. That RNA polymerase may play an unusually significant role in the adaptive mechanisms of streptomycetes is suggested by the striking complexity of the agarose gene (dag A) of Streptomyces coelicolor A3 /2/. The dag A gene is preceeded by four promoters with distinct -10 and -35 regions. RNA polymerases have been isolated that mediate in

Table 1

Functional differentiation of RNA polymerase core enzyme in E. coli

Core enzyme[a]	Sigma subunits	Holoenzyme	Transcribed genes
	σ^{70} <rpoD>	$E\sigma^{70}$	Log-phase genes
	σ^{32} <rpoH>	$E\sigma^{32}$	Heat-shock genes
$\alpha_2\beta\beta'$	σ^{24} <rpoE>	$E\sigma^{24}$	Extracellular protein genes
	σ^{54} <rpoN>	$E\sigma^{54}$	Nitrogen-regulated genes
	σ^{28} <rpoF>	$E\sigma^{28}$	Flagella-Chemotaxis genes
	σ^{38} <rpoS>	$E\sigma^{38}$	Stacionary-phase genes

[a]RNA polymerase core enzyme of E. coli binds one of the six different species of σ subunits with promoter recognition activity.

vitro transcription from dag AP2-RNA polymerase containing σ^{28} and dag AP3-RNA polymerase containing σ^{52} /7, 8/. By cloning genomic DNA fragments hybridizing with consensus sequences for sigma factor genes, Tanaka et al. /57/ isolated four separated genes, designed hrd A, B, C, D (homologs of rpo D genes from S. coelicolor A3(2)). A homologous portion with 13 amino acid in the rpo D genes was found in E. coli and B. subtilis and was named the "rpo D box" /57/. Disruption of hrd C and hrd D caused no marked change in phenotype. Whereas disruption of hrd B was apparently lethal. It was suggested that sigma factors product of hrd C and hrd D may support more specialized functions dispensable during normal growth /6, 8/.

The transcription of S. coelicolor dag A gene was studied also by Lonetto et al. /41/. The most upstream promoter Dag A-P4, was recognized by holoenzyme containing the major vegetative sigma factor, σ^{hrdB}, dag AP3 was recognized by E σ^{52} and Dag AP2 was transcribed by E σ^{28}. The Dag AP1 transcribing activity has not been characterized. The gene sig E, which encodes σ^{E} is involved in the transcription of dag AP2 was isolated σ^{E} (σ^{28}) is a member of distinctive subfamily of σ^{70} family of σ factors. These σS regulate genes encoding proteins with extracytoplasmatic functions - group of the ECP subfamily.

Differential expression of principal σ homologs of Streptomyces aureofaciens were described by Kormanec and Farkašovský /40/. The experiments described the presence of at least two tandem promoters directing expression of hrd A, hrd B and hrd D genes. The hrd E gene probably represents a new rpo D homolog. σ^{E} appears to control the last stage of Streptomyces differentiation -- spore maturation. Disruption of σ^{F} gene did not affect vegeta-

tive growth but did cause a whi mutant phenotype. In S. coelicolor one σ factor, σ^{whiG}, has been shown to be involved in differentiation /10/. whi G is epistatic to all other whi loci tested (whi A, whi B, whi H, whi I) and so plays a crucial role in triggering the initiation of sporulation /49/. We are however mainly interested in the transit of the exponential phase to the stationary phase. Several candidates have been suggested as signals for activation of the σ^S regulon. For example, subtle fluctuation in the ΔpH or $\Delta\psi$ may provide a common signal that regulatory sensors can transduce to cell's machinery, signifying starvation. The concentration of both ppGpp and AppppA drastically change as a result of a variety of stress including starvation. Another metabolite that accumulates towards the end of the logarithmic growth phase in some media and is subsequently metabolized is acetate. Although they are controversial, some reports suggest that acetate activates σ^S regulon /53/.

Regulatory mechanisms

One of the main signal mechanism is the "Two component signaling proteins" which transfer the environmental signals into the cell by using a pair of regulatory proteins - sensor transmitter protein which is localized in a cytoplasmic membrane and a cytoplasmic response regulator protein.

Sensor transmitter proteins are normally histidine protein kinases that autophosphorylate in their C-terminal transmitter domain after they receive the appropriate signal. Phosphate is transferred from ATP to the active site histidine. The phosphorylated protein then becomes the donor (transmitter) in a phosphotransfer reaction; the acceptor (receiver) is a cytoplasmic response regulator protein that forms a second member of the two-component phosphorelay pair.

Another important group of signal molecules is represented by nucleic acid-binding proteins which similarly as the two component system can be divided on some positively acting regulatory proteins which bind to DNA at recognition sites near the promoter and interact there with RNA polymerase to form an open complex initiating transcription and on a group of regulatory proteins which activates transcription by binding at an enhancer site well separated from the promoter.

For the regulation of the intracellular concentrations of primary metabolites and though mediatorially for biosynthesis of secondary metabolites

are also very important the negatively acting DNA binding proteins. The classical repressors of gene expression were first described in E. coli. These proteins bind to DNA sequences to/or overlapping a promoter recognized by E σ^{70}, so that the repressor makes direct contact with the polymerase and interferes with its function. Repressor of this type are commonly responsible for feedback control or primary biosynthetic pathways.

Negatively acting DNA binding proteins are used to suppress a wide range of metabolic activities that are redundant until changing physiological conditions make their functions appropriate. Some of the proteins involved in this type of regulation act only to suppress transcription, while others can either activate or repress depending upon the context. These regulatory proteins called also as the "Bifunctional DNA-binding proteins" work not only on the site at which they bind but also on the participation of other regulatory factors in modifying gene expression and the extend to which effectors modify their function. The best-known and most widely encountered is the adenosine -3'5'-cyclic monophosphate (cAMP) receptor protein (CRP).

Autoregulatory factors

One of the important steps in a regulatory cascade for differentiation and secondary metabolism in streptomycetes included autoregulatory factors, which are low-molecular-weight chemical substances effective at extremely low concentrations and are essentially required as intrinsic factors for triggering secondary metabolite formation and/or morphogenesis. These properties of autoregulators are akin to those of hormones in eukaryotic organisms. A-factor, is representative of such autoregulators.

Characteristic of autoregulators

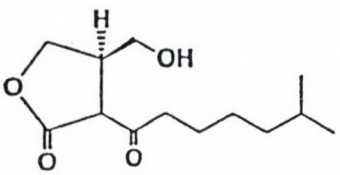
A-Factor

A-factor /2-(6'-methylheptanoyl)-3R-hydroxymethyl-4-butanolide/ (Fig. 1) was originally discovered by Khokhlov et al. /38/ in the culture broth of Streptomyces griseus as a factor that induced streptomycin (Sm) production in a mutant strain of S. griseus. They also found that A-factor was essentially required for aerial mycelium formation /39/. Genetic studies showed that some Sm-nonproducing S. griseus strain obtained by conventional

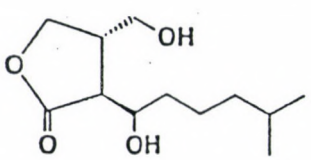
mutagenesis actually required a certain diffusible factor for recovery of both Sm production and sporulation /24/. On the basis of Khokhlov's group, A-factor was chemically synthesized and it was found that the R-form of A-factor at a concentration as low as 10^{-9} M completely restored the wild-type phenotype /24/. It was also found that a decreased level of resistance to Sm in the A-factor-deficient mutants was restored to that of the wild type strain through the stimulatory production of Sm-6-phosphotransferase /25/. In addition to these phenotypes, A-factor induced the production of a diffusible yellow pigment that contains an aminosugar moiety /30/.

Consistent with the pleiotropic effect of A-factor, the profiles of total proteins of S. griseus, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, are dramatically different in the presence and absence of A-factor. More then 10 proteins widely ranging in molecular size are produced only in the wild type strain, while a few proteins are present specifically in an A-factor-deficient mutant strain. The addition of A-factor to the mutant during growth made its protein profile become the same as that of the wild-type strain. Although the exogenous supply of an excess of A-factor slightly stimulates biomass formation in the cultures of both an A-factor-deficient and the wild-type strains, the yields of Sm per gram of mycelim do not change. However, Sm production begins earlier by one day when A-factor is added at the time on inoculation. These observations reflect the role of A-factor as a trigger of Sm production and sporulation but not as an enhancer for yield of Sm. In relation to this, A-factor is produced just prior to Sm production and it rapidly disappears before Sm production reaches a maximum, which may suggest the presence of an A-factor-inactivating enzyme because A-factor itself is rather stable chemically.

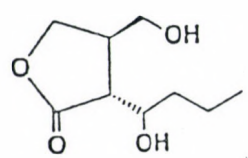
A-factor production is widely distributed among strains pf streptomycetes /24/. The autoregulatory role of A-factor, however, has been observed almost exclusively in the Sm-producing S. griseus. The response to A-factor has so far been found only in anthracycline production in S. griseus and in nosiheptide production in Streptomyces actuosus /46/. On the other hand, when the cultured media of various Streptomyces spp. were analyzed as to the A-factor activity for the S. griseus mutant, an active spot with a R_f value different from that of A-factor was detected using thin-layer chromatography /4/. We assume that A-factor and its analogs act as a chemical signal in a wider variety of species. If a mutant deficient in A-factor of a certain strain could be obtained, then the effect of A-factor on antibiotic production and cell differentiation might be seen just by



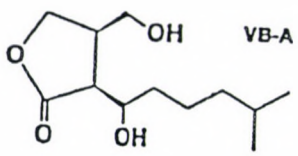
A-factor from *S. griseus*

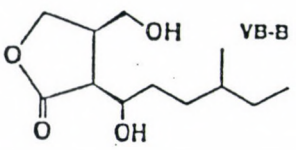


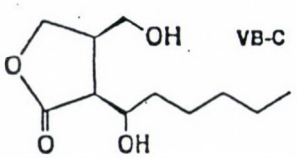
Factor I from *S. viridochromogenes*

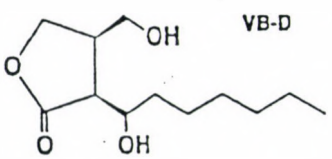


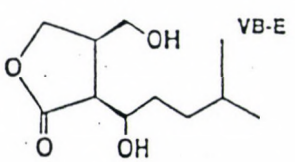
IM-2 from
Streptomyces sp. FRI-5











Virginiae butanolldes from
S. virginiae

Fig. 1. Chemical structures of autoregulatory factors with a butyrolactone ring

A-Factor Homologues

Virginiae butanolides

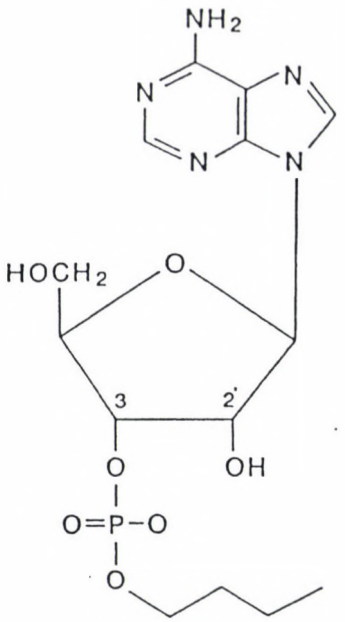
Yanagimoto et al. /60/ reported the presence of a regulatory factor for virginiamycin production in Streptomyces virginiae. Further studies by Yamada et al. /59/ revealed that the factor actually consists of a mixture of compounds, named virginiae butanolides (VBs) (Fig. 1). Although they are very similar to A-factor, no effect of VBs on morphogenesis of the producing strain has so far been observed. A-factor is not active for virginiamycin production in this strain, and vice versa; VBs show almost no effect on Sm production in S. griseus. The A-factor family possessing a β-keto group at the 6-position and the VB family with an OH group at this position are strictly discriminated from each other, but specificity within each of the families depending on the difference in the acyl side chains is rather loose.

I Factor

Grafe's group has been working with a mutant strain derived from Streptomyces griseus ZIMET 43682 whose anthracycline production and sporulation are restored by A-factor /21, 22/. A wide screening test with this mutant strain, based on the assumption that regulatory compounds are not necessarily specific for a given strain, led to the identification of I-factor from Streptomyces viridochromogenes (Fig. 1). The factor possessing an OH group at the 6-position showed an inducing activity similar to, but about 10 times less than that of A-factor. In regard to the fact that these A-factor analogs have an OH group at the 6-position, it still remains unclear whether these compounds actually function as a chemical signal for secondary metabolism and morphogenesis in the original strains. Genetic studies including the isolation of mutants deficient in these substances are obviously necessary.

IM-2

Sato et al. /52/ isolated an A-factor analog, named IM-2, which triggered blue pigment production in Streptomyces sp. FRI-5 at a concentration of 0.6 ng/ml. The structure was determined to be 2,3-trans-2(1'-hydroxybutyl)-3-(hydroxymethyl)butanolide (Fig. 1).



B-factor

Fig. 2. Chemical structure of autoregulatory factor B

B-factor

B-factor was isolated from commercial yeast extract on the basis of the observation that the addition of yeast extract to the culture of a mutant strain of Nocardia sp. restored rifamycin B production. B-factor purified from yeast extract is a butyl ester of 3'-AMP (Fig. 2) and is active at a very low concentration of 10 ng/ml /37/. An intrinsic substance with potent B-factor activity was also isolated from the parental Nocardia strain. The structural similarity of B-factor to 3'-5'-cAMP and guanosine oligophosphates, both of which are important in the regulation of multiple cellular functions in a wide variety of organisms, leads us to assume that B-factor or its homologues exert some physiological significant function in general.

Factor C

Szabó's group /55/ reported in the same year as Khokhlov et al. /38/ the discovery of factor C from Streptomyces griseus. The factor restored sporulation of mutant strain in the submerged culture. Factor C produced at a late stage of growth is a protein of molecular mass of 34 500 /5/ that is localized mainly in the membrane fraction. The factor C activity was distributed in wide variety of Streptomyces spp. Immunoblot experiments showed that the non-spore forming mutant, S. griseus 52-1, still produced a small amount of factor C. On addition of factor C to the mutant strain, it disappeared rapidly from the medium, but the sporulated cells thus obtained contained a large amount of factor C. Factor C therefore seems to induce further production of itself in a larger amount than when it is added. Szabó et al. /56/ proposed the hypothesis that factor C affects a cell membrane receptor and by changing its function evokes a chain of events leading to differentiation. For further elucidation of the mechanism of regulation displayed by factor C, genetic studies, including the cloning of the factor C gene, are obviously necessary.

AIB factor

In Streptomyces cimnamonensis mutant strain resistant to butyric and isobutyric acid (precursors of monensin A) a new factor AIB has been described /48/. The factor positively effects growth, sporulation and monensin production. AIB factor eliminates the toxic effect not only of isobutyrate but also of acetate, propionate, butyrate, 2-methylbutyrate, valerate and not only in S. cinnamonensis but in many other Streptomyces species. The positive effect was not found in submerged cultures. It is assumed that AIB-factor exerts its effect only in early stages of germination on solid media.

Novak et al. /45/ described an excreted diffusible factor in sporulating, avermectin-producing strain of Streptomyces avermitilis. The factor induced the formation of aerial mycelium and spores by a nonsporulating mutant on solid medium.

Biosynthesis of butyrolactone-type regulators

It is proposed that the afsA gene cloned from S. griseus as the gene complementing the A-factor deficiency of mutant strain /30/ codes for an enzyme catalyzing the condensation between the β-keto acid and the glycerol

derivative. The afsA gene exhibits the following properties: (a) A trimmed 1.2-kb fragment encoding a single open reading frame of 301 amino acids confers A-factor production to A-factor-deficient mutant strains of S. griseus. (b) The 1.2-kb fragment shows a remarkable gene dosage effect on A-factor production when it is carried on a high-copy-number plasmid, and (c) the 1.2-kb fragment can confer A-factor production with a marked gene dosage effect to all the Streptomyces strains so far tested /31/. We suppose that almost all Streptomyces spp. contain the glycerol derivative as a common metabolite and a pathway for polyketide biosynthesis that endows the β-keto acid as a common metabolite as well. We therefore speculate that the AfsA protein is one of the key enzymes responsible for the condensation, although further studies are obviously needed to clarify the function of AfsA.

A-factor is the only autoregulator whose biosynthesis is genetically studied. So far the observations can be summarized by suggesting that the afsA gene is on an unstable extrachromosomal element such as a plasmid or a transposon in S. griseus. However, the attempts to detect plasmid DNA physically by conventional and pulse-field agarose gel electrophoresis have failed so far. On the other hand, the afsA gene in Streptomyces coelicolor is mapped at a fixed position on the chromosome, and the mutation frequency of afsA is as low as those for auxotrophic markers /26/. In S. coelicolor, a mutation in afsA does not cause any detectable alteration in antibiotic production or morphogenesis.

As described before, A-factor production by S. griseus is enhanced exactly at the onset of streptomycin production. The unusual feature of transcription and translation of the afsA gene may be associated with its differential expression during the growth phases. The similarity in the functional features of A-factor to eukaryotic hormones directed the examination for the presence of a specific binding protein in S. griseus. As expected, a binding protein specific to A-factor was found in the cytoplasmic fraction, but not in the membrane fraction /43/. The binding protein has an apparent molecular weight of approximately 26 000, as determined by gel filtration chromatography. The absence of any binding protein in the membrane fraction is consistent with a model in which A-factor diffuses freely into the cell and requires no surface receptor. Isolation of mutants deficient in the A-factor-binding protein resulted from the incidental finding that a S. griseus mutant requiring no A-factor for its Sm production or sporulation had a defective binding protein /44/. This observation implied that the A-factor-binding protein in the absence of A-factor repressed the expression

of both the phenotypes in the wild-type strain. All these data suggested that the A-factor-binding protein played a role in repressing both Sm production and sporulation and that the binding of A-factor to the protein releases its repression. Functional involvement of the binding protein in A-factor regulation supported the idea that the protein is a real functional receptor and that A-factor can be termed a prokaryotic hormone.

A still unanswered question is how the biosynthesis of autoregulators are triggered and controlled in the life cycle of streptomycetes. In S. coelicolor, the expression of afsA probably encoding the key enzyme for A-factor biosynthesis is controlled by afsR /32/, whose gene product is phosphorylated by a specific membrane-bound kinase named AfsK /27/. The AfsR and AfsK proteins as a regulator and sensor, respectively, supposedly compose a two-component regulatory system. The AfsK sensor may recognize a certain environmental signal and transfer it, via protein phosphorylation, to the AfsR protein serving as a positive regulator for afsA (Fig. 3). Although no counterpart for afsR in S. griseus has yet been detected, a regulatory system similar to this AfsR-AfsK system may function as a switch for the afsA expression in S. griseus. In addition to the control of biosynthesis of autoregulators, a signal transfer to the downstream genes required for morphogenesis and secondary metabolism remains to be explored in detail. The γ-butyrolactone-type regulators conceivably transfer their positive signal in a manner similar to the relay of the A-factor-positive signal to the Sm biosynthetic genes. Because a change in the profile of phosphorylated proteins in the presence and absence of A-factor has been observed, a signal relay through phosphorylation of intermediate regulatory proteins may play an important role in the signal transfer downstream from the step involving autoregulators and their binding protein.

New compounds

The development of molecular biological approaches has brought new views on the classical biochemical and physiological studies. For the sake of objectivity it should be admitted that some expectations concerning the use of molecular genetics for the solution of secondary metabolite overproduction have not yet been fulfilled. This situation was noticed by Chater /11/. Genetics has spawned a dramatic expansion of the biotechnology industry in the direction of the products of single genes. On the other hand,

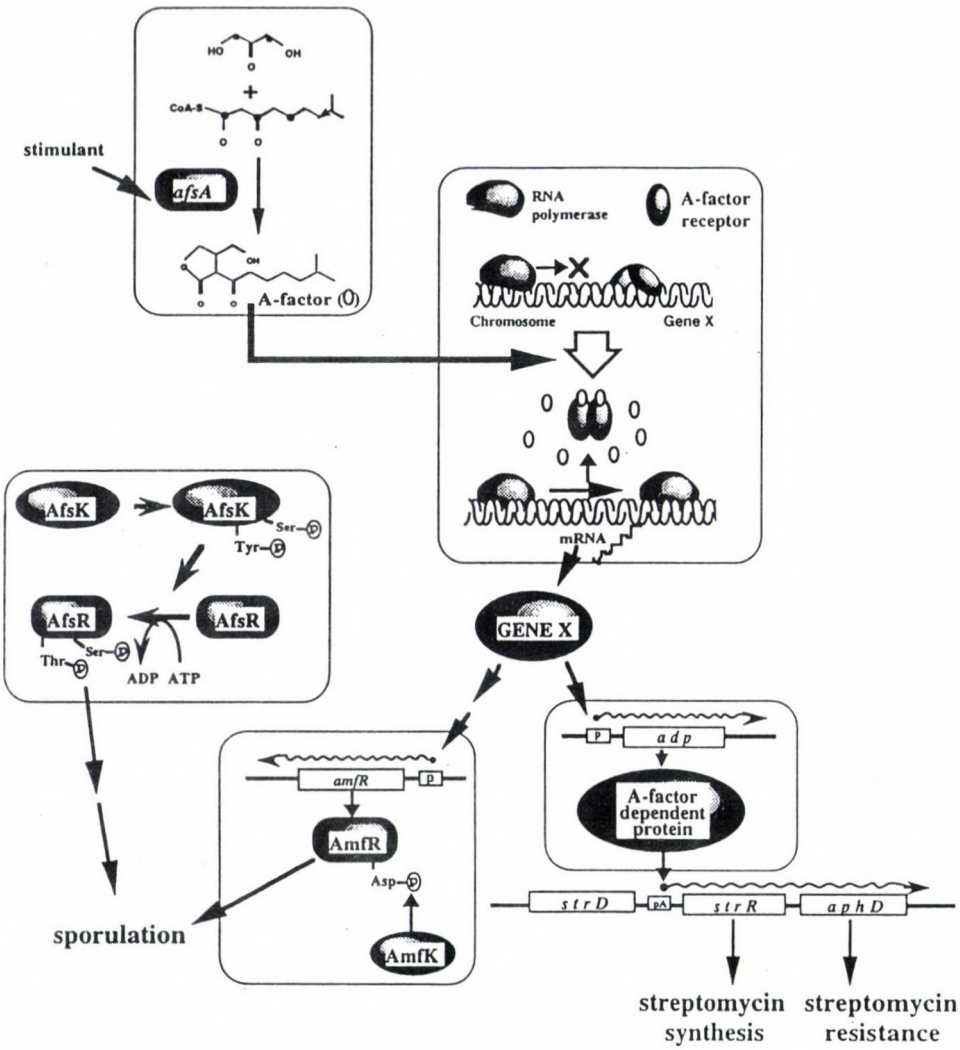


Fig. 3. The A-factor regulatory cascade. The afsA gene probably encodes a key enzyme for the biosynthesis of A-factor. The A-factor signal is transferred via the A-factor receptor protein to the Adp (A-factor dependent protein) product that serves as a transcriptional activator for strR and aphD. The aphD gene encoding a streptomycin resistance determinant is transcribed mainly by the read-through from the A-factor dependent strR promotor, which ensures prompt induction of the resistance in response to A-factor. The A-factor signal is also transmitted to the yellow pigment production genes and the genes for aerial mycelium formation. The expression of amfR encoding protein similar to response regulators of prokaryotic two-component regulatory systems is controlled by A-factor. The kinase able to phosphorylate Asp-54 of AmfR has not yet been identified. A Ser/Thr kinase and its target protein similar to the S. coelocolor A3(2) afsK/afsR system probably control aerial mycelium formation, because afsK suppresses the aerial mycelium-negative phenotype of an A-factor deficient mutant of S. griseus (after /33/)

antibiotics — some classical products of biotechnology — result from the concerted action of many genes, and it is therefore less straightforward to apply the new techniques to antibiotic production. Studies of cloned genes for antibiotic biosynthesis are now providing information that should allow the application combination of traditional and DNA methodology to the improvement of yield in antibiotic-producing Streptomyces species.

For the targeted preparation of new compounds the thorough knowledge of their biosynthesis is essential. This principle was well documented by Ikeda and Omura /34/ on the directed biosynthesis of avermectins and selected production of new useful compounds of this type. The great number of avermectin derivatives was found using different techniques, like biotransformation /20/, chemical mutagenesis /18/ and classical mutagenesis /20/.

Very fruitful was the work of Dutton et al. /16/ where so-called mutational biosynthesis was used to obtain new avermectins. Avermectins with a wide range of novel C-25 substituents have been prepared by feeding carboxylic acids or their biosynthetic precursors to a Streptomyces avermitilis mutant strain ATTC 53568. This organism lacks the ability to form isobutyric and S-2-methylbutyric acid from their 2-oxo acid precursors and thus is unable to produce natural avermectins unless supplied with these acids. The novel avermectins produced by mutational biosynthesis possess broad-spectrum antiparasitic activity.

The first successful use of DNA recombinant technology in streptomycetes producing antibiotics was described by Hopwood et al. /28/ and Omura et al. /47/. They succeeded in producing the "hybrid" polyketides mederrhodin A and dihydrogranatirhodin. These molecules were generated by transfering partial or complete biosynthetic gene clusters between different polyketide-producing strains and screening for new metabolites. Generation of novel molecules in this manner was largely empirical. Rational design of aromatic polyketide natural product by recombinant assembly of enzymatic subunits was described by McDaniel et al. /42/ and a rational approach for producing novel macrolides by generating analogues of the erythromycin polyketide lactone through deletion of active sites within the polyketide synthetase itself was published by Donadio et al. /14/.

The possibility to prepare the hybrid macrolide antibiotic in Streptomyces ambofaciens and Streptomyces lividans by introducing the cloned carbomycin biosynthetic genes from Streptomyces thermotolerans was described by Epp et al. /17/. Similar aim e.g. to prepare in single step fermentation the acyltylosines by genetically engineered strains has the work of Arisawa et

al. /1, 2/. They succeeded in transformation of tylosine producing strain Streptomyces fradiae by plasmids carrying genes from Streptomyces thermotolerans responsible for acylation of macrolides /3/.

Gene engineered technology was successfully used in preparing the cephalosporins intermediates as well. Here again the two step fermentation was modified into single step fermentation by introducing the expandase genes from Streptomyces into Penicillium producing isopenicillin N /51/.

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TREATMENT POSSIBILITY OF HYPERCHOLESTEROLAEMIA ASSOCIATED WITH HYPERTRIGLYCERIDAEMIA*

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Thirty patients were investigated with hyperlipoproteinaemia, 15 patients with non-insulin dependent diabetes mellitus (NIDDM) and 15 with primary hyperlipoproteinaemia. The patients took 250 mg acipimox (5-metyl-pyrazine-carboxylic acid 4-oxide) 3 times per day for 2 months. Serum examinations were performed before and after 1 and 2 months of acipimox administration. After treatment the cholesterol and triglyceride levels decreased in both groups. Patients with NIDDM had 11% increase of high density lipoprotein-cholesterol (HDL-C) level at the end of the first, and 18% increase at the end of the second month, while patients with primary hyperlipoproteinaemia did not change significantly. The low density lipoprotein (LDL) level did not change significantly in either groups of patients. The apolipoprotein A 1 (apo A 1) levels increased significantly during the second month of acipimox administration. Uric acid levels decreased in both groups, but significant change could be detected mainly in the NIDDM group. Serum glucose level did not change in the non-diabetic patients, while it decreased significantly in the NIDDM group.

Keywords: Primary hyperlipoproteinaemia — non-insulin dependent diabetes mellitus — acipimox

Introduction

The therapeutic effect of acidum nicotinicum on hypercholesterolaemia and hypertriglyceridaemia has been known since 1995, and it is widely used ever since. Nicotinic acidamin is an important element of pyrimidin nucleotides, and it has a significant role in oxidative reactions, thus acting in tricarboxylic acid cycle, fatty acid oxidation and pentose-phosphate metabolism. Its actions include inhibition of the release of free fatty acid (FFA) through decreasing hormone-sensitive lipase activity, inhibition of

*Dedicated to the memory of Professor Gábor Szabó.

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very low density lipoprotein (VLDL) (and consequently, LDL) formation. Due to these effects of nicotinic acid, in the previous years widely used in the treatment of type 2b, 3 and 4 hyperlipoproteinaemia. To achieve the effects of acidum nicotinicum it was necessary to administer high dosages, 2-3 g/day, which resulted in the abnormalities in glucose metabolism and the increase of fatty acid levels. These produced side effects in a great number of patients, so the application had to be limited /4, 7, 10/. In the past few years acipimox (5-metyl-pyrazine-carboxylic acid 4-oxide), a derivate of acidum nicotinicum has been more widely used, as it has a more significant lipid decreasing effect, and plasma half time is longer /7, 18/. The usage of acipimox has a special significance in the case of NIDDM, which is characterized by an increased glucose release from the liver resulting in the increased level of fasting blood glucose. Insulin-dependent glucose absorption is decreased in peripheral tissues, especially in the muscles, which may lead to postprandial hyperglycaemia, and significantly higher non-esterified fatty acid level can also be noticed. Walker et al. showed that the increase of fatty acid decreases insulin-stimulated glucose absorption in the muscles /1, 17, 24/. Acidum nicotinicum and its derivate called acipimox, when used in NIDDM, decreases the free fatty acid level /2, 19/. According to the earlier hypothesis it would lead to the increase of peripheral insulin effect and the improvement of blood glucose levels. This effect, however, can be noticed only with acipimox /20/. On the basis of earlier studies, as a rebound effect of acidum nicotinicum rebound develops in the fatty acid level, with a consequent decay of peripheral insulin effect and glucose tolerance /7/. The present study describes our experiment on the effect of acipimox which has been used for a 3 years in our country, on patients suffering from hyperlipoproteinaemia without diabetes and those with NIDDM. We evaluated the changes in glucose, lipid and lipoprotein levels as compared to the parameters before the treatment in both groups of patients.

Patients and Methods

We investigated 15 patients with primary hyperlipoproteinaemia and 15 with NIDDM. There were 9 males and 6 females in the primary hyperlipoproteinemic group (I) while there were 11 males and 4 females in NIDDM group (II). In the first group the mean age was 55.7 years, in the second 55.9 years. According to the serum lipid levels and lipid electrophoresis there were 10 patients type II.b, 2 patients type III, 3 patients type IV, hyperlipoproteinaemia classified by Fredrickson, 12 patients II.b and 3 patients IV type from NIDDM. Thirteen patients had macroangiopathy from NIDDM. It was

determined by color Doppler arteries of lower extremities, carotis Doppler, ECG, exercise ECG and angiography. The mean body mass index was 29, which did not change significantly during the examination period. We measured each of their serum glucose, haemoglobin A1C (HbA1 C), uric acid, cholesterol, triglyceride, HDL, LDL, apo A 1, apolipoprotein B 100 (apo B 100), apolipoprotein /a/ (apo /a/) level, liver and kidney functions and we performed lipoprotein electrophoresis. Patients with kidney and liver disease were excluded from later examinations. The patients were kept on a special lipid lowering diet. The carbohydrate intake was 180 g/day for NIDDM patients and glybenclamid medication was used to maintain the carbohydrate balance. The patients took 250 mg acipimox 3 times per day for 2 months. The serum examinations were done before the treatment and after 1 and 2 months of acipimos administration. Serum cholesterol and triglyceride determination were performed by the Boehringer enzymatic method. HDL determination was carried out by the phosphotungstate-magnesium precipitation method. The LDL cholesterol was calculated by the Friedewald formula /6/. Apolipoproteins determinations were performed by an immuno-nephelometric method, using Orion Diagnostic kit. We used phosphotungstic acid precipitating method for serum uric acid determination (normal range: 220-450 μmol/l). HbA1C was determined colorimetrically (normal range: 3.4-7.4%). Five patients had transitoric skin flush reaction and 1 had palpitations as side effects. These reactions disappeared after 2 weeks of drug administration.

Results

After the treatment cholesterol and triglyceride levels decreased in both groups. The decrease could be noticed after one month of treatment, and it continued in the second month, but it was slower. Interestingly, in patients with diabetes mellitus cholesterol and triglyceride levels did not change significantly in the second month. Patients with primary hyperlipoproteinaemia showed a slight increase of HDL-C during treatment, but it was not significant compared to the starting values. Patients with NIDDM had 11 per cent increase in their HDL-C levels at the end of the first, and 18 per cent increase at the end of the second month. In the primary hyperlipoproteinaemic group LDL cholesterol showed a slight decrease, but the change was not significant. Patients with NIDDM at the end of the first month had a slight, not significant LDL increase, which returned to the starting value by the end of the second month (Fig. 1). Apo A1, the main apoprotein element of HDL increased significantly ($p < 0.05$) in both groups of patients at the end of the first month. In the primary hyperlipoproteinaemic group was no significant change by the end of the second month compared to the values of the first month, while in the NIDDM group apo A1 serum level grew significantly compared to the first month levels. In the primary hyperlipoproteinaemic group apo B changed only slightly, in the first month in the NIDDM

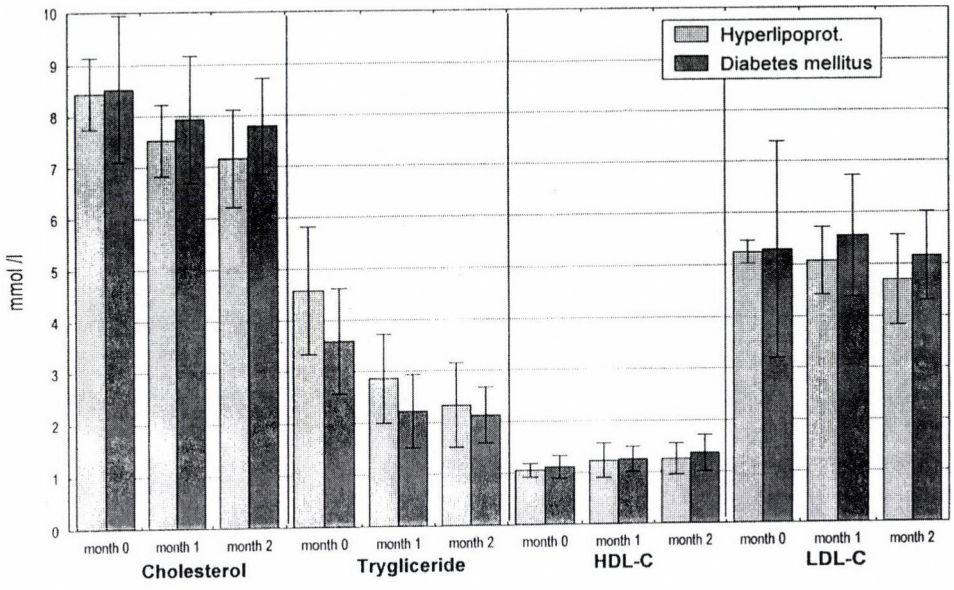


Fig. 1. Lipid parameter changes during acipimox treatment
Abbreviation: Diab.mell. = non-insulin dependent diabetes mellitus

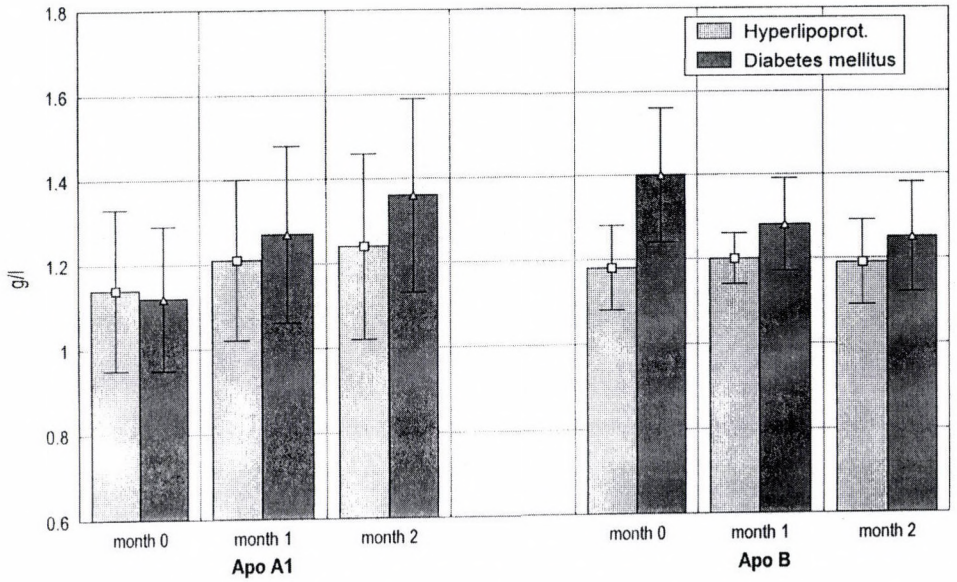


Fig. 2. The effect of acipimox on apolipoprotein levels

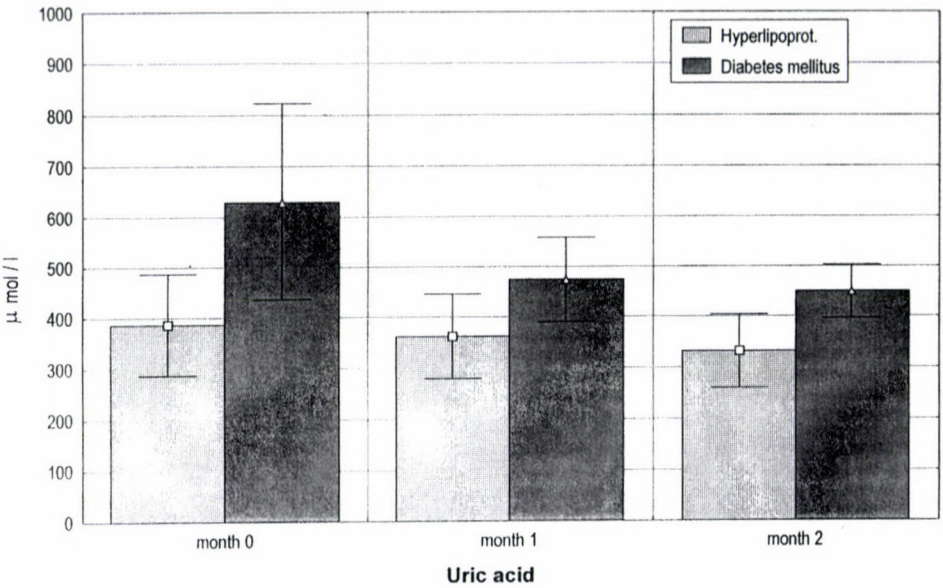


Fig. 3. The uric acid level changes

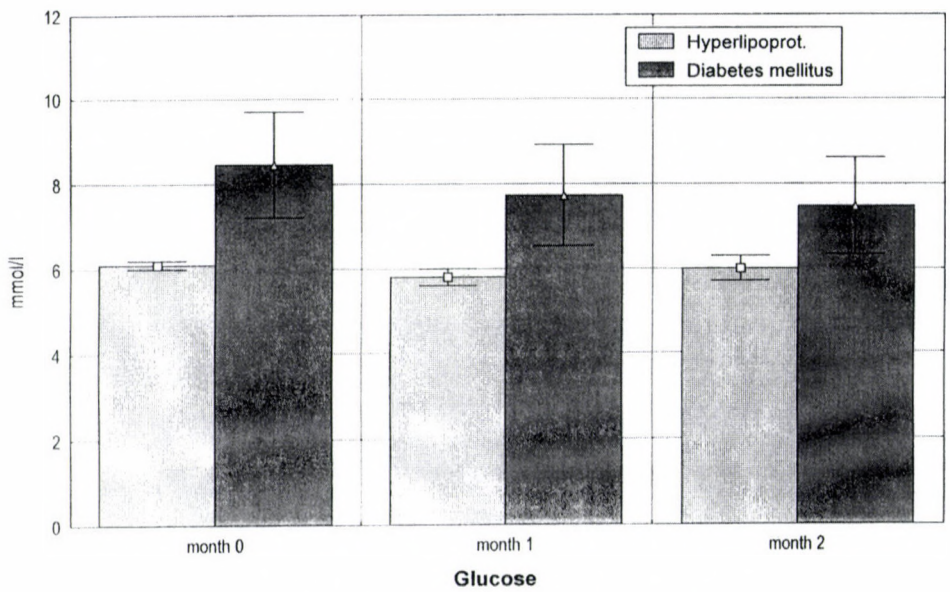


Fig. 4. The serum glucose levels in both groups

group, and the decrease continued in the second month (Fig. 2). Uric acid levels decreased in both groups, but a significant change ($p < 0.05$) could be detected mainly in the NIDDM group (Fig. 3). Serum glucose level did not change in the non-diabetic patients, while it decreased significantly ($p < 0.05$) in the NIDDM groups (Fig. 4).

Discussion

The application of acipimox (5-metyl-pyrazine-carboxylic acid 4-oxide), a derivative of acidum nicotinicum, has recently been focused on the treatment of hypercholesterolaemia accompanied with hypertriglyceridaemia /8, 12, 25/. Our findings show the decrease of triglyceride and cholesterol levels in both groups of patients. These effects can be explained by the inhibition of VLDL development due to a higher free fatty acid level. A faster decrease of the triglyceride levels in the group of diabetic patients can be explained by the fact that the glucose metabolism of the peripheral tissues is normalized by the decrease of free fatty acids and it provides a further support to the improving effect of acipimox /23/. No significant LDL changes have been detected in either groups during acipimox treatment. LDL subfractions have not been examined. Significant LDL decrease was not found even in the patients with higher starting LDL levels. Carlson et al. pointed out that nicotin acid analogues effect on LDL levels depends on the phenotype of hyperlipoproteinaemia. Patients with high LDL levels had approximately 10-15 per cent decrease of plasma LDL concentration as a result of the administration of nicotinic acid derivate, whereas patients with normal or low LDL levels may develop a higher level of LDL concentration after acidum nicotinicum treatment /3/. Levy et al. consider nicotinic acid to inhibit LDL synthesis, and the inhibitory effect is realized in the hepatic excretion of the decreased VLDL, the VLDL being a precursor of the LDL /13/. Griffin et al. showed 4 LDL subfractions, called LDL-I to LDL-IV, with density gradient ultracentrifugation. The main component in the healthy individuals is the LDL-II, whereas there is an increased level of LDL-III in patients with arteriosclerotic disorders, and that of LDL-I in women /9/. The level of total LDL usually does not change after the usage of nicotinic acid derivate, but within the LDL subfraction the amount of small and dense LDL III decreases, whereas amount of LDL-I increases /21/. Franceschini et al. found that after acipimox treatment the cholesterol-ester content of LDL grew by

25.4 per cent, whereas triglyceride content decreased by 46.2 per cent. The modification increased affinity of LDL to specific LDL receptors /5/. HDL level increase is considered to be one of the arteriosclerosis-decreasing factors, especially HDL 2 inhibits the development of arteriosclerosis. Consequently, lipid decreasing therapy aims at the increase of protecting HDL cholesterol level in addition to the decrease of LDL cholesterol. During acipimox treatment we found that HDL levels did not change significantly in the group of patients with primary hyperlipoproteinaemia, whereas they grew significantly in the group of diabetic patients. The present study has not analysed the change of the particular HDL subfractions, so the rate of HDL2 and HDL3 cannot be presented here. It is possible that the decrease of triglyceride levels has an important role in the development of HDL level increase, as the inverse relation between HDL cholesterol and triglyceride is known from earlier studies /14, 15/. In addition we found that in patients with non-diabetic hyperlipoproteinaemia blood glucose levels did not change significantly compared to the starting level, whereas the blood glucose levels content decreased in the diabetic group after acipimox treatment. The slight decrease of uric acid levels reinforces the other favourable metabolical effects. Skin flush caused by acipimox could be found only in a few patients, and it lasted only temporarily. Pontiroli et al. found intensive flush formation in 33 patients out of 74, which showed partly dosage dependence, and could be explained partly by individual sensitivity /16/. These results call attention that in hypercholesterolaemia complicated with hypertriglyceridaemia acipimox, the derivate of acidum nicotinicum may serve as a good tool to achieve the therapeutic goal.

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MULTIMODAL ELECTROPHYSIOLOGICAL EXAMINATIONS IN PATIENTS SUFFERING FROM VARIOUS TUMORS OF THE PINEAL REGION*

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Multimodal electrophysiological examinations: blink-, glabella- and masseter-reflexes, as well as brain stem acoustic, somatosensory and visual evoked potentials were examined in thirteen patients with clear consciousness suffering from extraaxial, chronic, expanding processes in the tectal region. According to the data, the authors came to the conclusion that several modalities were often required to make a correct diagnosis or to the localization of the space occupying processes. Functional disturbances of the whole of the lower brain stem, but especially of the mesencephalon and of the lower pons were found in cases of expanding processes surrounding the tectum.

Keywords: Multimodality evoked potentials — brain stem reflexes — pineal region — tectal region

Introduction

The expanding processes of the pineal region cause usually disturbances in the central nervous system. These may be local neurological sings, diaschisis or most frequently clinical signs of raised intracranial pressure caused by obstructive hydrocephalus. The aim of this study is to investigate whether electrophysiological recordings of blink (BR), glabella (GR) and masseter reflexes (MR), as well as brain stem acoustic (BAEP), somatosensory (SEP) and visual evoked potentials (VEP) are useful tools for establishing the diagnosis, or not. We focused our attention mainly on the question of whether electrophysiological examinations are suitable to establish the diagnosis of mesencephalic damage.

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*Dedicated to the memory of Professor Gábor Szabó.

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Material and Methods

Thirteen patients (4-70 years, mean 39) with clear consciousness suffering from expansive tumor of the tectal region were investigated. The procedures were approved by the local Medical Ethics Committee. The number of patients and the results of histology are shown in Table 1.

Table 1

Diagnosis	No. of patients
Pinealoma	4
Meningioma	4
Lipoma	1
Exophytic glioma	1
Epidermoid	1
Arteriovenous malformation	2

Various examinations were performed in the following distribution among the 13 patients: BR in 13, GR in 7, MR in 7, BAEP in 12, SEP in 8 and VEP in 11. Seven patients disclosed mental symptoms, ataxia could be observed in 6. Papilledema was in 6 patients, but only in one with loss of visual acuity.

Electrophysiological methods

BR

Before examining blink reflex, the function of the facial nerve was checked in all patients. The supraorbital nerves were percutanously stimulated and the action potentials were recorded from the orbicularis oculi muscle on both sides. The early R1, and bilateral late R2 responses were examined.

GR

The glabella reflex was evoked by a gentle hit with the reflex hammer onto the glabella which also started the oscilloscope without any appreciable delay. Registration and evaluation of the potentials was similar to the BR.

MR

It was evoked also with the reflex hammer by hitting the genital process at a slightly opened mouth. The responses were recorded from the masseter muscle on both sides.

All brain stem reflexes were evaluated with the aid of the superposition technique.

BAEP

The auditory stimuli used to evoke BAEPs were 0.1 ms clicks with alternating polarity, delivered at a regular rate of 20 Hz. The stimulus in-

tensity was 60-70 dB above the hearing threshold and the contralateral ear was masked with white noise (30 dB below the click stimulus). Two runs of 2048 average were made. The BAEP's were recorded from the mastoid process in front of the vertex reference. We measured the interpeak latencies (IPLs) and the 3/1, 5/1 and 5/3 quotients of the amplitudes.

SEP

We stimulated the median nerve at the wrist and the SEPs were recorded in two places: at the spinous process of C 2 (N 14) and above the contralateral centroparietal cortex (N 20), in front of the vertex reference. Two-hundred fifty-six records were averaged. The time difference between these two latencies was regarded as central conduction time (CCT).

VEP

A LED stimulator with 16 red box arranged in a network manner was used to evoke the VEPs. The evoked potentials were recorded with a mid-occipital electrode and with a mid-frontal reference. Hundred twenty-eight responses were averaged. The latency and amplitude of the major positive response (P100) and the latencies of the preceeding and following negative peaks (N1 and N2) were measured.

Results

BR

The M response evoked by percutan stimulation of the facial nerve was normal in all patients. In 4 patients out of 13, normal BR were observed. The duration of R1 responses was increased in the other 9, and the amplitude was decreased in 4 patients. The latency of the R1 was increased only in one patient. The pathological changes of the R2 responses were always bilateral. After removing the tumor, the pathological BR become normal again. On the basis of BRs data, we could establish that the expansive processes in the region of the lamina quadrigemina may cause functional disturbance of the pons.

GR

The GR was normal in 5 patients out of 7. We found prolonged latency of R1 only in one, increased duration of R2 and decreased amplitude of R2 in two patients.

MR

We found pathological MR, which mean different latencies between the two sides, increased latencies and decreased amplitude and duration of the potentials on one or both sides in all patients but one. The MR was lacking

on one side in one patient and bilateral in another patient. The patient who suffered from exophytic cystic astrocytoma disclosed normal MR.

BAEP

The BAEP was normal in 2 patients out of 12. The size of the tumor was small in both cases.

Increasing of the 1-3 and 1-5 IPLs and of the 5/3 amplitude quotient, and decreasing of 3/1 amplitude were typical of most of the pathological BAEP. Increased 3-5 IPL was in two patients and increased 5/1 quotient was in another patient. From these data, we suppose that the focus of the functional damage may have been in the region where the third wave of the BAEP is generated. The damage was not serious in every case because we found usually a slightly increased IPLs.

SEP

The latencies and CCTs were normal in half of the patients. In the other half the CCT was pathological at least on the one side. We found decreased amplitude of the N 14 and N 20 waves only in one patient. High amplitude cortical responses were recorded in most cases which was surprising. Some patients disclosed increased amplitude of the early response. We found elongated CCTs and increased amplitudes of the cortical responses in half of the cases. We consider the pathological CCT with the normal cervical latency was caused by direct compression of the sensory pathways.

VEP

Four patients out of 11 produced normal VEP. In the others, we found prolonged P100 and sometimes prolonged N1 and N2 latencies and increased amplitudes of the cortical wave complex. The amplitude of the P100 response was decreased in one case. The parameters of VEP showed similar changes on both sides. There were no correlation between the pathological changes of the VEP and the degree of hydrocephalus and papilledema.

Discussion

So far the usefulness of several kinds of electrophysiological methods were described only for patients who suffered from multifocal neurological diseases /8, 12, 17, 20, 29/. There are few studies, involving more than two

electrophysiological modalities, on the damage of the brain stem caused by expanding processes or other diseases /5, 23, 24, 27, 28, 30, 33/.

The relay sites of the BR, GR, MR and SEP pathways are well known /16, 25, 25/ whereas serious doubt has been lately thrown on the classical model of the BAEPs /6, 7, 20/. Since generators of evoked potentials may be localized in different positions, multimodal electrophysiological techniques are required for the localization of the one-center lesions in the brain stem.

In order to achieve a correct diagnosis as to the level of the lesion, it is important to know that the relay centers, for the cervical SEP, the R2 responses of BR and GR are found in the most caudal position of the brain stem. Rostral to these loci, are the generators for the early deflection of the BAEP, then that for the R1 response of the BR and GR, and finally the relay center for the MR and VEP occupy the most rostral position.

The pathological R1 response is generally regarded as indicating caudal pontine lesions /18/, but the central representation of the R2 responses still under debate /4/. There is, however, an agreement on the indispensability of the motor trigeminal nucleus in the appearance of the R2 response /2, 19, 25/. In our material, we have found a less damaged GR than BR despite the lack of any reference to probable difference in the afferent or efferent limbs of these reflexes. According to literary data, vertical pontine and mesencephalic lesions results in pathological MR /26/. Disregarding a single case in which a small cystic exophytic astrocytoma did not expand into a space occupying event, the MRs were pathological in the majority of our patients. In this single case, all electrophysiological parameters were normal.

With intact acoustic organ and VIIIth cranial nerve, pathological BAEP responses indicate damages rostral to these structures. Changes in the BAEP due to mass dislocations or tonsillar herniation have already been described /33/. The observed BAEP changes, while they may have been quite significant, they were not alarming; they merely supported that the functional disturbance might be around the site of generation of the 3rd wave.

The SEP does not always allow to make a correct diagnosis at the level of the lesion, and this compels us to extend the technique of examination. In the majority of the cases, the pathological change of the SEP appears in the form of side differences accompanied by symmetric elongation of the CCT. The increase in the amplitude was unexpected in cortical responses, although similar observation has been described /10/. The localization of the tumor,

as it compressed the somatosensory tracts, could be accounted for the increase in the latency and decrease in the amplitude; if an increase in amplitude is observed this may be interpreted as a result of irritation. This phenomenon was recorded in more than half of the patients and almost always on both sides. The observation that patients with normal latencies also produced an increase in amplitude suggests again the effect of irritation or damage of certain inhibitory fibers, and in such cases the first sign of a pathological SEP could be an increased amplitude.

In the interpretation of VEP, one has to make distinction whether the pathological changes are due to the direct compression, or a diffuse damage of the visual pathway due to increased intracranial pressure have been observed in infants, children and adults /1, 9, 13, 31, 34/. On the basis of our own experience we are inclined to believe that the direct compression of the visual pathway must also be in the background, since the VEP of our patients with hydrocephalus remained pathological following ventricular drainage. Brain stem reflexes and evoked potentials recorded from patients with paratectal space occupying processes are interpreted as indicating mesencephalic or caudal pontine disturbance with unambiguous mesencephalic dominance.

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PRESYMPTOMATIC DIAGNOSIS OF FAMILIAL COLON POLYPOSIS*

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The gene responsible for familial adenomatous polyposis (FAP) has recently been mapped, identified and this makes the presymptomatic molecular diagnosis of the disease possible. It can be performed by direct mutation analysis or indirect haplotype analysis. In families where several affected individuals are available the indirect haplotype analysis is the easiest way for performing presymptomatic diagnosis of persons at risk. Among Hungarian families we have performed haplotype analysis using D5S346, a highly polymoprhic dinucleotide CA repeat marker located 30-70 kb downstream from APC gene with the combination of restriction endonuclease RsaI site polymorphism. Marker regions were amplified by polymerase chain reaction (PCR) and basen on the above-mentioned polymorphic systems, the haplotype at the APC locus was determined. We believe that haplotype analysis of individuals at risk in large FAP families containing several affected members is a rapid, efficient, and highly valuable method for presymptomatic diagnosis of familial colon polyposis.

Keywords: Familial adenomatous polyposis — FAP — presymptomatic diagnosis — haplotype analysis — PCR

Introduction

Familial adenomatous polyposis (FAP) is a dominantly inherited condition characterized by hundreds of adenomatous colonic polyps that usually develop in early adulthood /4/. One or more of these adenomas inevitably progress to adenocarcinoma if they are not removed. Extracolonic manifestations also occur, such as duodenal adenomas, osteomas, epidermoid cysts, dermoid tumors and congenital hypertrophy of the retinal pigment epithelium (CHRPE) /1, 16/. The offspring of a patient with FAP has a fifty per cent chance of inheriting the gene defect. Although the clinical manifestations

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of the syndrome have been known for many years, the diagnosis still relies largely on the detection of numerous colorectal polyps. Frequent examination of at risk subjects by sigmoidoscopy beginning at the age of ten years /5/ is therefore recommended in order to detect the early stage of the disease and carry out prophylactic colectomy. If the risk of individuals could be estimated without clinical signs of the disease, the frequency of screening procedure would be reduced in low risk subjects, and high risk patients can be regularly examined by sigmoidoscopy until the appearance of polyps in the colon.

The first step toward this aim was achieved when the inheritance of FAP was linked to a small region of chromosome 5 (5q21) /2, 11, 13/. Subsequently, the putative tumor suppressor gene called APC (adenomatous polyposis coli) was identified by positional cloning /10, 12/. It codes 2843 amino acids and includes a large coding region divided into 15 exons containing 8535 nucleotides. Germline mutations of APC gene are responsible for FAP. Since most of the characterized germline mutations of this gene spread over most of the exons, one must usually scan the entire open reading frame of this large gene to find the specific mutation in any particular family in spite of the fact that 20% of the mutations is localized within about 900 nucleotides in the mutation cluster region in exon 15. Therefore, screening for a specific mutation in the APC gene is usually not the first approach for identifying carriers of the gene defect. We believe that the presymptomatic diagnosis must rely on demonstrating of the coinheritance of the disease-causing allele with linked genetic markers. Naturally, the search for finding a specific mutation in every case should be part of the diagnostic process, although sometimes it fails to identify such an alteration in the APC gene.

For indirect haplotype analysis several APC linked genetic markers are now available. There are some highly informative cytosine-adenine (CA) repeat markers proximal and distal to APC gene, and some intragenic polymorphisms that can be used in the linkage analysis. The most informative CA repeat polymorphism linked to APC gene is a DNA sequence lying 30-70 kb from that. It shows a length polymorphism with high heterozygosity and therefore it is very useful in indirect presymptomatic diagnosis. The diagnostic value of this marker, as every linked markers, depends on the size of the investigated family. In a large family, which contains numerous clinically proved affected persons, the informativeness of this marker is very high while in small families the usefulness of linkage analysis can be very low. In addi-

tion to CA repeat polymorphism an intragenic RsaI site polymorphism was detected in order to construct the haplotype, and reduce the change of a misdiagnosis.

Materials and Methods

DNA isolation

Figure 1 shows the pedigree one of the investigated family. Most of the affected members and risk persons in the families were also examined by appropriate techniques for the presence of CHRPEs. Some of the affected individuals have already undergone prophylactic colectomy or have been operated because of colorectal cancer.

Whole blood was collected in EDTA containing tubes from all available family members. DNA was isolated from lymphocytes from 200 µl of EDTA anticoagulated venous blood by the use of Ready-Amp Genomic Purification System (Promega). Four microlitres of this ssDNA was used for PCR amplification.

Polymerase chain reaction (PCR) and gel electrophoresis

All PCR amplifications were performed in a Thermal Cycler 480 (Perkin Elmer). PCR conditions for the amplification of the D5S346 locus from total genomic DNA were as follows: 4 µl of purified DNA, 1 µM of each primer (LNS-CAI and LNS-CAII) /17/, 200 µM of each deoxynucleoside triphosphate, 1.0 mM $MgCl_2$, and 1.5 U Taq polymerase (Promega) in Taq polymerase reaction buffer (Promega) in a total volume of 50 µl. The following PCR cycles were performed on this reaction mixture: one initial denaturation cycle of 95 ^{o}C for 4 min, 36 cycles of 94 ^{o}C for 1 min, 58 ^{o}C for 1 min, 72 ^{o}C for 1 min. For RsaI site polymorphism /15/ we used a PCR mixture consisted of the following reagents: 4 µl of purified DNA, 1 µM of each primer (C3 and C24), 200 µM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, and 1.5 U Taq polymerase (Promega) in Taq polymerase reaction buffer (Promega) in a total volume of 50 µl. After initial denaturation at 95 ^{o}C for 2 min, denaturation, annealing, and extension were carried out at 94 ^{o}C for 1 min, 51 ^{o}C for 1 min, 72 ^{o}C for 1 min for 36 cycles. PCR products are normally 97 bp in size. Twenty microlitres of PCR products were then digested with 10 U RsaI restriction endonuclease at 37 ^{o}C for six hours. Allele with RsaI site was cut for two fragments (54 bp and 43 bp) by the enzyme.

After pCR of LNS CA repeat polymorphism products were run on 8% polyacrylamide gel. Six microliters of each sample were loaded onto the gel and electrophoretic separation was performed at 160 V for six hours. The alleles on the gel were visualized by silver staining. We used that staining because it was found that the amount of products not enough for ethidium bromide detection as reported earlier. For the detection of the RsaI digested PCR products a 8% polyacrylamide gel was used with ethidium bromide staining. An example of RsaI genotype of some family members is seen in Fig. 2.

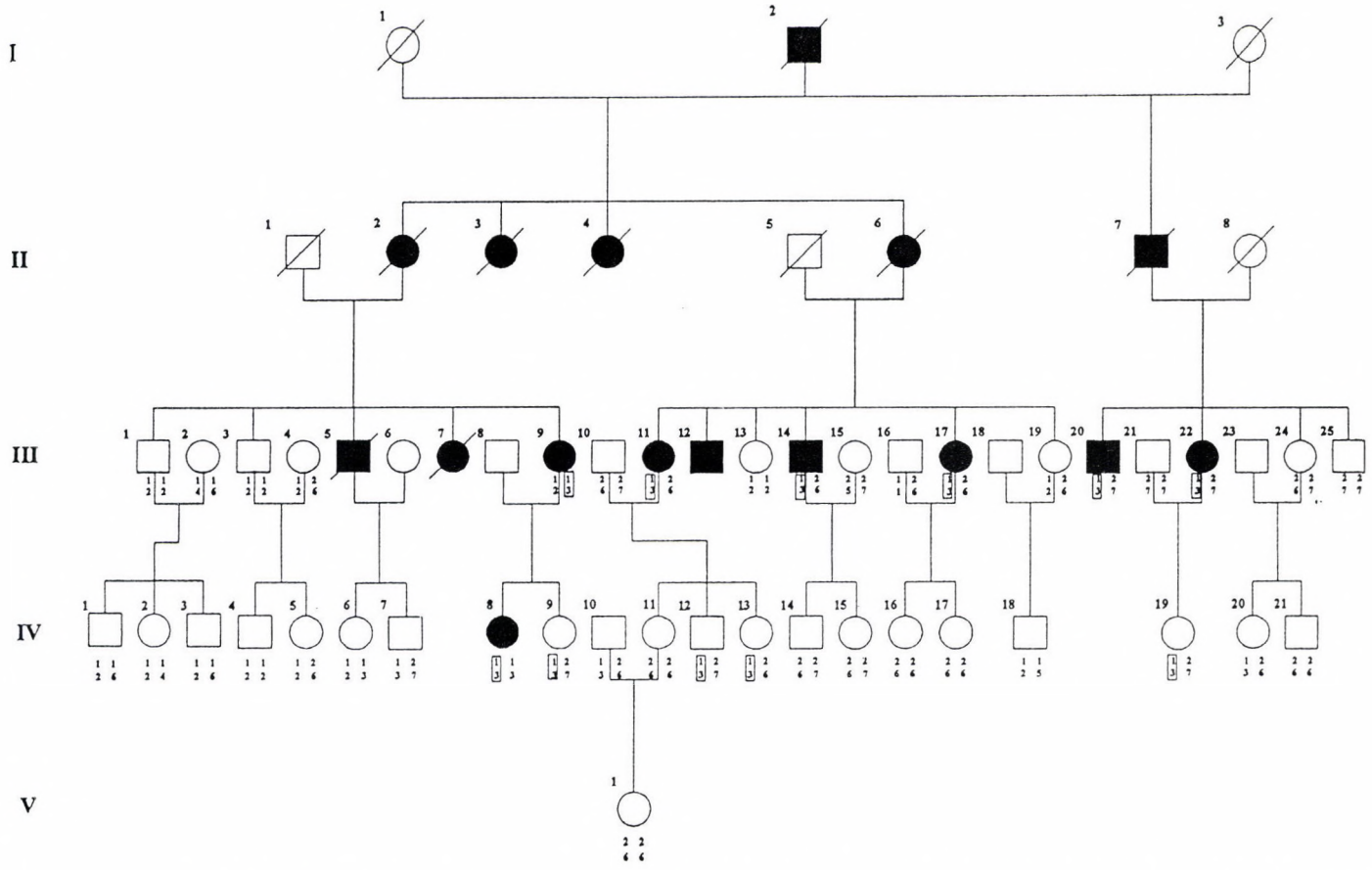


Fig. 1. Pedigree of a large family. Haplotype is indicated below symbols, determined by RsaI site polymorphism (upper) and LNS CA repeat polymorphism (lower). The haplotype associated with the disease /1—3/ is indicated in boxes

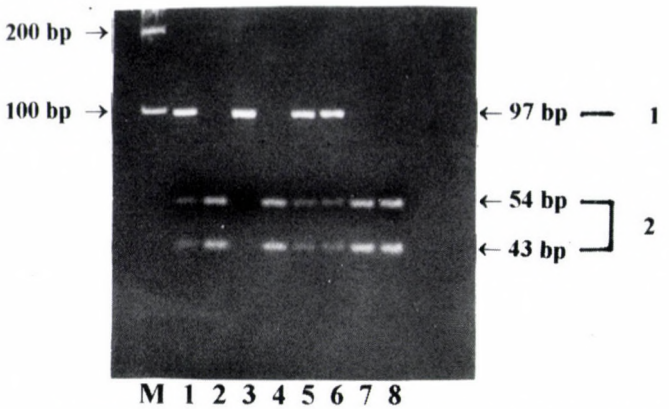


Fig. 2. RsaI site polymorphism of some family members. 1 - IV/13, 2 - III/25, 3 - III/13, 4 - III/10, 5 - III/11, 6 - IV/10, 7 - V/1, 8 - IV/11, M - 100 bp ladder. Ordered numbers on the right side indicate the genotype of alleles

Results

Alleles of D5S346 were designated by numbers according to their relative size. Alleles of Rsal site polymorphism were marked as allele 1 without Rsal site, and allele 2 with RsaI site. Using haplotype analysis we carried out presymptomatic diagnosis of at risk members of Hungarian families. The haplotype was constructed from the CA repeat length polymorphism close to APC gene and the intragenic Rsal site polymorphism. Forty members of the family in Fig. 1 were examined by this method. Seven clinically affected persons were in the family and it was found that the disease causing allele must be linked with the haplotype /1—3/. Out of further twenty-eight at risk individuals only four affected members were detected and twenty-two individuals did not inherit the wrong allele. In the case of two members (IV/6, IV/7) the same haplotype was detected as in the affected members but the examination was not informative, because the parents were not available for the test. It can be seen from the haplotypes that the D5S346 CA repeat marker was informative by itself in all cases. The Rsal polymorphism was informative in eleven cases by itself, without the CA repeat marker. Presymptomatic diagnosis was performed similarly in other families as well.

Discussion

Familial adenomatous polyposis is an inherited tumor disease that usually requires surgical removal of the entire colon. Therefore presymptomatic diagnosis is desirable in persons at risk for the disease. The time optimal of presymptomatic is before the age of ten years or sometimes it can be performed even prenatally. Experiences /5/ show that diagnosis in appropriate time and subsequent operation enables an efficient prevention. Patients at risk, have to undergo regular rectosigmoidoscopy beginning at the age of ten years /6/. Presymptomatic diagnosis of at risk patients can be performed using ophthalmological examination for the presence of CHRPE /7/ or in a more accurate way using molecular genetic methods.

Using molecular genetic methods, it has recently become possible to detect carriers of a mutated APC gene /3, 8, 14/. Among molecular genetic techniques, a PCR based method, such as CA repeat length polymorphism or a restriction site polymorphism, is usually the most effective way for analysing DNA of an at risk patient for FAP. Using this method we have shown in this article that in a large FAP family with many affected members like in our case, only one closely linked highly polymorphic marker can usually be informative, and its combination with an intragenic polymorphism reduce the chance of misdiagnosis because of crossing over during meiosis. Because of the large size of the APC gene, the identification of an mutation responsible for the disease may be difficult. Therefore indirect genotype analysis, such as used by us, saves time and effort compared to direct mutation analysis. In some other studies the informativeness of linked markers to APC gene was investigated and limitations of these markers were indicated /9, 15, 18/. However, the number of members in the investigated family was much more lower than in our family. However, we could not find such a large FAP family in the literature, as presented here. In our study, the CA repeat polymorphism has shown in high heterozygosity among Hungarian patients, and this fact proves its usefulness in presymptomatic diagnosis of FAP in Hungary. Considering the location of the D5S346 CA repeat marker from the APC gene being 30 to 70 kb downstream, one must not ignore the rare possibility of recombination. This chance can be decreased using an intragenic marker like RsaI site polymorphism.

Based on our experience we believe that haplotype analysis of individuals at risk in FAP families containing several affected members, is a rapid, efficient, and highly valuable method for presymptomatic diagnosis of

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MOLECULAR BIOLOGY AND ROLE OF HISTAMINE IN PHYSIOLOGICAL AND PATHOLOGICAL REACTIONS. A REVIEW

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Histamine is known to exhibit a wide variety of both physiological and pathologic functions in different tissues and cells, this amine is one of the most general mediators of the mammalian organism. Extracellular histamine action is believed to be mediated by binding to its specific cell-surface receptor(s) resulting in the converting the extracellular signal to intracellular events. Moreover, numerous studies suggest basic role for intracellular histamine as one of the intracellular messenger moieties. The functional consequences of the action of histamine involve many physiological and pathophysiological events, including allergy, neurotransmission and cell growth.

Keywords: Histamine — biogenic amine — proliferation — antihistamine

Introduction

Histamine is one of the most studied biogenic amines. Its importance has been discovered in the last century. Its different effects (including those involved in allergic reactions and also its recently recognised involvement in cell proliferation) are partly well-known, agonists and antagonists are available not only in clinical applications but also for research.

The biosynthesis of histamine

Histamine is produced by the L-Histidine-decarboxilase (HDC). The genomic sequence of the enzyme is known, it is located as a single-copy gene on the 15th chromosome /30/. The mRNA is processed by a post-translational

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modification, thus several isoenzymes can be found. In the liver of rat embryos the HDC mRNA is transspliced with an androgen-binding protein (ABP). The HDC-ABP protein can also be detected. The biological role of the process is unknown, but due to the homology between the human and rat HDC, this might occur also in human tissues /27/. The highest activity of HDC and histamine-content can be found in hystiocytes and mast cells, where the histamine is stored in cytoplasmatic granules.

The specific irreversible "suicide" inhibitor of the enzyme is a ligand-analogue, the alpha-fluoro-methyl-histidine (alpha-FMH).

Histamine is degraded through diamine oxidase and histamine-methyl-transferase.

Receptors and effects

The extracellular histamine acts through three, G-protein associated family of receptors. The activation of the H1 receptor which stimulates phosphoinositol degradation and Ca mobilisation, can be inhibited with classical antihistamines (e.g. mepyramine, chlorpheniramine, etc.) used frequently in allergic diseases. The selective agonist of the receptor is 2-methyl-histamine.

The H2 receptors upon binding histamine generating cAMP as second messenger can be antagonised with cimetidine, ranitidine, etc. These drugs play an important role in the therapy of gastric ulcer. There are a few selective agonists of the H2 receptor such as 4-methyl-histamine and impromidine.

The newly discovered H3 receptors acting also through G-proteins can be inhibited with thioperamide and agonised with R-alpha-methyl-histamine. The therapeutical importance of the H3 agonists and antagonists is not fully defined yet.

All three classes of receptors can be divided to sub-classes upon the receptor-ligand binding properties but the functional importance of the sub-classes is unknown yet.

The intracellular histamine binding sites

Beside the extracellular receptors specific intracellular binding sites have been discovered /2/, these are found in the nucleus and the microsomes. In the microsomes low affinity (micromolar), in the nucleus low

and high affinity (femtomolar) binding sites were described /5/. The L-histidinol and a phenyl-methyl-phenoxy-aethylamine (DPPE) are competing for this binding sites with histamine. The direct effects of the receptor-binding seems to be closely related to cellular proliferation, but the mechanism is not discovered, either the involvement of the microtubular system, arachidonic acid/thromboxane or a metabolism connected with putative p450 molecule(s) is likely.

The effects of histamine

1. Central Nervous System (CNS)

The main source of histamine in the CNS are the mast cells and the histaminergic neurons in the hypothalamus. Most of them are located in the tuberomammillary nucleus (TM), of the posterior hypothalamus. The role of the histaminerg neurons and histamine receptors is not clearly defined, but the experiments suggest that histamine plays an important role in the CNS.

H1 receptor and its antagonists

The first discovered effect was the sedating side effects of the first-generation H1 receptor antagonists. The ethylen-diamine derivates (e.g. mepyramine, chloropyramine, etc.) are used as antiallergic drugs, but their most important side effect is sedation and somnolence. Beside these the phenothyasine and ethanol-amine derivates are used as depressive and antiemetic drugs. They have not only antihistamine but also antimuscarine effects. These effects are based on neuron depolarisation through H1 receptor, which can neither be inhibited with H2 and H3 antagonists nor can be altered with H3 agonist R-methyl-histamine. The cause of the depolarisation is the inhibition of the non-voltage-dependent K+ "leakage" /23/. The same mechanism can explain the anti-Parkinson effect of some classical antihistamines, however, these drugs are recently less frequently used in the therapy of Parkinson disease.

The histaminergic neurons

The histaminergic neurons of the hypothalamus take part in the regulation of food and water uptake /26/. Activation of H1-receptor or inhibition of the H3-receptor in the ventromedial hypothalamus (VMH) or the paraventricular nucleus suppressed food intake. The antagonists of H1 and H2 re-

ceptors in concentration corresponding to the receptor binding decrease water intake.

The lesions of the above-mentioned TM nucleus is likely to facilitate the performance rats in a variety of learning tasks suggesting an inhibitory effect of the histaminergic neurons /13/.

Alpha-FMH inhibiting histamine production injected into the ventromedial nucleus -- a known centre of regulating food intake -- increases, the H3 antagonists decrease the appetite in rat. H1 antagonist similar to alpha-FMH increase the food intake in animals on low-protein diet.

Histamine plays a role in the regulation of activity and circadian rhythm. H3 antagonist thioperamide increases the locomotor activity in mast cell deficient mice. This effect can be antagonised with methyl-histamine, H1 and H2 antagonists. In H1 deficient mice the decrease of locomotor activity is found /14/.

Neuro-endocrine system

As an effect on the hypothalamic neuro-endocrine system H1 and H2 antagonists prevent the ACTH mediated corticosteroid releasing effect of beta-endorphine. Histamine through H3 receptors facilitates the prolactin secretion and reduces the GH secretion. The histaminergic neurones of the tuberomammillary nucleus have dual effect on oxytocin synthesis and release: they increase the synthesis -- this can be supressed with H1 receptor antagonists -- and inhibit the release of oxytocin through H2 receptors /17/.

2. Immune system, haemostasis

The first known and most studied effect of histamine is its immunological allergological effect. Histamine is released from the granules of the mast cells and basophyl leukocytes. Besides the antigen stimulation via cross-bound IgE antibodies and anaphylactic complement fragments histamine release can be evoked by many drugs. For instance, quinine, morphine, heparin, atropine as well as physical effects, such as sunlight or heat exposition may trigger the liberation of histamine. In vitro the most potent drug inducing histamine release is metoxyphenyl-amine solved in formaldehyde: "48/80".

Inflammation

The inflammatory reactions caused by histamine are mostly related to H1-receptors. Pretreatment with H1 antagonists can prevent the development of odema after treatment with dithranol, 48/80, capsaicin, etc. On the other hand, histamine through H2 receptors inhibits the secretory activity of mast cells and basophil cells and through this the activation of lysosomal enzymes and its release itself. The histamine release induced by Concavaline A can be enhanced by H3 antagonist, which supports the possibility of a H3 mediated self-regulation.

The role of the intracellular receptor

In mouse spleen cells alpha-FMH -- after incubation time (necessary for depletion of histamine pool) -- blocks the DNA synthesis after treatment with a mitogen, Concavalin A /4/. Some antihistamines in the concentration of saturating the intracellular receptor have the same effect. It means that the binding of antihistamines to the intracellular receptors correlates with the antiproliferative potential. Concavalin A acts not only through histamine but also through Ca because at the concentration of DPPE and verapamil inhibiting thimidine incorporation cannot prevent the reaise of concentration of Ca and histamine, respectively.

The Concavaline A-mediated histamine release in mast cells can be inhibited with DPPE, and this effect cannot be overcome with alpha-FMH because preformed histamine in mast cells released from its huge pool.

On the other hand, the reactions for 48/80 or protein kinase C activator TPA are ambiguous and not significant. So DPPE and intracellular histamine has an controversial role in the regulation of histamine release.

Other effects

Histamine potentiates the activation of natural killer cells suppressing the inhibitory effect of monocytes. The basis of this phenomenon is the down regulation CD16 (FcγIII receptor) and CD56 (an immunoglobulin like adhesion protein) surface antigens on NK cells which are induced by monocytes /12/.

Histamine can either up- or downregulate acute phase proteins (e.g. complement proteins) depending its action on H1 or H2 receptors /25/. Moreover, histamine directly enhance the interleukin-6 production in various cells acting through H1 receptors /22/.

The H1 and H2 receptors have opposing effect on the IL-6 binding of B-lymphocytes: H1 receptors stimulate, H2 receptors inhibit the effect /21/.

Collagen and phorbol-acetate in vitro evokes platelet aggregation. Both increases the histamine content of the platelets. Alpha-FMH pretreatment inhibits both the concentration rise and aggregation, while DPPE inhibits aggreagation only, but not the increase of histamine content. In platelets permeabilised with saponine the external histamine can overcome the inhibiting effect of both alpha-FMH and DPPE /18, 19/.

3. Heart and circulation system

Peripheral vessels

In peripheral vessels histamine causes vasodilatation on both H1 and H2 receptors. While through H1 receptors a rapid, but short effect can be observed at lower histamine concentrations, which is related to the endothel derived relaxing factor; H2 receptors related to the adenyle-cyclase cause a late-onset, long-term relaxation.

Although the responses to histamine, in contrast to other vascular regulatory mechanism are not fading with age in man, the effects based on H2 receptors can be more easily suppressed.

On muscular arteries the histamine (through H1 receptors) shows a constrictor effect. The explanation of this phenomenon is the enhancement of Ca influx on the smooth muscle cells. The vasoconstriction can prevent blood pressure lowering effect the peripheral vesselns in rodents, where the vasoconstriction is extremely strong.

Heart

Histamine on H1 and H2 receptors has a direct positive inotropic and chronotropic effect on the heart, and potentiate the arrythmogen agents.

In the field of the central regulation of circulation, intracerebroventricular administration of H2 histamine receptor antagonist cimetidine prevented the decreases of blood pressure and heart rate to a subsequent treatment of clonidine suggesting a role of histaminergic neurons in the regulation of blood pressure and heart rate /1/.

In the recent years the non-sedating antihistamine terfenadine was found to cause various cardiac side effects (e.g. arrhytmia, cardiac arrest, sudden death, etc.). In consequence of this side-effects terfenadine was withdrawn from the OTC market in many countries /16/.

4. Gastrointestinal tract

Gastric acid secretion

The most known and pharmaceutically most important effect of histamine is the stimulation of K-H pumps in the stomach, the HCl excretion. The other physiological regulator of the acid secretion are gastrin and acethylcholine, but their effect is related to histamine. This phenomenon can be explained in different ways:

-- these agents act through different receptors, but gastrin and acethylcholine have an effect only in presence of histamine;

-- gastrin and acethylcholine induces the release of histamine from mast cells and this effect stimulates the K-H-ATPase. The important role of histamine in the gastric acid secretion is also supported by the fact that in the treatment of gastric and duodenal ulcer H2 blocks (Histodil, Ulceran, Quamatel) are the drugs of first choice.

The Hic antagonist DPPE antagonises the stress and alcohol-induced ulcer in animals. This antagonism is more potent than the one of the H2 blockers. This effect can be caused by the 10-fold increase on prostaglandin synthesis due to DPPE therapy. This induction can be suppressed by acethylsalicilic-acid /10/.

H3 receptors have an opposite effect on gastric acid secretion, however H3 receptors are located likely only in the myenteric plexus, where regulate the transmitter release of the non-adrenerg-non-cholinerg neurons.

5. Tumorgenesis, tissue regeneration

The role of histamine in tumor growth is based on various data of increased histamine level described in many neoplasmas (e.g. colorectal cancer, melanoma, fibrosarcoma, etc.). The same phenomenon can be found in rapidly developing embryonal and regenerating tissues /15/.

The role of histamine in the cell proliferation is supported by the enhancing effect of the exogen histamine /3, 29/ and the inhibitory effects of the antagonists /9, 24/. The in vivo antitumor activity of H2 receptors is likely to be mediated through the modification of immune response. In contrast the Hic antagonist DPPE causes a concentration-dependent decrease of the size of human colorectal cancers /8, 28/ although data showing a tumoe stimulation effect were also found /7/. Beside the antineoplastic effect DPPE and L-histidinol potentiate the chemotherapeutics (e.g. antimeta-

bolites), and have a protective effect on normal, slowly proliferating cells. These effects correlate with histamine binding. In conclusion: the antimetabolites (fluoro-uracil, doxiribicin, cysplatin) in ineffective concentration were found to be effective in human breast and colon carcinomas, while in leukaemic mice the selectivity of the same therapy was achieved by the protection of normal colonies. The explanation of this phenomenon is based on spculation yet.

The first possibility is, that DPPE inhibits the timidine and leucine incorporation DNA and protein synthesis in normal cells at micromolar concentration, but increases that of in breast carcinoma cells and transformed lymphocytes. That is, the slowly proliferating cells get typically in G_0, the fastly proliferating cells get in S phase, which is the effective phase of chemotherapeutics /6/.

Second, the DPPE by connecting to intracellular histamine biding sites has an effect on eicosanoid metabolism, forming cytoprotective prostaglandins and malignant cells less sensitive to.

The success of the DPPE in vitro led to clinical investigations. Although the cysplatin resistant ovarian cancers did not have positive response to DPPE administered alone, the cysplatin sensitivity significally increased. Hormone resistant prostatic cancer patients at the maximum tolerable dose of DPPE (6 mg/kg) combined with cyclophosphamide in 50% showed a 50% drop of PSA, correlating with the oncological status. The observed side effects (nausea, ataxia) were consequent with the level of DPPE.

In human melanoma cell lines H1 and H2 receptor antagonists administered alone had no significant effect on cell proliferation, but combinations of H1 and H2 blockers with DPPE strongly decreased cell proliferation /11/. The tyrosinase activity was found to be raised by H1/H2 antagonists alone, and this effect was suppressible with H2 agonist nordimaprit. Nordimaprit did not inhibit the enzyme, but shortens its half-life. Prolonged nordimaprit therapy caused the cells became amelanotic /20/.

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GENETIC DIVERSITY IN PROTEUS PENNERI

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DNA of thirteen Proteus penneri strains derived from four European countries (nine strains from Germany, two strains from United Kingdom, one strain from Turkey, one strain from Hungary) was examined by random amplified polymorphic DNA-PCR (RAPD-PCR) method. RAPD with primer AACGCGCAAC gave different patterns, which suggests a DNA sequence microdiversity within this species. The method provides a fast, economical and reproducible means for typing P. penneri.

Keywords: Proteus penneri — PCR — genetic microdiversity

Introduction

Proteus penneri, formerly known as "indol-negative" P. vulgaris or "P. vulgaris biogroup 1" /1/ has been isolated from a variety of clinical sources and associated with infections of the urinary tract and wounds /2/. A variety of DNA-based methods have been used to identify individual isolates of human pathologic microorganisms including plasmid profiles, chromosomal restriction patterns, Southern blotting and restriction of specific polymerase chain reaction fragments. Independent clinical isolates were often not distinguished from one another in these tests. This could reflect a clonal population structure in several bacterial species /3/, or limitations in the method used for strain identification. In this study we investigated the use of RAPD for typing of this bacterium species.

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Materials and Methods

Twelve strains of P. penneri have been kindly supplied by Dr. B. W. Senior (University of Dundee, UK) /4/. One strain was isolated from wound infection in Hungary and identified by standard biochemical methods /5/.

Chromosomal DNA was isolated according to the method of Owen and Borman /6/ with the modification that 100 µl overnight cultures were used for DNA preparation.

PCR was carried out in 50 µl volumes containing 20 ng of P. penneri DNA, 20 pmoles of primer, 200 µM each of dATP, dGTP, dCTP and dTTP (Pharmacia), 1 U of Taq DNA polymerase (Pharmacia) in 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0 with primer AACGCGCAAC. A Techne GeneE HL-1 thermal cycler was used for amplifications. The cycling programs were 30 cycles of 94 °C 1 min, 36 °C 1 min, 72 °C 2 min, preceded by an initial denaturation step (4 min at 94 °C) and followed by a final elongation step (10 min at 72 °C). After PCR 20 µl aliquots of products were electrophoresed in 1.5% agarose gels containing 1 µg/ml ethidium bromide, 1X Tris acetate running buffer, and photographed under UV light. The 1 kb DNA ladder (Gibco BRL) was used as size marker in all gels.

Results and Discussion

Figure 1 shows the results of RAPD analysis. With primer AACGCGCAAC RAPD-PCR resulted in strain-specific arrays of up to about 11 bands in the 0.5 kilobase (kb) to 6 kb size range for P. penneri. A band of about 1.3 kb size in different intensities was characteristic for all P. penneri strains studied while 11 strains gave an about 0.9 kb band, too (Fig. 1). The patterns differed from each other at least from one to several bands. The patterns of strains E1125, E1152 and E1263 (lanes 5, 6, 7) seemed to be identical, however, the examination of the gels by eye revealed subtle differences.

RAPD-PCR method revealed a significant DNA diversity among P. penneri strains. We have found no completely identical patterns in the strains examined. This could reflect genomic microdiversity /7/ that can represent allelic variation in this bacterium. All P. penneri isolates were repdoducibly typable with this method and could be divided into two main groups. For Group I (Fig. 1, lanes 1, 3, 5, 6, 7, 8, 10, 11, 13) bands of 0.9, 1.3 kb and several other bands were characteristic. Group II strains presented only two to five bands (Fig. 1, lanes 2, 4, 9, 12).

According to our results, RAPD is probably a choice for large-scale epidemiological studies of P. penneri. It is a quick, economical technique with high reproducibility and typability, and provides a degree of discrimination. The value of such epidemiological studies lies in the recent evolu-

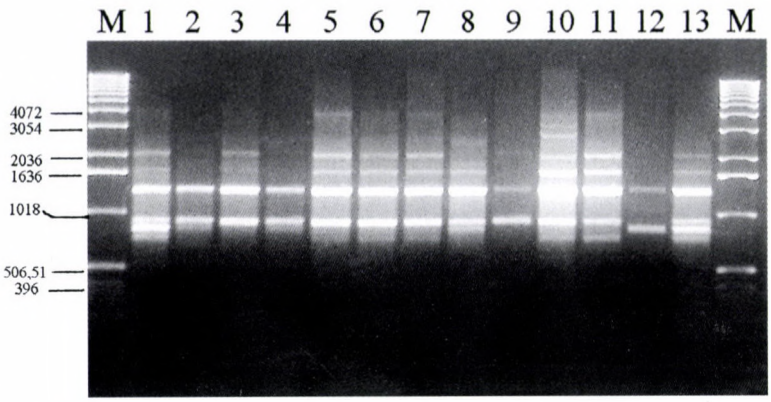


Fig. 1. RAPD analysis of P. penneri strains. DNAs were prepared and amplified as described in Materials and Methods and were run in a 1.5% agarose gel. DNAs were amplified with primer AACGCGCAAC. M: 1 kb DNA ladder. Lanes: **1**, strain E46, **2**, strain E100, **3**, strain E180, **4**, strain E587, **5**, strain E1125, **6**, strain E1152, **7**, strain E1263, **8**, strain H1209, **9**, strain H1360, **10**, strain 05665V, **11**, strain 16803Z, **12**, strain T77, **13**, strain 357. The first 9 strains derive from Germany, two from UK (lanes **10**, **11**), one (lane **12**) from Turkey, one (lane **13**) from Hungary

tion of P. penneri as a bacterial pathogen in hospitals, including nosocomial outbreaks, as well as in the community.

Acknowledgements

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PHOSPHOFRUCTOKINASE INTERACTS WITH MOLECULAR CHAPERONINS GroEL and GroES

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For studying the possible interaction between the chaperonins and phosphofructokinase (PFK), bacterial chaperonins GroEL, GroES, and the PFK were co-purified from chaperonin over-expressing, heat treated E. coli strains. GroEL interacted with PFK in the presence of Mg^{2+}, leading to a gradual decrease in the activity of the enzyme. This type of GroEL-PFK interaction was overcame by the GroES. On the effect of the addition of ATP to the GroEL-PFK complex, the decreased activity of the enzyme was recovered suggesting release of the GroEL-bound enzyme. Contrary to this, in a complete refolding system containing GroEL, GroES, ATP and Mg^{2+}, activity of heat treated bacterial PFK gradually increased showing, that GroEL and GroES together play a role in the folding and/or assembly of PFK. Similar effect of refolding system was also observed on rabbit muscle PFK. The data show that PFK can interact with GroEL, which results in binding of the enzyme; by contrast, GroEL and GroES together has a folding effect leading ultimately to increase of the activity of the enzyme.

Keywords: Phosphofructokinase — chaperonins — GroEL — GroES

Introduction

In the past decade it became clear that a family of macromolecular cellular components exists which modulates and promotes protein folding and/or assembly and disassembly, and facilitates the elimination of misfolded peptides /4, 5/. Belonging to these proteins called molecular chaperons, chaperonins GroEL and GroES, products of the GroE operon, are present in all prokaryotes and in organelles of eukaryotic cells. GroEL is arranged as two stacked rings of seven subunits of 60,000 Dalton protomers, and GroES forms a single ring of seven subunits of 10,000 Daltons. GroEL interacts with GroES in ATP dependent manner; the ATP-ase activity of GroEL is inhibited by GroES in the absence of unfolded protein as substrate /6/.

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In vitro GroEL has been found to form complexes with certain unfolded proteins, like ribulose bisphosphate carboxylase, pre-β-lactamase, dihydrofolate reductase, citrate synthase, rhodanase, and GroEL itself /6, 8/. Hydrolysis of ATP by GroEL promotes the release of protein chain from the complex if GroES is not present. So far positive folding effect of chaperonins has been demonstrated on enzymes exhibiting spontaneous refolding after chemical denaturation /11/.

In the present work the possible interactions between the chaperonins and PFK were investigated. Since the use of relatively drastic chemical denaturants, like guanidine or urea, led to irreversible loss of activity of the enzyme, other approaches were chosen to induce damage of the PFK. Since peptide aggregation or misfolding frequently occurs in vivo during protein synthesis at elevated temperatures, in the present works the PFK was isolated from E. coli exposed to higher temperatures. In addition, the enzyme was heat-exposed during the purification, and in certain experiments, ATP (a known protector of PFK) was also withdrawn from solutions of the purification procedure to allow conformational changes leading to decrease of the activity of the enzyme.

Materials and Methods

Purification of GroEL, GroES and PFK

Over-expressing strains of E. coli (POF 39 and PGTI R18) were generous gifts of Constantine Georgopoulos, University of Utah, Salt Lake City, and Leila Gierasch, University of Texas, Southwestern Medical Center at Dallas. The E. coli strains grown in 5600 ml LB medium containing 50 μg/ml ampicillin. After reaching the OD 0.4 the cultures were exposed to 42 °C for 120 min to promote the expression of chaperonins by the heat stress /2/. The purification procedures of GroEL and GroES were done essentially as described /7/. After harvesting and sonication of cells the supernatants were treated with ammonium sulphate twice. The dissolved 1.6-2.2 M ammonium sulphate cut was heat precipitated at 50 °C in water bath for 30 min. The extract was further purified on a 500 ml column of Sephacryl S-400 (S 400) equilibrated with 50 mM Tris-Cl buffer containing 125 mM NaCl, 2.5 mM $MgCl_2$, 2 mM ATP (buffer A), and 1 mM NaN_3. The same buffer without NaN_3 was used for elution. The fractions (5 ml each) containing the chaperonin proteins were concentrated with Amicon concentrators, and the concentrated proteins (200 μl) were purified to GroEL and GroES with Dionex HPLC equipped with a Mono Q column as with using the solutions and parameters as described /7/. The pure chaperonins giving single bands with SDS-PAGE were stored at 4 °C in 50 mM Tris-HCl, pH 7.5.

The fractions of S-400 effluent containing PFK were combined, concentrated, and were purified by affinity chromatography with an ATP sepharose column as described /9/, with except that the enzyme was eluted with 50 mM

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Tris-Cl, pH 7.5 containing 2.5 mM ATP and the enzyme was stored in 50 mM Tris-Cl, pH 7.5, 2 mM $MgCl_2$ and 2 mM dithiothreitol (DTT). The specific activity of the enzyme obtained with this procedure was approximately lower with two orders of magnitudes as compared with other methods /9/. Mammalian PFK was purified from rabbit muscle as described /10/.

Analytical procedures and methods

The PFK activity was determined at the optimum condition by measuring the rate of the formation of fructose-1,6-P at 25 °C /10/: in 1 ml of final volume the assay mixture contained 50 mM Tris-HCl, pH 8.0, 1 mM fructose-6-P, 0.1 mM EDTA, 6 mM $MgCl_2$, 4 mM $(NH_4)_2SO_4$, 0.15 mM NADH, 2.0 mM DTT, enzyme mixture containing 0.4 unit of aldolase, 2.5 units of triose-phosphate isomerase, 0.4 unit of α-glycerophosphate dehydrogenase and the sample to be assayed. The reaction was initiated by the addition of ATP giving final concentration of 1 mM and the absorbance at 340 mm was recorded continuously with a Gilford spectrophotometer. The citrate synthase activity was determined by direct measurement of the thioester bond of the acetyl-CoA. The purity of chaperonins were controlled by SDS PAGE, and was compared to that which was purified in laboratory of Leila Gierasch (Department of Pharmacology, University of Texas, Southwestern Medical Center at Dallas). To test the biological activity of chaperonins the refolding system of citrate synthase was used /1/. For SDS PAGE the Pharmacia Phast-system was used. All experiments were repeated at least twice and the measurements were done in duplicates, results are shown as means ± SE.

Results

Interaction between PFK and GroEL

With the usual chemical denaturants like 6 M guanidine HCL or with 8 M urea /1/ prompt and irreversible denaturation of PFK was observed and we could not find any effect of the chaperonins on the denatured PFK. We assumed that the heat treatment of the bacteria during the culture, and heat treatment of the enzyme during the purification; or elimination of the protective ATP from the purification media and from the storage media might promote the degradation and/or the misfolding of the PFK, which can generate target proteins for chaperonin action.

For the study of the interaction of GroEL and PFK, activity determinations were done in incubation mixture containing GroEL and the bacterial enzyme (Fig. 1). To avoid the ATP-induced increase of the activity of the stored enzyme, in these experiments the PFK was incubated overnight in storage media containing 2 mM ATP and the ATP was washed out with Amicon microconcentrators immediately before the experiment. During the incubation at room temperature activity of PFK gradually decreased (Fig. 1), and the

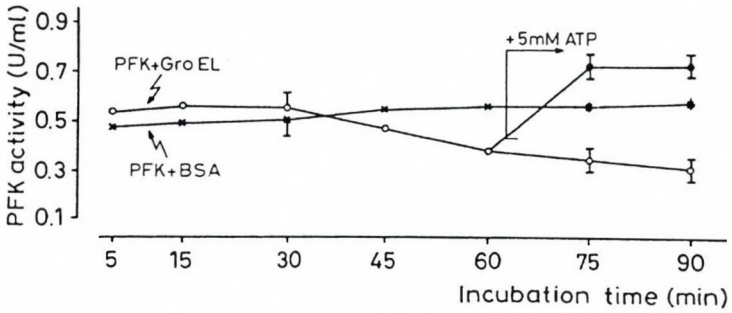


Fig. 1. Decrease of activity of PFK caused by the interaction of the enzyme with GroEL. Forty μl of bacterial PFK (8 mg/ml storage buffer, activity 21.8 U/ml) was mixed either with 4 μl of GroEL (-o-, protein 3 mg/ml in 50 mM Tris-Cl pH 7.5); another 40 ul with 4 μl of BSA (-x-, protein 2.9 mg/ml in 50 mM Tris-Cl pH 7.5) at room temperature. After 60 min incubation ATP was added giving final concentration of 5 mM (-●-, GroEL-PFK plus ATP; -x-, BSA-PFK plus ATP). The PFK activity of the incubation mixture was measured as described using an aliquot directly for the assay; the results were calculated based on the linear OD change after the lag-period

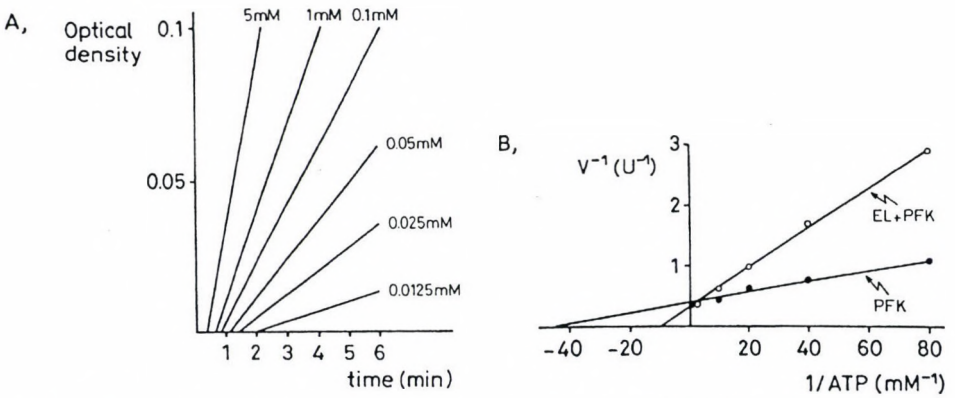


Fig. 2. Kinetics of the PFK interated with GroEL. Panel A: GroEL (7 μl, 3.3 mg/ml) and 7 μl PFK were mixed with 10 μl buffer (50 mM Tris-Cl pH 7.5, 2 mM $MgSO_4$) and 10 mM DTT. After 120 min incubation at room temperature the PFK activity of the incubation mixture was measured using 1 μl aliquots and different final ATP concentrations in the cuvettes, and the OD change was recorded at 340 nm. The original activity of the incubation mixture was 8.8 U/ml; as assayed immediately after mixing the GroEL, PFK and the buffer solution; this value decreased to 3.5 U/ml after 120 min storage as assayed using 5 mM final ATP concentration in the cuvette. Panel B: the same experimental setup was used for the determination of the Lineweaver–Burk diagram of PFK for $/ATP^{-1}/$. Key: -●- PFK without GroEL (equation curve: y = 0.0338x + 0.284), -o- PFK with GroEL after 120 min of incubation as described above (equation: y = 0.0079x + 0.407); data of -o- are from experiments 2/A. (No difference of V_{max} is seen in the figure since in the experiments with non-GroEL reacted PFK the amount of enzyme was chosen to be 3.3 U/ml, giving the same V_{max})

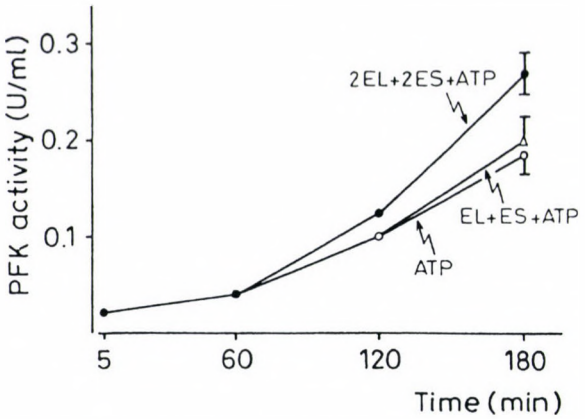


Fig. 3. The chaperonins GroEL and GroES together promote the repair of PFK activity. PFK obtained during ATP free S-400 purification of chaperonins showed only 0.046 U/ml PFK activity. 40 µl of enzyme incubated with 40 µl of buffer (50 mM Tris-Cl, pH 7.5, 4 mM $MgSO_4$, 4 mM KCl, 4mM DTT and 10 mM ATP) alone (-o-); in the presence of 6 µg (0.007 nmoles) GroEL and 1.48 µg (0.02 nmoles) GroES (-Δ-); or in the presence of 12 µg (0.014 nmoles) GroEL and 2.96 µg (0.04 nmoles) GroES (-●-) at room temperature and the PFK was assayed at the given times using 15 µl aliquots of the incubation mixture

PFK with decreased activity showed a lag-priod during the assays. The presence of molar excess of GroES to GroEL prevented the interaction of GroEL with PFK (not shown; similar experiment with non-ATP-pretreated PFK is shown in Fig. 5, curve -x-). The decreased activity of PFK restored after addition of ATP to the GroEL-PFK mixture (Fig. 1). Similar change of PFK activity was not observed if the enzyme was incubated with bovine serum albumin having similar molecular weight as the GroEL (BSA, Fig. 1) or stored alone in the same incubation buffer (not shown). Addition of ATP alone to PFK had no effect on the activity of the enzyme under these conditions.

Duration of the lag-period in assays of GroEL interacted PFK was negatively correlated with the concentration of ATP used in the PFK assay mixture (Fig. 2A). This lag-period was seen only if the original activity of the PFK decreased. Kinetic analysis of data showed (Fig. 2B) that the apparent K_m for ATP increased from 20 to 120 µM.

Effect of GroEL and GroES on the activity of PFK

When the chaperonins and PFK were purified on S-400 column as described with except that the ATP used for the protection of the enzyme was eliminated from the media (neither the equilibrating medium nor the elution buffer contained ATP), the protein profile of the effluent remained the

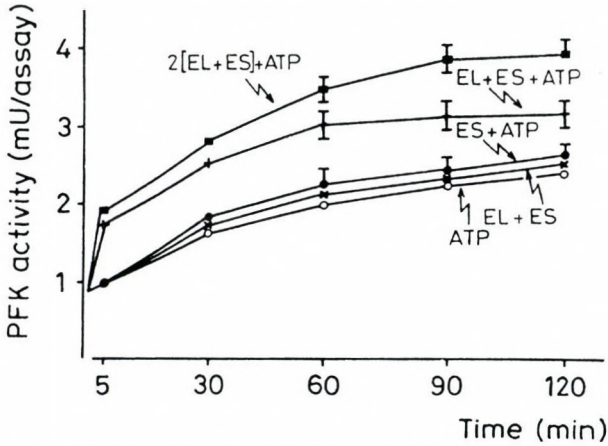


Fig. 4. Effect of chaperonins GroEL and GroES on the activity of bacterial PFK in the presence of ATP and Mg^{2+}. Bacterial PFK (specific activity 26.2 U/mg) was incubated in Tris-Cl 50 mM, pH 7.5, 5 mM KCl, 5 mM $MgSO_4$, 5 mM DTT, with or without ATP (6.7 mM final concentration) and different amounts of chaperonins in the final volume of 14 µl. Key: -o-, ATP without chaperonin; -x-, GroEL (1 µl of 13.5 mg/ml protein, total of 0.016 nmol) and GroES (2 µl of 1.7 mg/ml protein, total of 0.049 nmol); -●-, GroES with ATP; -+-, GroEL, GroES with ATP; and -■-, 2 volume of GroEL, GroES (1x amounts are as indivated above) and ATP. The incubations were done at room temperature in a final volume of 14 µl, and at the indicated times 2 µl aliquots were used directly for PFK assay. Activity of PFK at 0 min was 0.91 ± 0.03 mU/assay

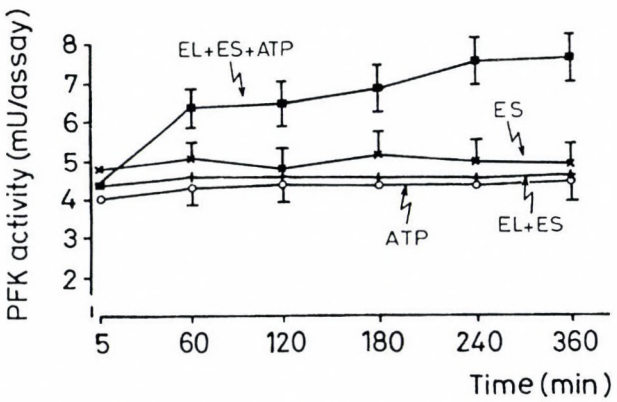


Fig. 5. Effect of GroEL and GroES on rabbit muscle PFK in the presence of ATP and Mg^{2+}. Purified rabbit muscle PFK (6 µl, 5 U/ml, 0.4 nmol/ml) was incubated in Tris-Cl, 25 mM pH 7.5, 5 mM KCl, 5 mM $MgSO_4$, and 5 mM DTT with or without ATP (2.5 mM final concentration) and chaperonins in a final volume of 125 µl. Key: -o-, ATP without chaperonins; -x- GroES (18 µl of 0.15 mg/ml protein, total of 0.039 nmol); GroEL (12 µl of 3 mg/ml protein, total of 0.043 nmol) and GroES -+-; -x-, GroEL, GroES and ATP (amounts as above). Incubations were done at room temperature and 20 µl samples were used directly for PFK assay. GroEL alone had no effect on PFK activity during this period, however, in long-term incubations (> 6 hours) the activity decreased, which was reversed by ATP similarly to the bacterial PFK (Fig. 1). PFK activity at 0 min was 4.41 ± 0.39 mU/assay

same, but only trace (< 2% of usual) of PFK activity was detected as compared to experiments when ATP was used. Addition of ATP to this fraction the activity of PFK increased by the time showing an ATP induced reconstruction of activity (Fig. 3). On the effect of chaperonins GroEL and GroES this recovery of activity was considerably higher and depended on the concentrations of chaperonins used (Fig. 3).

Possible effects of GroEL and GroES were tested with PFK isolated from the heat-treated bacteria but in the presence of ATP in the solutions (except the storage medium), so the enzyme might suffer only heat-induced damage. Increase of the activity of the enzyme was the highest in the presence of the complete refolding system consisting of GroEL, GroES, ATP and Mg^{2+} (Fig. 4). The same effect was observed when activity of purified mammalian (rabbit muscle) PFK was monitored in incubations containing the chaperonins GroEL, GroES, ATP and Mg^{2+} (Fig. 5).

Discussion

In the present work interaction between GroEL and PFK was demonstrated. The decrease of the activity of the PFK in the presence of the GroEL was a likely result of the binding of damaged protein molecules having remainder activity. The lag-period, and the behavior of the kinetic parameters of the PFK also provide evidence for binding of the enzyme; the GroEL-substrate interaction is known to be associated even with destabilization of the secondary structure of the substrate /11/. A biological meaning of the formation of the GroEL-PFK complex alone may be related to the sequestering function of the GroEL. This sequestering function includes the trapping of proteins in misfolded form, enabling them to assume productive conformation /3, 6/.

After substrate-GroEL interaction the subsequent events leading to the release of the bound peptides are different. Binding of Mg-ATP to GroEL is sufficient to release some substrate proteins, but others are not released unless GroES is present. For example, rubisco is released from GroEL only in the presence of GroES, ATP and K^{+} /11/, but for the liberation of several other proteins from the complex, the presence of ATP is only necessary /6/. The data of the present study show that for the release of PFK the Mg-ATP is sufficient.

The GroES molecule forms a cap on one surface of the stacked rings of the GroEL cylinder, and the interaction also causes tructural changes of the GroEL ring-complex /3/. The substrate binding site of the GroEL complex is in the central hole of the stacked-protein rings on the opposite end of the GroEL. In the current study the presence of the GroES prevented the interaction of the GroEL and the PFK. Structural changes on GroEL due to the interaction with GroES probably can affect the PFK binding sites and binding characteristics of the complex.

Besides the sequestering function probably the more important biological meaning of chaperonin action in general is the promotion of the development of correctly folded structure of certain polypeptides. Again, in the present series of experiments the PFK underwent heat exposure or was ATP-unprotected, which could positively induce damage in the enzyme. Albeit the incubation of the partially inactive enzyme with ATP alone led to a recovery of the activity, which is a known phenomenon, incubation of the enzyme with the same ATP concentration the increase of the PFK activity was unequivocally higher in the presence of a complete refolding system consisting of GroEL, GroES and Mg^{2+} in all experiments. These data show that under certain circumstances the bacterial PFK interacts with chaperonins and on the effect of GroEL and GroES in the presence of ATP and Mg^{2+} the interaction results in an increase of the activity of the enzyme.

Despite of the results obtained on bacterial PFK, the in vivo biological significance of the findings obtained with mammalian PFK is not so clear. In mammals the PFK and the chaperons corresponding to the Gro family are located in different compartments, the former in the cytosol while the latter in the mitochondria. The results obtained with muscle PFK are in consistence with those observed with bacterial PFK, however, the enzyme was heat-treated only during the purification procedure /10/. The data suggest that as the sequences of chaperons are highly conserved during evolution /4/, the sequences in the mammalian PFK required for recognition and action of chaperonins are conserved as well.

Based on the results of the present work, it can be concluded that the bacterial (and possibly the mammalian) PFK belongs to the substrate family of the chaperonins. Probably a multiple type interaction can physiologically occur. This includes the trapping or sequestering of the misfolded enzyme by GroEL and folding/assembly of the misfolded enzyme, and combination of them.

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PITUITARY ADRENOCORTICOTROPHIC (ACTH) CELLS DURING REPRODUCTIVE CYCLE IN A VESPERTILIONID BAT, SCOTOPHILUS HEATHI

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Present study described the pituitary adrenocorticotropic (ACTH)-cells during different phases of reproductive cycle of Indian Vespertilionid bat, S. heathi. ACTH-cells were identified by histochemical, immunocytochemical and electron microscopic techniques. These cells shows round to polygonal in shape and be easily distinguished by histochemical staining. The body weight attains peak during recrudescence phase due to accumulation of fat. The mean areal fraction occupied by ACTH-cells were higher in recrudescence and winter dormancy as compared with other reproductive phases. The size and granulation pattern of these cells showed variation in relation with the seasonal fat cycle. The possible significance of ACTH-cells during periods of recrudescence and winter dormancy, which is also coincided with period of delayed ovulation in this species, were discussed.

Keywords: ACTH-cells — immunocytochemistry — electron microscopy — bat

Introduction

Reproduction in bats is of special interest due to several unique adaptive specialization, which have been reviewed time to time in the past /4, 14, 17, 18, 28, 29, 31/. Certain species of bats have exceptionally high circulating level of cortisol and corticosterone that are matched in vertebrates only by squirrel monkeys /10, 12/. The basis for the unusually high level of glucocorticoides in bats is presently unknown. The major function of adrenocorticotrophic hormone (ACTH) is to regulate the synthesis and release of glucocorticoids in the adrenal cortex. Glucocorticoids exert multiple metabolic effects and have been linked to the development and maintenance of obesity /20/. Corticosterone was shown to be necessary for in-

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creased food intake and gain of body mass /16/. In ground squirrels, cortisol levels increased during the season of fat deposition /9/. Several bat species including S. heathi have also shown the seasonal variations in body weight mainly due to changes in the accumulation and metabolism of white adipose tissues /2/. Coinciding with the period of increased body weight during recrudescence and winter dormancy, S. heathi has been shown to produced exceptionally high androstenedione, which was suggested to be responsible for delayed ovulation in this species of bat /1/. This suggest that in S. heathi reproductive cycle is uniquely influenced by seasonal fat cycle. Since ACTH is also known to regulate androgen synthesis in the adrenal /22/, it would be interesting to find out the pattern of ACTH synthesis and release in S. heathi during reproductive cycle. Immunoreactive ACTH level is measured only in Pteropus hypomelanus using human ACTH polyclonal antibodies and were reported to be well within the mammalian range /34/. Changes in adrenocorticotrophs (ACTH-cells) in relation to reproductive cycle has been earlier been reported only in two species, Miniopterus schreibersii /24/ and Macrotus californicus /29/. In S. heathi seasonal changes in gonadotrophes and lactotrophs has recently been demonstrated in relation to delayed ovulation /30/. The objective of the present study was to characterize the ACTH-cells using histochemistry, immunocytochemistry (ICC) and electron microscopy. Furthermore, the variations in ACTH-cell, identified using ICC in the pituitary, was also studied during different phases of reproductive cycle of S. heathi.

Materials and Methods

Thirty-six adult females of S. heathi were collected from varanasi, India (25^{0} N, 83^{0} E) during November 1990 to December 1992. Bats were transported to the laboratory and were sacrificed by decapitation within two hours of their capture. The body weight and amount of white adipose tissue deposited were recorded for each bat. The pituitary gland along with brain were fixed in Bouin's fluid for 24 h, and then preserved in 70% alcohol. The tissues were dehydrated in ethanol, cleared in xylene and embedded in paraffin wax. The serial section were cut at 6 μm.

Histochemistry

Several standard histochemical staining procedures, such as Alcian blue Periodic acid Schiffs-Orange G (AB-PAS-OG), Lead hematoxylin (PbH) and Herlant tetrachrome, were employed to identify ACTH-cells.

Immunostaining

ACTH-cells were identified using peroxides anti-peroxides technique /33/. The hACTH antisera (NIDDK, USA lot No. AFP-6242891) at 1:500 dilution were used in this study. The section were deparfinized and treated with 3% H_2O_2 in 10% methanol for 5 minute in order to inactivate endogenous peroxides activity. The detailed methods are described elsewhere /30/.

Electron microscopy

Bats were sacrificed by perfusion fixation. The fixatives consist of 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Tissues were postfixed in 2% osmium tetraoxide in 0.1 M phosphate buffer in 3% sucrose. After dehydration in graded series of ethanol, tissues were embedded in araldite. The ultrathin section from selected area were cut on LKB ultramicrotome, mounted on copper grid stained with uranyl acetate and lead citrate and examined in Phillips EM 400.

Morphometric Analysis

Bats were divided into five groups as described earlier /30/. At least 3 bat each collected during Quiescence (August—October), Recrudescence (November), Winter dormancy (December—January), Breeding (February) and Pregnancy (March—July) were used for morphometric analysis. Pituitaries from these bat were serially section at 6 μm and ACTH-cell were localized using PAP immunocytochemistry. Percentage area occupied by ACTH immunoreactive cells in relation to total area of the pars distalis (PD) in serial section were estimated using standard point counting techniques /34/. The details of the morphometric procedure employed in this study was recently described in detail /30/. The section used for morphometric analysis were selected by a systematic random scheme /35/. The size of ACTH cells were measured by an ocular micrometer on pituitary section subjected to immunopositive cells. The dimensions of the stained cells types appearing largest in serial section was measured. At least 15 different cells were measured for each animals. The pituitaries from at least 3 females from each group were examined for morphometric analysis. The data were analyzed by ANOVA and Duncan's.

Results

Body weight

Body and white adipose tissue weight exhibit significant variation during reproductive phases (Fig. 1a, b). Body weight attained a peak during November due to accumulation of white adipose tissue. The body weight gradually declines during winter and a basal level of body weight attained during February (Fig. 1a, b).

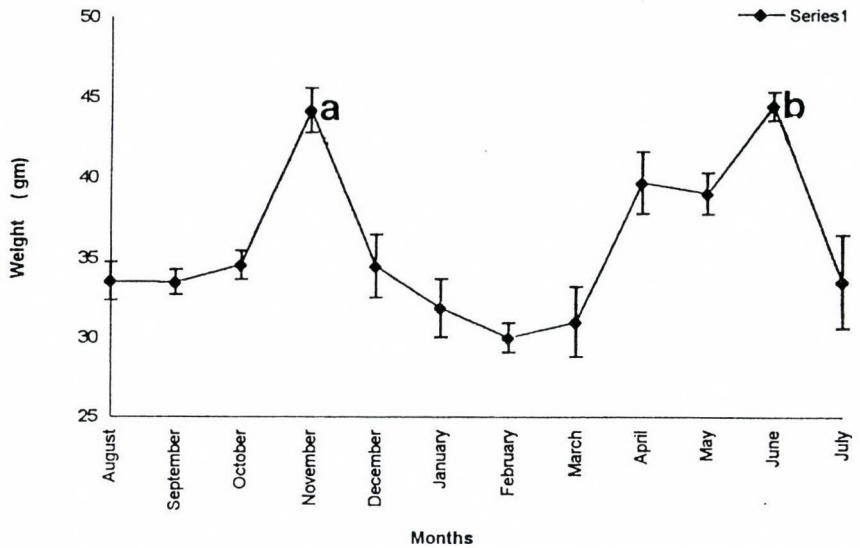


Fig. 1a. Seasonal changes in body weight of mature female S. heathi

Values are (± SE) a) P < 0.001 vs. other month of the year; b) P < 0.001 vs. Other month of the year (body weight increase due to pregnancy)

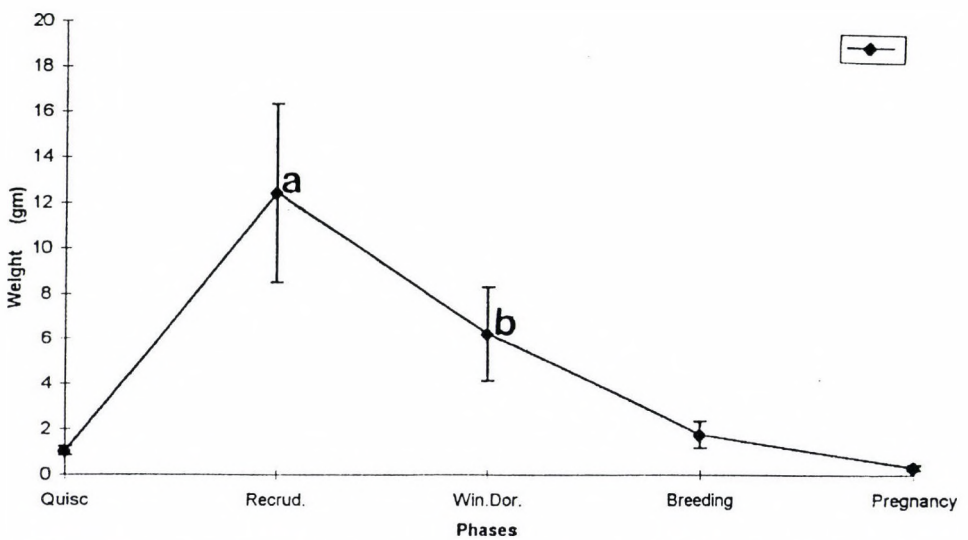
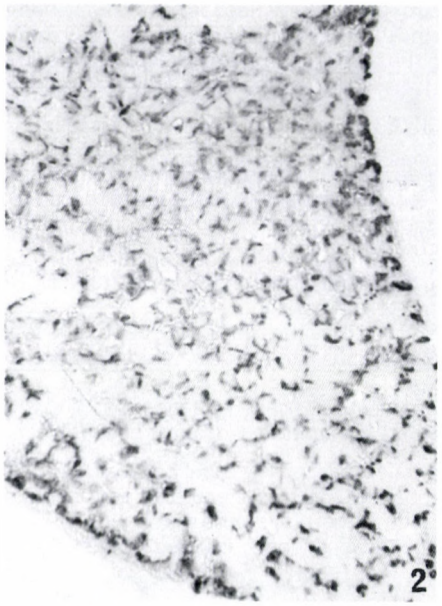
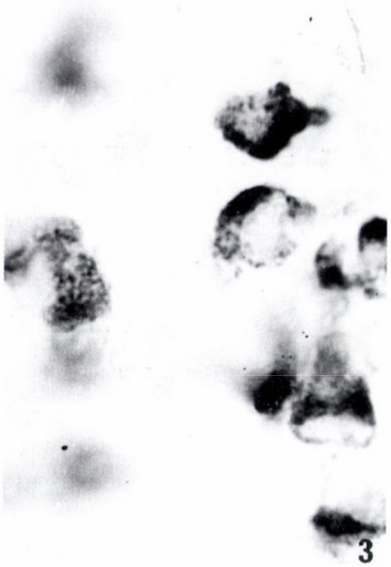
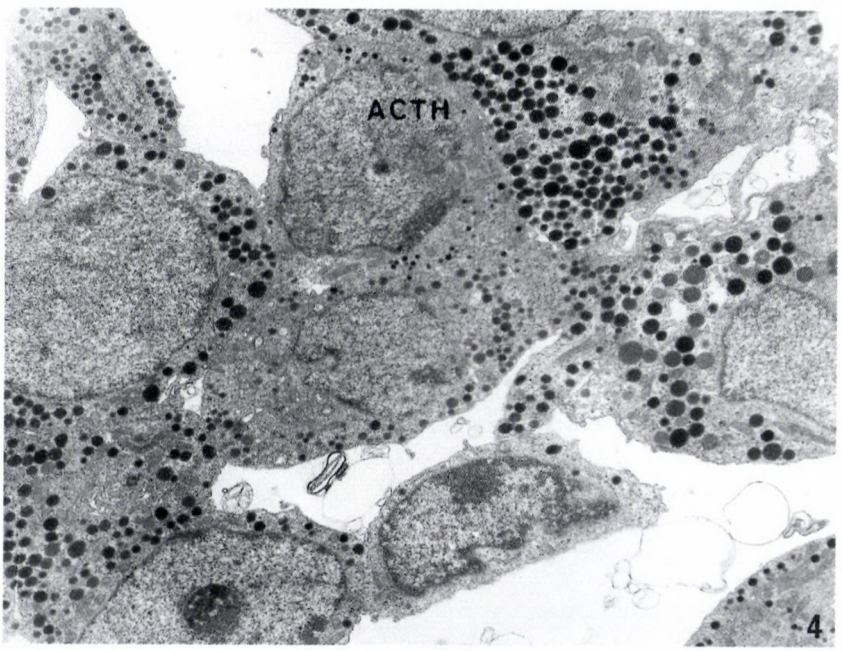


Fig. 1b. Seasonal changes in weight of white adipose tissue during different reproductive phases in female S. heathi

Values are (± SE) a) P < 0.001 vs. Quiescence, Winter dormancy, Breeding and Pregnancy; b) P < 0.001 vs. Quiescence, Breeding and Pregnancy







Figs 2—4. All figure are transverse section of the anterior pituitaries of female S. heathi

2. Section of parts distalis showing distribution of ACTH cells stained immunocytochemically with antisera against h ACTH, x 60. 3. ACTH-cells stained immunocytochemically with antisera against h ACTH, x 650. 4. Low power electron micrographs of pars distalis of female S. heathi during breeding phase showing ACTH-cells, x 5600

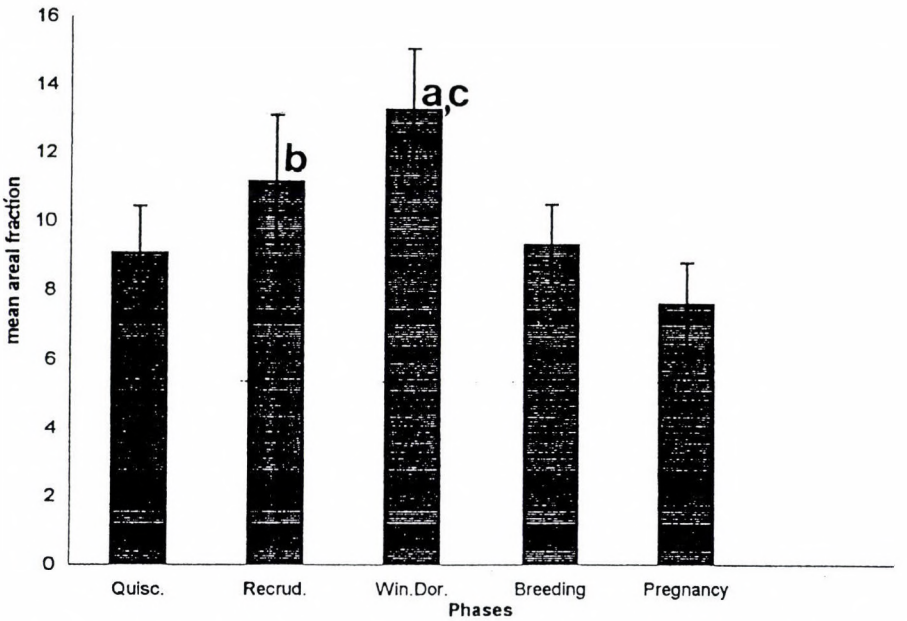


Fig. 5a. Seasonal changes in mean areal fraction occupied by ACTH-cells during different reproductive phases in S. heathi

Values are mean (± SE) a) $P < 0.05$ vs. Quiescence, Breeding and Pregnancy; b) $P < 0.05$ vs. Pregnancy; c) $P < 0.05$ vs. Recrudescence

Morphological features of ACTH-cells

ACTH-cells were identified in the PD by histochemistry, immunocytochemistry and electron microscopy. Immunoreactive ACTH-cells using corresponding antisera were mainly observed singly or in small clusters in antero-ventral region of the PD (Fig. 2). These cells showed round or oval to polygonal in shape (Fig. 3). The ACTH-cells did not show any reactivity with antisera against hβ LH, hβ FSH, hβ TSH and h PRL. These cells could also be easily seen by histochemical staining like PbH, with which they stained dark blue or black (Fig. 6).

The ultrastructural examination of ACTH cells revealed the presence of small secretary granules of about 100-190 nm in diameter (Fig. 4). The nuclei were ovoid to irregular in shape. The mitochondria with spaced cristae were also seen.

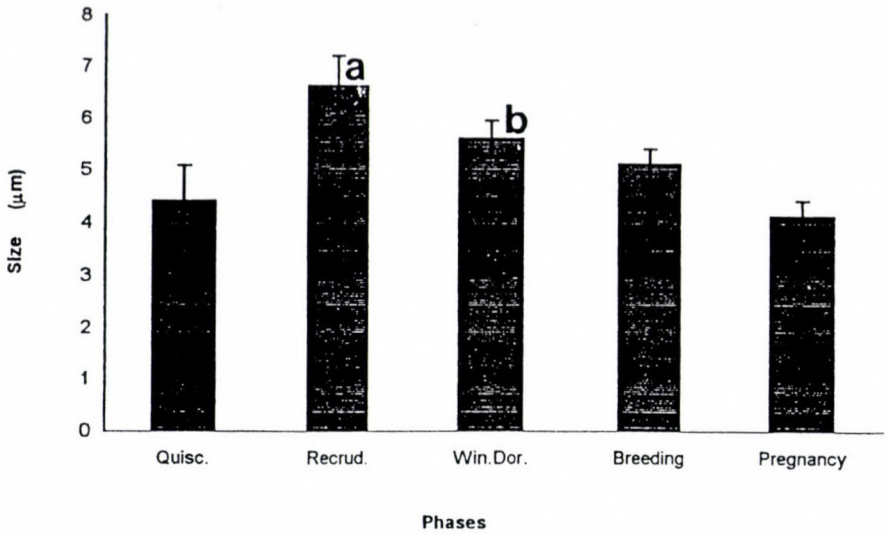
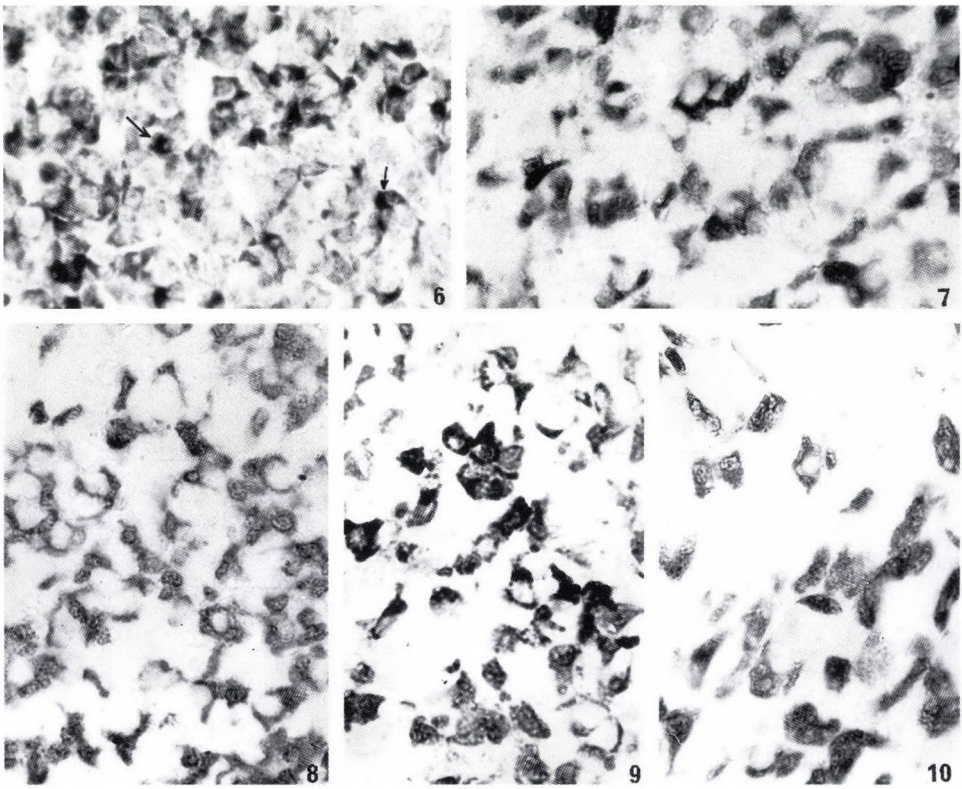


Fig. 5b. Seasonal changes in size of ACTH-cells during different reproductive phases in S. heathi

Values are mean (± SE) a) $P < 0.05$ vs. Quiescence, Winter dormancy and Breeding; b) $P < 0.05$ vs. Pregnancy

Seasonal variation

The mean area fraction, size and granulation pattern of ACTH-cells clearly showed variation during reproductive cycle (Fig. 5a, b). The areal fraction occupied by ACTH-cells were significantly higher during recrudescence and winter dormancy as compared with quiescence and pregnancy. During quiescence ACTH-cells were small and showing degranulation (Fig. 7). The ACTH-cells were large and showing intense immunoreactivity during recrudescence (Fig. 8). The cell size remained large and showing a sign of mild degranulation during winter dormancy (Fig. 9). The cells were large and granulated during breeding phase (Fig. 10) and were slightly smaller and degranulated during pregnancy.



Figs 6—10. All figures are transverse section of anterior pituirary of female S. heathi 6. Section of pars distalis stained with PbH showing ACTH-cells (arrows), x 240. 7. ACTH-cells during non-breeding phase (August) stained ICC with antiserum against hACTH showing degranulation of cells, x 275. 8. ACTH-cells during recrudescence phase (November) stained ICC with antiserum against hACTH showing large and granulated cells, x 275. 9. ACTH-cells during winter dormancy (December) stained ICC with antiserum against hACTH showing cells are intense immunoreactive, x 275. 10. ACTH-cells during breeding phase (February) stained ICC with antiserum against hACTH showing cells are large and granulated, x 275

Discussion

The identification of ACTH-cells in the PD of S. heathi using histochemical, ICC and electron microscopy broadly agrees with the findings in other bat species /7, 24, 29/. Herlants /19/ identified these cells in Myotis myotis as classical acidophils by trichrome methods. These cells again shown to be amphophilic in nature in Rousettus leschenaulti /8/. In the present study these cells were identified by PbH techniques. These PbH positive nature of ACTH cells are consistent with report in certain teleost fishes /5/, Slender loris /3/, North Indian langoor /13/ and in certain bats /7, 8, 29/. The localization of ACTH-cells was also confirmed by ICC techniques /24/. Patil /27/ could not identified these cell type in three species of Hipposiderid bats.

Ultrastructural characteristics of ACTH-cells includes small size of the granules and mitochondria with spaced cristae. These characteristics corresponds to those reported for other bat species /8, 29/, in rat /26, 32/, rabbit /11/, cow /17/ and mink /25/.

The present study showed significant ($P < 0.05$) variations in the size and mean areal fraction of ACTH-cells during different reproductive phases. Seasonal variation in ACTH-cells has earlier been studied only in two bats species, Miniopterus schreibersii /24/ and Macrotus californicus /29/. ACTH-cells were small and showing intense degranulation during quiescence. This may indicate increase in release of ACTH during this period. This also coincided with the period of increased feeding of insects in S. heathi during these months /30/. This is followed by conspicuous hypertrophy and increase in granular content of ACTH-cells during recrudescence in November. This increased immunoreactivity and areal fraction of ACTH-cells coincided with the period of maximum fat accumulation in S. heathi /2/. The intensity of immunoreactivity and size of ACTH-cells declined during winter dormancy and breeding phases as compared with recrudescence phase. This decline in size and areal fraction of ACTH-cells coincided with the period of fat mobilization in S. heathi. This also corresponds with low circulating level of corticosterone in male S. heathi during November /21/. Seasonal variation in ACTH-cells immunoreactivity observed in present study thus showed some relationship with seasonal fat cycle in S. heathi /2/.

Increased areal fraction of ACTH-cells during recrudescence and winter dormancy also coincided with the high circulating androstenedione concentrations shown in S. heathi /1/. Previous in vitro study also showed stimula-

tory effect of ACTH on androstenedione production from the adrenal in S. heathi, although at the significantly lowered rate compared with the effect of LH on ovarian androstenedione synthesis by the ovary /1/. Mikami et al. /24/ showed maximum immunoreactive ACTH-cells during hibernation. Gustafson and Belt /15/ suggested active adrenal cortex during hibernation than during active phases. Further study is required to find out the physiological significance of high immunoreactive ACTH-cells during the period of recrudescence and winter dormancy in S. heathi.

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EFFECTS OF ADMINISTRATION OF TESTOSTERONE ON SEMINAL VESICLE ACTIVITY IN THE CATFISH CLARIAS BATRACHUS DURING PREPARATORY PHASE: A STUDY CORRELATING CHANGES IN TESTIS AND SERUM SEX STEROID LEVELS

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In the catfish Clarias batrachus administration of testosterone (0.25, 0.5 and 1.0 μg/g BW for 30 days) during the preparatory phase increased significantly gonadosomatic index (GSI), seminal vesicle-somatic index (SV-SI) and concentrations of proteins, fructose, hexosamines, and sialic acid in the SV and testis in a dose-dependent manner. These stimulatory effects could be correlated positively with the increase of serum testosterone level. Serum estradiol-17β was also significantly high in the testosterone groups.

Keywords: Seminal vesicle — testis — catfish — total protein — fructose — hexosamine — sialic acid — testosterone — estradiol-17β

Introduction

Seminal vesicle (SV) is a unique feature of the male reproductive system of catfishes, gobies and blennies and has been described as lateral outgrowths of the sperm duct or as modification of the testis /1, 11--13, 15, 25, 30, 38/. It secretes a protein-carbohydrate-lipid-rich complex into the lobular lumina in which sperm is stored /13, 22, 38/. Earlier investigations have demonstrated annual correlative changes in both SV and testis /15, 22, 25/. Sundararaj and his coworkers in a series of experiments in Heteropneustes fossilis have demonstrated androgen influences of the SV activity under gonadotropin control /21, 31, 34/. In all these investigations, the SV activity was assessed qualitatively by morphometric and histological parameters. While sensitive quantitative biochemical parameters have been used extensively in the study of mammalian sex accessory gland functions, no such studies are available in fishes. Rai /24/ has reported significant

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annual variations in total proteins, hexosamines, sialic acid and fructose whose levels could be correlated with spermatogenesis in the testis and secretory activity in the SV of C. batrachus. In order to test whether those annual changes were under androgenic control, the present study was undertaken. In this investigation, we examined the effects of exogenously administered testosterone on SV and testicular levels of total proteins, hexosamines, fructose and sialic acid, and correlated with changes in gonadal hormone levels.

Materials and Methods

Collection and maintenance

Catfish (60-65 g) were collected in the preparatory phase (March) of the annual reproductive cycle. Since the identification of sex was difficult externally, the body cavity was opened surgically under MS 222 anesthesia to expose the gonads. Males were identified and acclimated for 15 days under natural photoperiod and temperature conditions. They were treated with benzanthine penicillin (1,00,000 IU/litre) for an hour daily for three days to prevent skin infection. The fish were fed goat liver during acclimatization and experiments.

Experimental design

Acclimatized fish were divided into five groups of 60 each. Group 1 (initial control) fish were sacrificed at the start of the experiment. They were weighed and blood was collected by caudal puncture. The blood samples were kept for 1/2 h at 4 ^{o}C to clot and centrifuged at 4000 rpm. Serum was separated and stored at -20 ^{o}C for measurement of hormones. The fish were sacrificed by decapitation immediately after the blood collection. The testis and seminal vesicle (SV) were removed quickly and weighed. The tissues were stored at -20 ^{o}C for estimation of various biochemical parameters. Group 2 (parallel control) fish were injected with 0.1 ml propylene glycol as vehicle control. Groups 3, 4 and 5 were injected with testosterone (Sigma) intraperitoneally daily for 30 days in a dose of 0.25, 0.5 and 1.0 µg/g body weight, respectively. The steroid was dissolved in propylene glycol. After termination of the treatments, the fish in all the groups were weighed, serum collected, and sacrificed by decapitation. The tissues (SV and testis) were removed quickly, weighed and stored at -20 ^{o}C for biochemical assays.

Study parameters

The weight of the SV and testis were expressed as percentage of the body weight to calculate seminal vesicle—somatic index (SV-SI) and gonadosomatic index (GSI).

Levels of total proteins, fructose, hexosamines and sialic acid in the SV and testis were measured spectrophotometrically according to the methods of Lowry et al. /17/, Mann /19/, Elson and Morgan /9/, as modified by

Davidson /7/ and Warren /39/, respectively. Readings were noted from standard curves prepared from serial concentration of bovine serum albumin, fructose, mixture of glucosamine and galactosamine and n-acetyl neuraminic acid, respectively.

Radioimmunoassay (RIA) of testosterone and estradiol-17β (E_2)

E_2 and testosterone RIAs were carried out according to the procedure of Abraham /1/. /2,4,6,7,^{3}H(N)/ E_2 (sp. act. 87.0 Ci/nMol) and /1,2,6,7,^{3}H(N)/ testosterone (sp. act. 92.1 Ci/nMol/ were purchased from NEN, Boston, MA, USA. E_2 antibody was a generous gift of Professor G. D. Niswender, Colorado State University, Fort Collins, Co., USA and testosterone antibody was a generous gift of the Indian Council of Medical Research, New Delhi. Details of the steroid RIA were described earlier /29/. The sensitivity of the assay was 10 pg/ml. E_2 antiserum cross-reacted with estrone (4%) and estriol (0.5%). The testosterone antiserum cross-reacted with 5α-dihydrotestosterone (3%), Δ^4androstenedione (4.3%), 5α-androstanediol (1.9%), progesterone (1.1%), androstane-3α-diol (1.5%), cortisol (0.096%), androstane-3β-diol (2.5%), estradiol-17β (0.061%) and dehydroisoandrosterone sulphate (0.72%). The intra- and interassay coefficients of variation (n = 5, mean ± SD) for E_2 were 2.28% (SD ± 0.011) and 5.3% (SD ± 0.025), respectively and those for testosterone were 1.43% (SD ± 0.014) and 2.40% (SD ± 0.023), respectively.

Statistical analysis

Data were expressed as means ± standard error of means (SEM). They were analyzed by one-way analysis of variance (ANOVA), followed by Newman–Keuls' test for multiple group comparisons.

Results

Changes in SVSI and GSI

An overall significant variation was noticed in the SV-SI ($F = 59.38$; $p < 0.001$; one way ANOVA) and GSI ($F = 78.79$; $p < 0.001$). The administration of testosterone caused a dose-related increase in the SV-SI and GSI. The increase compared to control values was significantly high for all dose groups except that in the 0.25 μg group. A comparison of the different dose groups also showed significant differences (Newman–Keuls' test, $p < 0.05$) (Fig. 1).

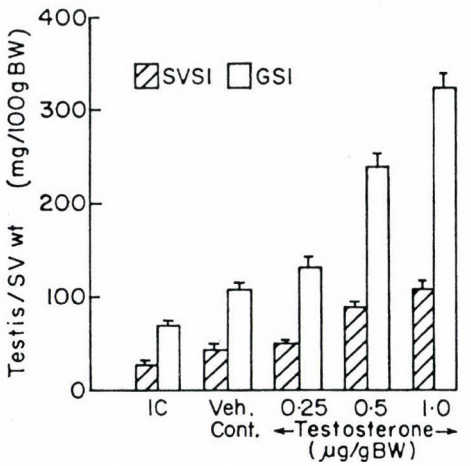


Fig. 1. Shows changes in seminal vesicle-somatic index (SV-SI) and gonadosomatic index (GSI) in C. batrachus after testosterone treatment (means ± SEM, n = 5). IC — initial control; Veh. Cont. — vehicle control; one-way ANOVA — Newman—Keuls' test

Changes in serum hormone profiles

One-way ANOVA showed overall significant changes in the hormone profiles: E_2 ($F = 10.90$; $p < 0.001$) and testosterone ($F = 46.47$; $p < 0.001$). The administration of testosterone elevated significantly the levels of both testosterone and E_2. The E_2 level did not vary significantly between the different dose groups. The testosterone level was significantly high in the 1.0 µg group compared to the low dose groups (Newman—Keuls' test, $p < 0.05$) (Fig. 2).

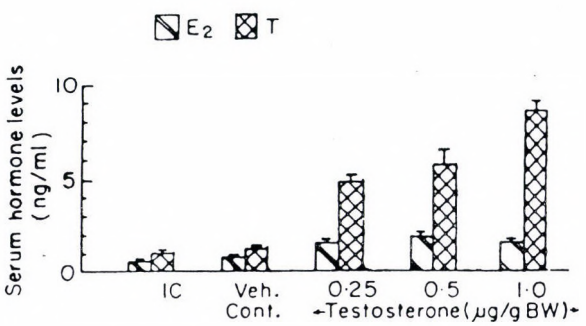


Fig. 2. Effect of testosterone treatment on serum levels of estradiol-17β (E_2), and testosterone (means ± SEM, n = 5). IC — initial control; Veh. Cont. — vehicle control; one-way ANOVA — Newman—Keuls' test

Changes in biochemical parameters

Total proteins

One-way ANOVA showed an overall significant variation of the protein levels in the SV ($F = 25.11$; $p < 0.001$) and testis ($F = 29.57$; $p < 0.001$). The testosterone treatment increased significantly the protein concentration. Newman–Keuls' analysis showed that the increase was not significantly different among the different dose groups (Fig. 3A).

Fructose

One-way ANOVA showed significant changes of the fructose level in the SV ($F = 44.14$; $p < 0.001$) and testis ($F = 26.66$; $p < 0.001$). The administration of testosterone elevated the sugar level significantly. However, the increase was not significantly different among the treated groups (Newman–Keuls' test, $p < 0.05$) (Fig. 3B).

Hexosamines

One-way ANOVA showed an overall significant variation of the hexosamine levels in the SV ($F = 22.45$; $p < 0.001$) and testis ($F = 71.74$; $p < 0.001$). The testosterone injections increased significantly the amino-sugar level. However, the SV hexosamine level did not vary significantly among the treated groups (Newman–Keuls' test, $p < 0.05$). In the testis, the increase in the 0.5 and 1.0 µg groups is significantly higher than that of the 0.25 µg group but not significantly different between them (0.5 and 1.0 groups) (Fig. 3C).

Sialic acid

One-way ANOVA showed an overall significant variation of sialic acid levels in the SV ($F = 130.91$; $p < 0.001$) and testis ($F = 1200.64$; $p < 0.001$). The steroid treatment increased significantly the sialic acid levels only in the 0.5 and 1.0 µg groups. The increase in the 1.0 µg group was higher than all other groups (Newman–Keuls' test, $p < 0.05$) (Fig. 3D).

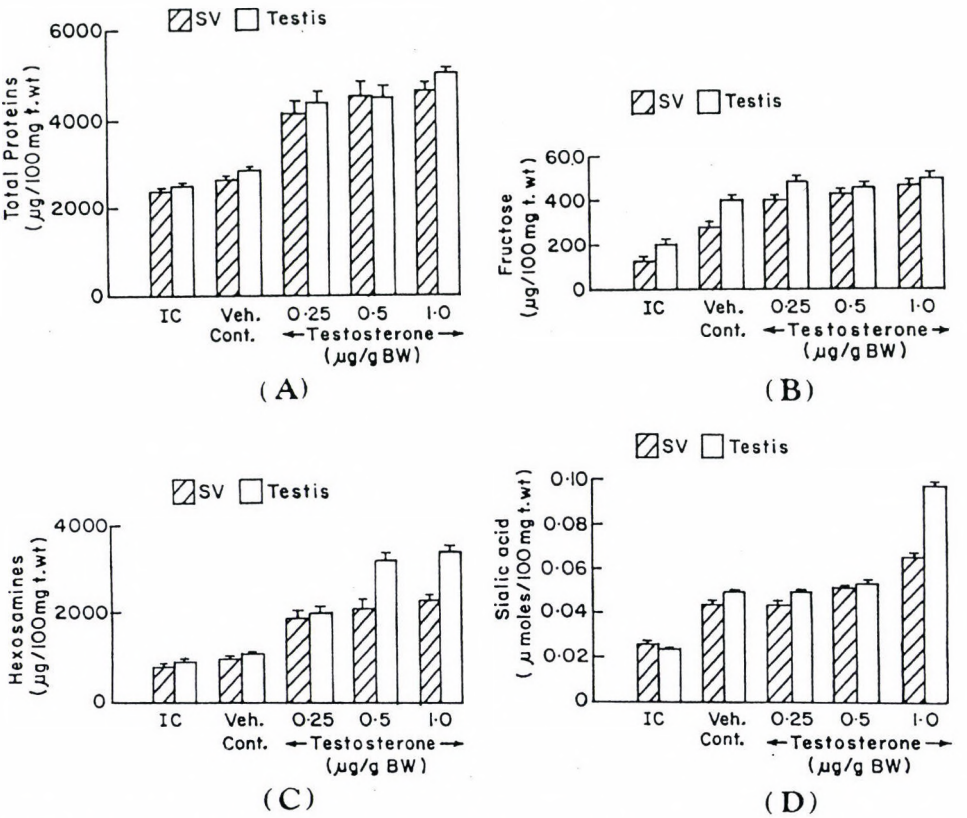


Fig. 3. Effects of testosterone treatment on seminal vesicle (SV) and testicular concentrations of total proteins (A), fructose (B), hexosamines (C) and sialic acid (D) in C. batrachus (means ± SEM, n = 5). IC — initial control; Veh. Cont. — vehicle control; one-way ANOVA — Newman—Keuls' test

Discussion

The administration of testosterone increased the circulating titres of testosterone and E_2. The two-fold increase in the E_2 level, although not commensurate with the testosterone level, may be due to increased aromatization of testosterone at the testis or extratesticular sites like the brain and pituitary /4/. Aromatization may be a mechanism to maintain the testosterone level under physiological limits and plays an important role in the positive feedback of testosterone on gonadotropin secretion /8/. In goldfish, E_2 has been reported to have an inhibitory effect on androgen production Trudeau et al. /35/ which, in turn, may explain the inhibitory effect

of E_2 on spermatogenesis /3, 10/. The elevated E_2 level apparently did not inhibit testicular and SV activities in the catfish. The stimulatory influence of testosterone and other androgens on spermatogenesis have been well documented /16, 24, 26—28, 32, 33/. While a majority of studies on the role of androgens were addressed to spermatogenesis, there have been practically no studies that have dealt with the control of testicular exocrine activity and metabolism. Costa /5/ has observed that testosterone stimulated protein and RNA synthesis in rainbow trout testis. In our study, the testicular concentrations of proteins, fructose, hexosamines and sialic acid increased in a dose-dependent manner, suggesting a stimulatory effect of testosterone on the secretion of these constituents.

The administration of testosterone has stimulated the SV in a dose-dependent manner as evident from the increased SV-SI and concentrations of the biochemical correlates. Thus, the SV is an androgen-responsive organ in the catfish, as has been reported in H. fossilis /21, 31, 32/. In the past only gravimetric (weight) and histological (including measurement of vesicular luminal area) indices have been used to assess the SV activity. Sundararaj and Goswami /31/ have shown that in castrated or hypophysectomized catfish H. fossilis, testosterone propionate alone produced only a slight stimulation of the secretory epithelium; but it in combination with prolactin stimulated the SV activity (a case of synergism) markedly. The present study was conducted in gonad-intact fish in the preparatory phase when the pituitary-gonadal axis was responsive to increasing photoperiod and temperature and only low titres of gonadotropin, testosterone and E_2 were noticed in circulation /24/. Further, we used only low doses of testosterone in contrast to a high dose used by Sundararaj and Goswami /31/. The differences in the response of testosterone treatment in either studies might be due to differences in the endocrine state of the fish, the dose of testosterone used, or to the sensitivity of the parameters: histological vs. biochemical used for assessing secretory activity of the SV. The biochemical parameters have been used for the first time in an experimental study of this nature. The dose-dependent increase in the concentrations of proteins, fructose, hexosamines and sialic acid could be associated with the secretory activity of the SV /18, 20, 23/. In mammals, testosterone stimulates mitotic activity of male accessory sex glands resulting in their growth and differentiation /6, 36/. Similarly, androgens also stimulate their secretory activity increasing the levels of secretions such as proteins, hexosamines, fructose, sialic acid etc., that contribute to the composition of seminal

fluid /18, 20, 23/. Although embryologically, the catfish SV cannot be homologised to any one of the accessory sex glands because of its origin from the genital ridge in common with the testis /11, 24/, the present data show that it is an androgen target organ and serves an analogous function related to nutrition and maintenance of spermatozoa during brief storage. To this end, it has an epididymis-like role. The testicular gland of lennies serves a role in sperm maturation /14/. Recent studies also show that the catfish SV is not only involved in sperm storage, but also enhance sperm survival, fertilization ability and as a source of pheromones, participates in spawning activity /37, 38/.

Acknowledgements

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ORGAN CULTURE AS A MODEL OF STUDYING FOLLICULAR DEVELOPMENT AND FUNCTION OF POSTNATAL MOUSE OVARIES

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Ovarian organ culture was used to study the influence of various gonadotropin hormones (100 ng FSH, 100 ng LH, 100 ng LH added together with 100 ng FSH; 1 i.u. hCG or 10 i.u. PMSG) on growth and development of follicles as well as on steroid secretion by ovaries of postnatal, 15-day-old mice. Ovaries were aseptically removed and single organs were placed on a piece of lens paper which was supported by a stainless steel grid in the small organ culture dish. The cultures were maintained in medium M199 supplemented with 5% of calf serum in a CO_2 incubator at 37 °C. The morphological changes and steroid secretion measured by appropriate RIAs were studied. The stages of follicular development and the incidence of particular types of follicles were scored. Progesterone and estradiol were detected in the medium by radioimmunoassay. Differences between control and gonadotropin stimulated ovaries were found in the number of the ovarian follicles in more advanced maturation stages. There was increased number of multilaminar and antral follicles in FSH and FSH plus LH treated cells. The adding of gonadotropin hormones to the culture medium stimulated significantly progesterone secretion. The most significant effect was observed in media of cultures treated with LH and hCG. As to estradiol secretion the highest stimulatory effect was seen in cultures supplemented with FSH alone, together with LH and with PMSG.

The organ culture techique applied in the current study could be a suitable model of studying the interaction of various factors as well as its effect on ovarian differentiation and on selection of dominant follicles. This system allows maintaining the structural integrity of the whole ovary, thus in the physiological functional status of the organ.

Keywords: Immature mouse ovaries — organ culture — steroid secretion

Introduction

The development of ovarian follicles in mammals is a continuous and complex process whose regulation is not fully understood. Little is known about the mechanisms initiating and regulating the development and function

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of immature or preantral follicles. Most often 21-day-old rats and mice are routinely used in experiments including superovulation. At this stage the ovaries already fully respond to gonadotropic stimulation. In sheep, viable foetuses were obtained after in vitro fertilization of oocytes recovered from FSH-treated juvenile specimens /1/. A better understanding of early folliculogenesis is needed to mimic follicle development in vitro. The organ culture creates good physiological condition for ovarian function and may serve as a model of studying various factors that may be involved in the successful development of large Graafian follicles that can finally ovulate.

The aim of this investigations was to show the response of ovaries from immature 15-day-old mice to gonadotropins kept in an organ culture medium. The answer obtained from this pilot experiment should show whether such ovaries could be a model of studying ovarian differentiation and hormonal regulation of follicular function in vitro. It would be worthwhile to know whether oocytes from immature ovaries have capacity to mature, get fertilised and develop into a viable embryo.

Materials and Methods

Swiss mice were killed by cervical dislocation at day 15 of age. Ovaries were aseptically removed and single organs were placed on a piece of lens paper which was supported by a stainless steel grid in the small organ culture dish /10/ (Fig. 1). The grid and the lens paper was moistened but not submerged in the medium. Ovaries were kept in medium M199, supplemented with 5% of calf serum at 37 ^{o}C in a humidified CO_2 incubator. Ovaries were cultured in control medium without hormones or in a medium containing either 100 ng FSH, or 100 ng LH, or 100 ng LH plus 100 ng FSH, or 1 i.u. hCG or 10 i.u. PMSG. Highly purified ovine LH (NIH-LH-S-8), FSH (NIH-FSH-S-17), hCG, PMSG (Biogonadyl, Polfa, Poland) were used. Medium M199 and penicillin were products of Laboratory of Sera and Vaccines (Lublin, Poland).

On terminating the cultures, the ovaries were fixed in Bouin's solution, dehydrated, embedded in paraffin wax, serially sectioned at 5 µm thick and stained with hematoxylin and eosin. The stages of follicular development and the incidence of each type of follicles were scored. From each ovary, at least five sections, were selected. Total number of primary (A), multilaminar (B) and antral (C) follicles were counted.

On day 3 and 6, culture media were collected and frozen (-20 ^{o}C) until further steroid analysis.

Steroids analysis

Progesterone and estradiol were detected in the medium by radioimmunoassays described elsewhere /9/.

A highly specific antibody raised in sheep against 11a-hydroxy-progesterone hemisuccinate coupled to bovine serum albumin was used. The cross-

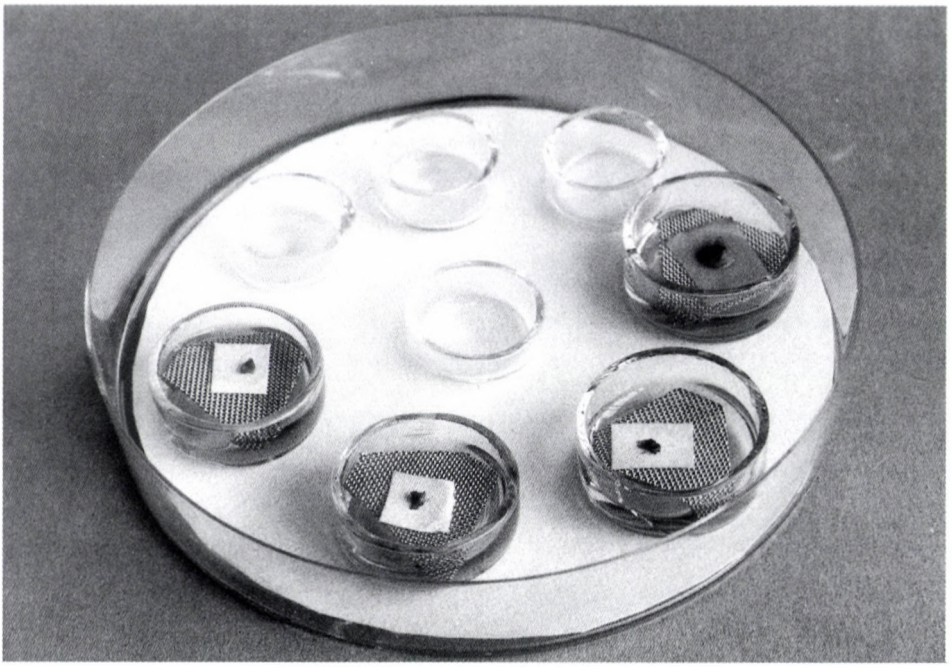


Fig. 1. Small organ dishes with a grid inside and piece of lens-paper on it

reaction with pregnenolone was 2.9%. All other tested steroids showed less than 1% cross-reaction. /1,2,6,7-3H/ progesterone (Radiochemical Centre, Amersham, England, sp.act. 80 Ci/mmol) was used as the tracer. The limit of sensitivity of the assay was 50 pg. The coefficients of variation within and between assays were 15% and 2.5%, respectively.

A highly specific antibody directed against oestradiol 17β-6-oxime-BSA antigen was raised in rabbits. It gave negligible cross-reactions with estrone (0.8%), estriol (0.8%) and 16-keto-estradiol 17b (1%). All the other tested steroids showed a cross-reaction level lower than 0.001%. /2,4,6,7,16,17-^{3}H/ oestradiol (Radiochemical Centre, Amersham, UK) was used as the tracer (140 Ci/mmol). The limit of detection of the assay was 5 pg. The coefficients of variation within and between the assays were less than 7.5% and 8%, respectively.

Statistical analysis

Significant differences between the concentrations of steroids in the control and experimental groups were calculated using analysis of variance (ANOVA) with Duncan's new multiple range test. All data points are expressed as means $\pm$ SE from at least three different experiments (n = 3) each in triplicate, resulting in at least nine observations.

Results

Morphological observation

The control ovaries of 15-day-old mice contained mainly primary follicles consisting of one to two layers of granulosa cells enclosed within a theca (Fig. 2A). In the organ culture the follicles of both control and gonadotropin treated groups were in a good morphological condition (Fig. 2A--F). Differences between control and gonadotropin stimulated ovaries were in the number of the ovarian follicles at different maturation stages (Table 1). There was increased number of multilaminar and antral follicles in FSH alone and FSH plus LH-treated cultures (Figs 2B, D). LH alone caused luteinization of interstitial tissue (Fig. 2C). In PMSG-treated cultures granulosa cells in some follicles showed signs of atresia (Fig. 2F).

Steroids secretion

The rate of progesterone secretion by ovaries isolated from 15-day-old mice maintained in culture for 6 days in the presence or absence of gonadotropic hormones is shown in Table 1a. Control ovaries secreted as little as 0.8 ± 0.02 ng of progesterone/ovary during first 3 days of culture. The secretion of P4 increased up to 9.3 ± 2.8 ng/ovary during the next three days of

Table 1

Effect of exogenous hormones on development of mouse follicles in vitro

Treatment group	A	B	C	No. of ovaries examined
C (% of total)	86	12	2	
(n)	45.6 ± 4.5	6.0 ± 0.3	1.2 ± 0.3	8
FSH (% of total)	16	50	33	
(n)	7.0 ± 0.6	21.4 ± 1.2	14.5 ± 1.2	10
LH (% of total)	84	14	2	
(n)	60.1 ± 1.2	10.3 ± 0.9	1.5 ± 0.8	7
FSH + LH (% of total)	24	32	42	
(n)	14.5 ± 0.9	19.7 ± 0.7	25.8 ± 1.5	10
hCG (% of total)	31	57	12	
(n)	15.8 ± 2.1	28.5 ± 3.1	7.9 ± 0.9	9
PMS (% of total)	34	30	344	
(n)	20.9 ± 1.8	18.7 ± 1.2	20.9 ± 1.1	10

(n) — total numbers of follicles per section
A — primary follicles; B — multilaminar follicles; C — antral follicles

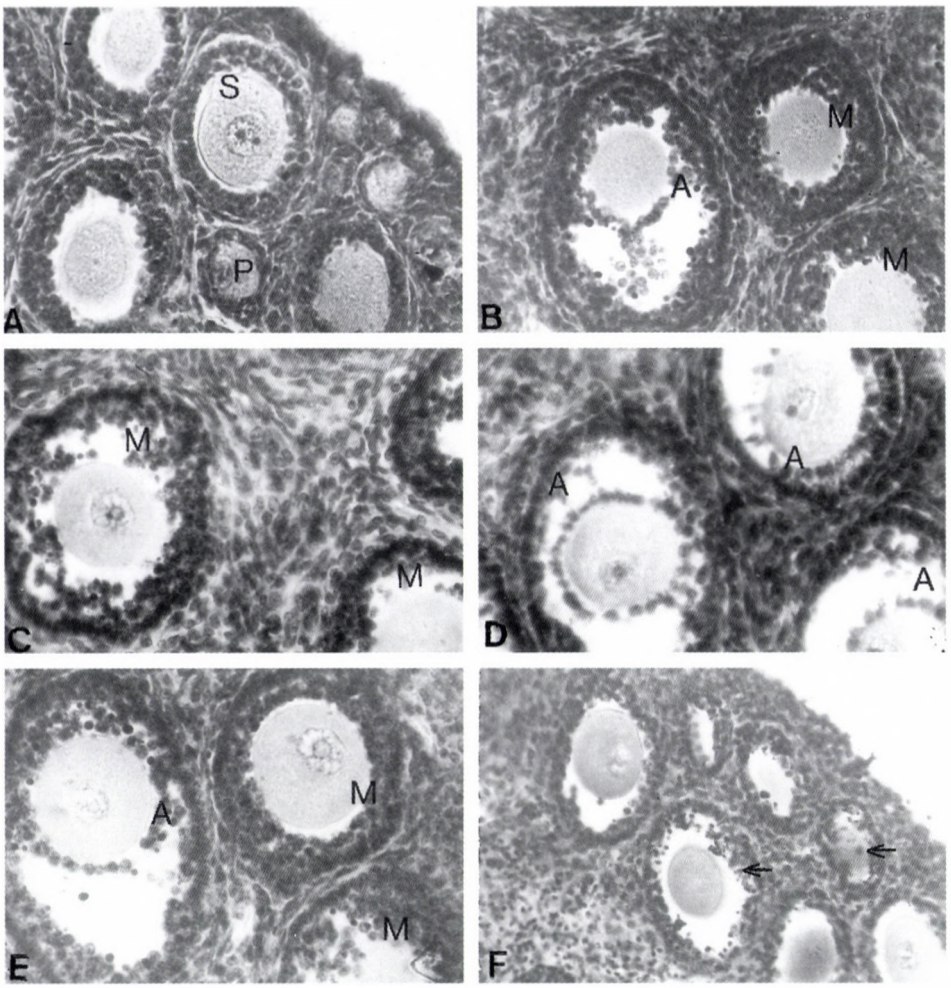


Fig. 2. Cross-section of (A) — control ovary. Mainly promordial (P) and secondary (S) follicles are seen, (B) — FSH treated ovary. More multilaminar primary follicles and antral follicles are visible, (C) — LH-treated ovary. Luteinization of the interstitial tissue is visible, (D) — FSH+LH treated ovaries. Large numbers of antral follicles are visible, (E) — hCG-treated ovaries. Large antral follicle is visible, (F) — PMSG-treated ovary. Cells in some follicles showed signs of atresia. Hematoxylin and eosin staining. Magnification x 32

Table 2

Progesterone (a) and estradiol (b) secretion by immature 15-days-old mouse ovaries

a)

	1st-3th day of culture ng/ovary	3th-6th day of culture ng/ovary
C	0.8 ± 0.02	9.3 ± 2.8
10 ng FSH	8.8 ± 1.2	14.2 ± 2.1
100 ng LH	25.4 ± 3.2	56.2 ± 9.1
100 ng FSH + 100 ng LH	32.5 ± 7.2	42.1 ± 2.7
10 i.u. hCG	17.7 ± 3.8	42.2 ± 7.2
10 i.u. PMSG	4.6 ± 1.2	28.1 ± 5.4

b)

	1st-3th day of culture ng/ovary	3th-6th day of culture ng/ovary
C	159.8 ± 5.1	480 ± 21
100 ng FSH	270.1 ± 12.3	754 ± 21.3
100 ng LH	110.5 ± 4.2	353.3 ± 9.7
100 ng FSH + 100 ng LH	415.1 ± 12.0	1027 ± 34.7
10 i.u. hCG	241.2 ± 6.4	766.2 ± 21.8
10 i.u. PMSG	134.1 ± 14.8	956.3 ± 23.7

culture. The treatment with gonadotropin hormones stimulated significantly progesterone secretion. During all days of culture the most significant increase in progesterone production was observed in cultured supplemented with LH alone (Table 2a).

The rate of estradiol secretion by immature 15-day-old ovary in control and gonadotropin stimulated cultures is shown in Table 2b. Control ovaries secreted 159.8 ± 5.1 pg. of estradiol/ovary during first 3 day of culture. The secretion of E2 increased up to 480 ± 21 pg/ovary during the next 3 days of culture. Gonadotropin hormones added to the culture medium stimulated significantly estradiol secretion. During all days of culture the most significant effect was observed in cultures supplemented with LH and FSH together (Table 2B).

Discussion

Our findings showed that the follicles explanted kept in an ovaries organ culture medium were in good morphological condition in both control and gonadotropin treated groups. Differences between control and gonadotropin stimulated ovaries were found in the number of the ovarian follicles in advanced maturational stages. There was increased number of multilayer and antral follicles in FSH and FSH plus LH-treated cultures. Estradiol secretion was most effectively stimulated by FSH and LH added together into culture medium and during the whole period of culture. It is known that both FSH and LH are required for normal follicular growth. It has also been reported that blood levels of FSH and LH are high in infant and juvenile mice and rats /3/. There is also evidence that ovaries of immature, juvenile rats can synthesise and secret estradiol in vitro /5/ and that estradiol plays an essential role in ovarian cell proliferation /7/. Oocytes obtained from ovaries of juvenile female calves, treated with pituitary gonadotrophins may then be fertilized and develop in vitro to the blastocyst stage at a rate that is similar to that of adult cow /2, 4/. However, in juveline goats, a lower rate of in vitro fertilization was observed when compared with adult animals /6/. In their experiment, donors were not primed with FSH before the collection of the oocytes. The possible influence of gonadotropins on the development of in vitro matured oocytes, obtained from juvenile animals was also observed after priming the donors in vivo with exogenous FSH /1/. These data support our observation. Moreover, follicular development is seemingly also regulated by intraovarian factors. it is likely that cell surface-related mechanisms, similar to those operating in other developing tissues may play a role in the organization of follicular cells during folliculogenesis. Whole ovaries of immature small animals or fragments of ovaries of large immature animals containing all compartments present in vivo, could be a model for studying the influence of various factors necessary for maturation and fertilization of oocytes in vitro to produce viable embryos. The technique presented seems to offer a suitable tool for this kind of investigation.

Acknowledgements

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SEROTONIN IMMUNOREACTIVITY IN THE PERIPHERAL NERVOUS SYSTEM OF OLIGOCHAETA

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The distribution of serotonin immunoreactive elements in the peripheral nervous system of three earthworm species (Lumbricus terrestris, Eisenia fetida and Tubifex tubifex) was studied using the peroxidase-antiperoxidase method. Most parts of the peripheral nervous system contain immunoreactive fibers. All segmental nerves as well as branches of the prostomial nerves show strong immunoreactivity. Muscles of the body wall contain serotonergic fibers, mostly between the circular and longitudinal layers. The best supplied area is the buccal musculature. All parts of the enteric nervous system contain serotonergic fibers, strongest staining was observed in the buccal and pharyngeal walls. Along the wall of the calciferous glands thin immunoreactive fibers were found. The excretory system, the metanephridial tubules also display immunoreactivitiy. Under the surface epithelium, a serotonergic subepidermal plexus was observed. No significant differences were observed between the species studied. On the basis of our morphological findings and functional studies performed by other authors it is suggested that serotonin plays an essential role in the function of the peripheral nervous system in Oligochaeta.

Keywords: Oligochaeta — peripheral nervous system — serotonin — immunohistochemistry

Introduction

It has long been known that serotonin is a widespread neurotransmitter and neuromodulator in the invertebrate nervous system /8, 12, 13/. The presence of serotonin has been shown in annelids, including also the oligochaete nervous system. Early studies using the formaldehyde-induced fluorescence technique with supporting chemical estimates have demonstrated that serotonergic neurons and fibers are widely distributed in the nervous system of the earthworm /6, 20, 21, 30, 35, 43/. Immunohistochemistry partly con-

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firmed these previous observations and greatly expanded the knowledge on the distribution of the serotonergic elements in Oligochaeta. One of the most detailed study is that of Spörhase-Eichmann et al. /37, 38/. These authors have described the pattern, number and morphological characteristics of serotonin-immunoreactive neurons in the central nervous system of Lumbricus terrestris, giving also statistical analysis of positional variations. Apart from early observations made by fluorescence methods mentioned above, no data are available on the serotonin-immunoreactive elements in the peripheral nervous system of the earthworm. Some preliminary studies of our laboratory have shown that serotonin immunoreactivity occurs also in the peripheral nervous system of Lumbricus terrestris /4, 5, 23/.

The co-existence of biogenic amines and neuropeptides in the same neurons is now well established not only in vertebrates but also in invertebrates. Recently we have started to analyze co-localization of serotonin and several neuropeptides in Oligochaeta. First we showed co-localization of serotonin and FMRFamide-like immunoreactivities in the central nervous system of Eisenia fetida /26/. Detailed maps of these substances described by immunohistochemistry provided the histological basis for co-localization studies /33, 37, 38/. Further co-localization investigations are in progress in our laboratory, which include studying co-existence not only in perikarya but also in neuronal fibers. This makes reasonable to describe the serotonergic elements also in the peripheral nervous system.

The aim of the present study is to show the distribution pattern of serotonergic elements in the peripheral nervous system of three earthworm species, Eisenia fetida, Tubifex tubifex and Lumbricus terrestris. For this purpose immunohistochemistry seems to be a suitable technique as also reported by Kemenes et al. /19/, who compared four techniques for mapping the distribution of serotonin in snail. These authors found that immunocytochemical method produced consistently higher numbers of neuronal soma and fibers than other techniques, and the distribution of these neurons showed a close correlation with biochemically measured serotonin.

Materials and Methods

Adult specimens of Eisenia and Tubifex were purchased from local supplier, Lumbricus was collected locally throughout the whole year. Results are given from the observation of 76 animals. The animals were supplied with water and leaves until required. Prior to decapitation some of the animals

were quickly anaesthetized in 10% ethanol. Small pieces were cut from different parts of the animals: anterior, clitellar or postclitellar segments. In some cases parts of the nervous system, intestines or genital apparatus were excised. Picric acid-formaldehyde solution was used for fixation. Penetration of the fixative was enhanced by treatment of the specimens with microwave irradiation (750 W, 10 s, 10 ml ice-cold fixative). Fixed pieces were embedded in paraffin or sectioned by cryostat. The serial frontal and sagittal sections were mounted on glass slides coated with 3-aminopropyl triethoxysilan (Sigma-Aldrich, Budapest) for better adhesion. Sections were treated with trypsin solution (0.12%, Reanal, Budapest). Unspecific background staining was inhibited by incubation in 1% normal sheep serum (20 min). Immunohistochemistry was performed using the peroxidase-antiperoxidase (PAP) method /40/. Anti-serotonin was a generous gift from Dr. T. Görcs /15/. Incubation with the antiserum (1:4000) was performed for 48 h at 4 ^{o}C. The rest of the staining procedure was carried out at room temperature. The sections were then incubated in anti-rabbit-gamma globulin (ARGG, sheep, 1:300) for 10 min, and PAP (1:600) for 10 min. ARGG was raised in our laboratory and PAP was obtained from Arnel (New York, NY, USA). The staining procedure starting with the treatment with ARGG was carried out twice (double PAP method). Bound PAP was visualized by 0.25% diaminobenzidine (DAB) in 0.05 M Tris-HCl buffer, 7.5 pH containing 0.01% H_2O_2 (20 min). The DAB end product was intensified with osmium tetroxide vapor. After completing the DAB reaction, some sections were intensified by the silver-intensification method described by Gallyas et al. /10/.

A method control was performed by omitting the primary antiserum. No immunoreactivity was observed following these tests, although blood vessels showed false reactions due to the endogenous peroxidase activity, which was blocked by 0.3% H_2O_2 on control sections. Antiserotonin is a well-defined serum not showing any cross-reactivity with any known antigen /15/. Unstained sections were also used as controls because the natural pigment in the body wall and the cuticle of annelids is similar to but distinguishable from the diaminobenzidine end product.

Results

Anti-serotonin antiserum resulted in intensive staining of the perikarya and neuropil in the central nervous system. In the peripheral nervous system mainly nerve fibers and exceptionally some perikarya stained. The background staining was low. The intensity of the immunological reaction varied individually. In silver intensified preparations the serotonergic elements showed more intensive staining, although the color of the silver-intensified end-product resembled the natural pigment of the animals to a higher degree.

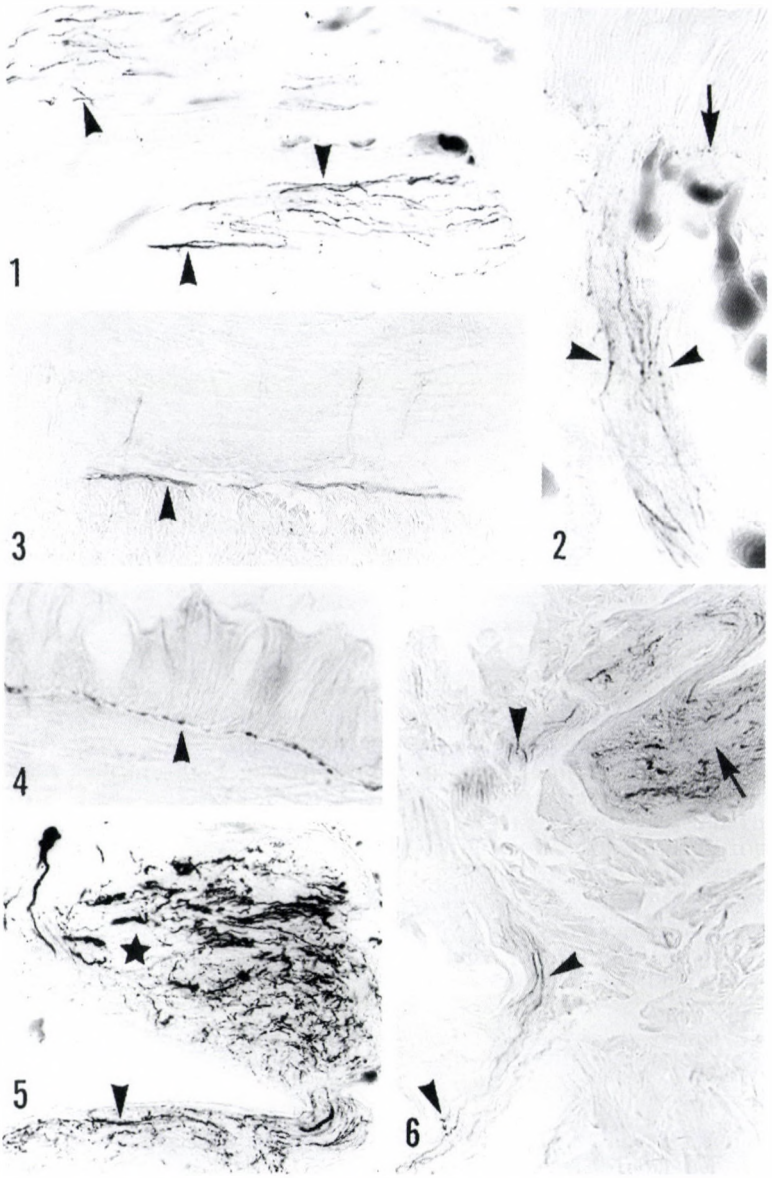
The intensity of staining showed inter- and intraindividual variations, but not significant differences in the distribution were observed between the three species studied. Therefore the distribution of serotonergic elements is described together in the oligochaete peripheral nervous system.

All segmental nerves showed intensive staining with many varicose fibers. The destination of these fibers could rarely be observed, in some cases they could be traced to the enteric plexus, to the metanephridian tubules or to the body wall musculature. Prostomial nerves originate from the cerebral ganglion, extend to the anterior part of the worm branching extensively along their course. The main branches were stained in almost every section, and smaller branches also showed consistent staining (Fig. 1). Thin serotonergic fibers could be traced from the prostomial nerves to the body wall musculature, where they showed radiate branching, the buccal musculature receiving the most intensively stained fibers. Thin branches from the prostomial nerves mainly longitudinally extend among the muscle fibers of the buccal wall before forming a subepithelial plexus (Fig. 2). The ganglionic sheath surrounding the ganglia contained no immunoreactive elements.

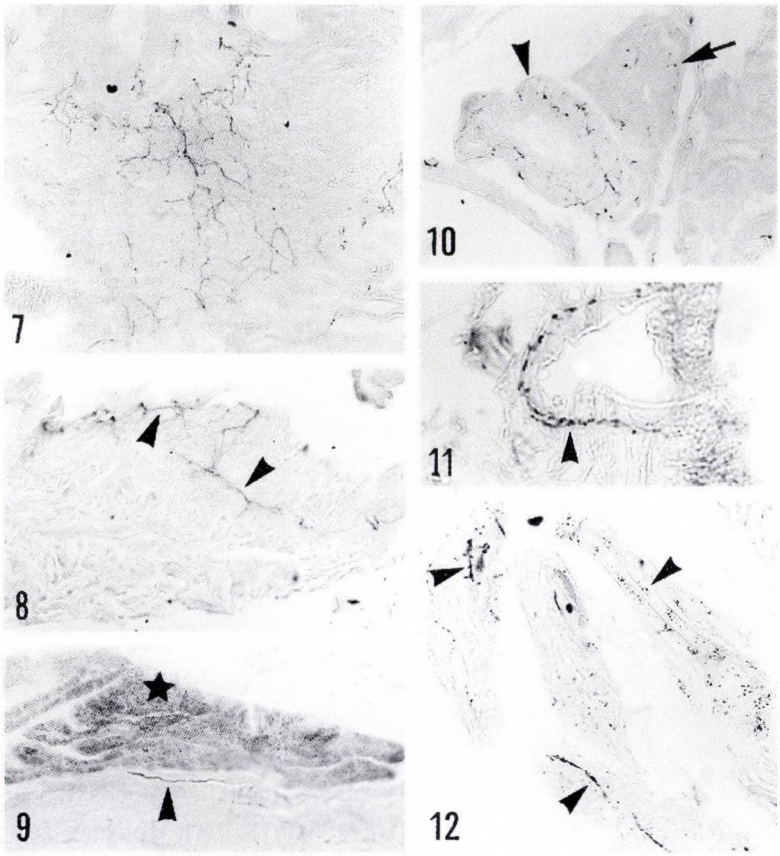
The body wall musculature contains many serotonergic fibers, especially between the outer circular and inner longitudinal layers (Fig. 3). Thin fibers extend among the muscle fibers of both layers, and are in connection also with the subepidermal plexus of the body wall. These immunostained fibers can easily be distinguished from the natural pigment of the animal which is inhomogeneous and shows no distinct contour lines. Less intensive staining was observed in the caudal segments of the animals.

No epidermal cells displayed immunoreactivity. A subepidermal serotonergic plexus was seen in all animals, being most intensive on the ventral part of the anterior segments (Fig. 4). A less extensive plexus was observed under the epidermis in the postclitellar segments. The subepidermal network seems to be in connection with the muscular plexus underneath, as well as with branches of the prostomial and segmental nerves. This serotonergic fibers surround the chaeta, probably innervating the muscle fibers around the chaeta.

Each part of the enteric nervous system contains serotonergic fibers. Many nerve fibers showed immunoreactivity in the stomatogastric ganglia which could be followed from the cerebral ganglion through the connectives to the stomatogastric ganglia (Fig. 5), from where they could be traced to the pharyngeal plexus (Fig. 6). Nerve cells displaying immunoreactivity were very rarely found in a few individuals. A subepithelial as well as an intramuscular fiber plexus was observed throughout the whole digestive tract. The staining intensity of these plexuses decrease gradually towards caudal parts of the alimentary tube, the richest serotonergic innervation being seen in the buccal cavity and the pharynx (Fig. 7). In both parts thin serotonergic



Figs 1–6. 1. Branches of the prostomial nerve showing serotonin immunoreactivity (arrowheads) in Lumbricus terrestris. Silver intensification, x 200. 2. Prostomial, nerve running in the buccal wall of Eisenia fetida (arrowheads) and forming a subepidermal plexus (arrow), x 200. 3. Serotonergic fiber between the circular and longitudinal muscle layers of the body wall (arrowhead) Lumbricus terrestris, x 100. 4. Subepidermal serotonergic plexus in a rostral segment (arrowhead) Lumbricus terrestris, x 200. 5. Serotonergic nerve fibers in the stomatogastric ganglion (arrowhead). Part of the cerebral ganglion is also shown (star). Silver intensification Lumbricus terrestris, x 175. 6. Serotonergic fibers from the stomatogastric ganglion (arrow) entering the pharyngeal plexus (arrowheads) Lumbricus terrestris, x 250



Figs 7—12. 7. Dense nervous plexus in the wall of the pharynx of Eisenia fetida. Tangential section, x 200. 8. Serotonergic fibers in the superficial and deep nervous plexuses of the pharyngeal wall (arrowheads) Lumbricus terrestris, x 200. 9. Serotonergic fiber (arrowhead) along the wall of the calciferous glands (star) Lumbricus terrestris, x 100. 10. Thin fibers in the narrow (arrow) and middle (arrowhead) tubes of the metanephridium Eisenia fetida, x 150. 11. The superficial opening of the metanephridium showing immunostained fibers (arrowhead) Tubifex tubifex, x 150. 12. Silver intensified serotonergic fibers in the metanephridian loops (arrowheads) Lumbricus terrestris, x 100

nerves branch among the crossing and interlacing muscle fibers. These branches could be seen to have connections with the body wall musculature. Under the enteric epithelium, dense superficial and deep serotonergic plexuses with thin varicose fibers were seen (Fig. 8). Both longitudinal and circular fibers were observed. The subepithelial plexus is most extensive in the dorsal part, but can also be found in the ventral and lateral parts. Similar, but less extensive network was found in the walls of the esophagus,

crop, gizzard and the intenstine except for the hindgut, where fibers were only exceptionally seen. This network was also observed in the pharyngeal evaginations and in the typhlosolis which is a dorsal evagination of the intestinal wall. No epithelial cells displayed any immunoreactivity. The calciferous glands are lateral swellings from the esophagus, in which the epithelium is thrown into marked foldings. Thin serotonergic fibers descend along the wall of these glands (Fig. 9).

The excretory system of the annelids, the metanephridium also contains many serotonergic fibers. The celomic opening the nephridiostome, which is a large ciliated funnel, contains a mainly longitudinally arranged plexus under the cliliated epithelium, and a circular plexus in the deeper layers (Fig. 10). This can be easily distinguished from the small pigmented droplets that are also often found in the metanephridian system. Thin fibers could be traced in the walls of the nephridian loops (Fig. 11) through the bladder to the superficial openings (Fig. 12). Thin, often varicose fibers were seen in the proximity of the blood vessels. In adjacent sections stained with the above-mentioned antisera no immunostaining was observed in the nephridian tubules.

The reproductive organs situated in the clitellum displayed no immunoreaction. The same negative results were obtained in excised parts of the sexual organs, such as the seminal vesicles.

Discussion

In the present study serotonin immunoreactivity was described in the peripheral nervous system of the earthworm. Rich serotonergic fiber network was found in most parts of the periphery. As far as the central nervous system is concerned, similar results were obtained to Spörhase-Eichmann et al. /37, 38/. The present documentation provides the first complete immunohistochemical description of the serotonergic elements in the annelid peripheral nervous system.

The function of serotonin in invertebrates has long been examined, although the results are greatly dependent on the species studied. From immunohistochemical data alone, no definitive conclusions can be drawn, only certain suggestions can be made on the basis of our results and observations made by other authors.

It is well known that serotonin is involved in neuromuscular regulation of molluscs. It plays a role in the regulation of heartbeat, swimming and different types of movements /28, 39, 41, 42/. In the leech it has also been shown to have effects on muscle contraction during swimming and feeding behavior /25, 27, 44/. It has been proposed to act as an inhibitory neurotransmitter in the central and peripheral nervous system of the leech /27, 36/. It is also suggested that serotonin modulates locomotion in the earthworm /11/. Our findings on immunopositive fibers in the body wall musculature and in all branches of the prostomial nerves in the earthworm well correlate with these supposed functions.

The subepidermal nerve plexus is believed to be a sensory plexus, which is formed from the processes of the epidermal sensory cells /3/. The consistent staining of the subepidermal plexus suggests that serotonin plays a role in the earthworm sensory processes. The thin fibers around the chaeta also support this hypothesis. An interesting finding was the lack of staining in the sensory cells of the epidermis. This may be due to the limitation of method, although in sections stained with other antisera we detected immunoreactive sensory cells /33, 34/. Epidermal sensory cells are extremely sensitive to different treatments, as it was reported by Gesser and Larsson /14/. They found that anaesthesia in ethanol brings about the disappearance of enkephalin immunoreactivity from the cell bodies but not from the sensory hairs. In our study sensory cells did not show staining in those animals either, where no anaesthesia was applied. The other reason might be low concentration of serotonin in the sensory cells. It is also possible that serotonin influences the mucus release from the epidermis, as it was reported in the leech /24/.

The stomatogastric system of the earthworm originates either from the cerebral or the subesophageal ganglia, and the nerves reach the gut directly or through the stomatogastric nerve cells. Besides the stomatogastric system, nerves innervating the enteric musculature also originate from the ventral cord ganglia. The enteric nervous system lacks of ganglionic arrangement in the earthworm, only solitary nerve cells and fibers are found /7/. We observed serotonin immunoreactive fibers in all parts of the enteric system. The most strongly staining area was the buccal cavity and the foregut. The buccal musculature is controlled by serotonin also in other invertebrate species /42/. The lack of serotonergic nerve cells in the enteric plexus, and the very few immunoreactive cells in the stomatogastric ganglia suggest that the serotonergic innervation of the enteric system is mainly

extrinsic in origin, like the majority of nerves innervating the gut /3/. These nerves originate mainly from the ventral nerve cord. In the ventral ganglia 5-10% of the nerve cells exhibit serotonin immunoreactivity, which is a high proportion compared to other invertebrates /37, 38/.

The calcium carbonate content of the calciferous glands is thought to neutralize the acids of the ingested soil, although other functions have also been ascribed to the glands. They are also believed to fix metabolic carbon dioxide, to provide buffer capacity for the blood and to excrete excess calcium /29/. The serotonergic fibers found in the wall of the glands may have an influence on these secretory activities, a similar effect of serotonin has been shown to be involved in the control of glandular secretion in the leech /24/.

In terrestrial annelids gut motility is involved not only in food uptake and digestion but also in burrowing. Digestive activity, mucus release have been supposed to be influenced by serotonin also in the leech /24/. In more complex behavioral responses such as the feeding behavior, serotonin has been shown to have an important effect in invertebrates /42/. The effect of serotonin on the enteric nervous system seems to be preserved throughout the phylogenesis, since evidences confirming this have been accumulated in the vertebrate gastrointestinal tract as well /9/. According to these observations and the present findings it can be supposed that serotonin, together with a set of other biologically active substances is involved in the gut motility and feeding behavior also in Oligochaeta.

The role of serotonin in the excretory system of the earthworm is not known. It has been observed that under different environmental circumstances (temperature, moisture) the number of monoaminergic cells show numeric and distributional changes /32/. The water loss is mainly directed via the urine /29/ and it has been suggested that the process of dehydration is controlled by aminergic and peptidergic neurons /29, 45/. We found weak but consistent staining in the nephridiostome and mainly the metanephridial loops in each segment. From these observations it can be suggested that serotonin may be involved in the regulation of water and osmotic balance.

Additional functional roles have also been attributed to serotonin. According to some observations, it participates in governing regeneration processes of earthworms /5, 22/. It has been shown that serotonin plays a role also in different types of learning in invertebrates /1, 18/. Investigations on the behavior of the earthworm have shown that earthworms are also

capable of a primitive type of learning /2/. Serotonin immunoreactivity has been found in the neural sheath of the blowfly and Helix nervous systems, thus proposed as a putative neurohormone /16, 31/. We found no immunoreactivity in the neural sheath around the ganglia in the earthworm, but the neurohormonal role of serotonin in the earthworm cannot be excluded.

No significant differences in the distribution patterns of the immunoreactive elements in the periphery were found between the three species studied, however, the intensity of staining showed inter- and intraindividual differences. Similar observations were made on the serotonergic cellular patterns in the central nervous system by Spörhase-Eichmann et al. /37, 38/. Such variations may be due to seasonal changes, as it was described in Helix pomatia. During winter, the number of detectable serotonin immunoreactive neurons in the snail is lower, which is consistent with biochemical measurements /16, 17/. Similar explanation is possible for our findings, although animals purchased at the same time also showed individual variations. Further physiological and ultrastructural inverstigations are needed to understand the mechanisms serotonin influences peripheral functions in earthworms.

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INVOLVEMENT OF CIRCADIAN RHYTHM IN RESPONSE TO ULTRASHORT PHOTOPERIODS IN BLACKHEADED BUNTING, EMBERIZA MELANOCEPHALA

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Groups of photosensitive adult female blackheaded buntings were exposed to various ultrashort days photoperiodic regimes for 60 days in which a fixed 3 h photophase was coupled with dark phases in cycles of 22 to 32 hours duration. One group of buntings was kept in long days (15L:9D) as control. Significant increase in ovarian weight and circulating plasma estradiol concentration was marked in the cycles of 30 h (3L:27D), and 32 h (3L:29D) photoperiodic schedules as well as in control group (15L:9D), whereas there was no response in the cycles of 22 h (3L:19D) and 24 h (3L:21D). It seems that the response to ultrashort day cycles is due to a phase advance or delay in photosensitivity of the response system repeatedly shows coincidence of the external photophase (3 h) with the photoinducible phase of an endogenous circadian rhythm. Therefore, the present result appears to be consistent with Bünning hypothesis suggesting the involvement of an endogenous circadian component in the female blackheaded bunting.

Keywords: Circadian rhythm — ultrashort — photosensitive — Blackheaded bunting

Introduction

The role of photoperiod in regulation of reproductive functions has been well defined in several temperate zone birds in comparison to tropical and subtropical avian species /7, 9, 11, 12, 23, 24, 30, 37, 38/. It has been suggested that many birds utilize an endogenous circadian rhythm of sensitivity to daily light as a physiological basis of photoperiodism /10, 14, 15, 39, 41/. Such concept was originally formulated by Bünning /5/ and it was experimentally performed in an avian species house finch (Carpodacus mexicanus) by Hamner /21/. This hypothesis involves the operation of an external coincidence model which predicts that a photoperiodic response occurs

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as a result of coincidence of light with the photosensitive (photoinducible) phase of an endogenous circadian rhythm.

Previously, a series of experiments have been performed involving the photoperiodic time measurement in several avian species including temperate and tropical zone birds which include exclusively males and very little attention has been paid towards the females /13, 19, 20, 24, 40/. The present investigation was carried out using female Blackheaded bunting, a strong migratory finch, which visits the Indian subcontinent during winter season. To our knowledge, no study has so far been performed to elucidate the role of endogenous circadian component and its role in plasma estradiol secretion in migratory avian species of subtropical region. Although the male blackheaded buntings have been studied earlier /22, 26, 27/. Keeping in view of the lack of information, it was planned to explore the possible involvement of endogenous circadian rhythm and its impact on ovarian growth and 17β-estradiol secretion in female blackheaded bunting using ultrashort day LD schedules which can be used to test the role of circadian component in photoperiodic time measurement.

Materials and Methods

Animal, capture and their maintenance

Blackheaded bunting (Emberiza melanocephala) is a palearctic migratory species which visits the Indian subcontinent during winter season /1/. Adult female buntings were captured locally near Varanasi (25° 18' N; 83° 1' E) in December 1995 when they were abundant. They were kept in outdoor aviary as stock. Prior to their use, they were allowed a fortnight to get acclimated to laboratory condition. Acclimatized birds were transferred to 8L:16D for two months to ensure their photosensitivity. The photosensitive birds were weighed and laparotomized to know that birds had maintained regressed ovarian condition (4 to 5 mg). Food and water were supplied ad libitum.

Experimental design

Twenty-five photosensitive female blackheaded buntings were divided into five groups (5 birds/group) kept in wire net cages (50 x 30 x 25 cm) and placed in photoperiodic chambers under different programmed LD cycles such as 3L:19D (T = 22 h), 3L:21D (T = 24 h), 3L:27D (T = 30 h), 3L:29D (T = 32 h) as experimental and 15L:9D as control. The reason for selecting these photoperiods was nothing but to test whether these ultrashort day LD schedules have any impact on circadian rhythm(s) of photosensitivity in blackheaded bunting. All photoperiodic chambers were illuminated by 20 W fluorescent tubes providing a light intensity of about 400 lux at perch level. The light was controlled by automatic time switches (in 24 h cycles) and in non 24 h

cycles, it was controlled manually. The ovarian weight and plasma estradiol concentration were measured on day 0, day 30 and day 60.

Sampling

Ovarian weight was assessed on day 0, day 30 and day 60 by laparotomy. At each laparotomy ovarian weight was assessed by comparing the size of ovary in situ with a standard set of known ovarian weight. The error by this method is less than $\pm$ 20% /36/. On day 0, day 30 and day 60 blood was collected in heparinized capillary tube, centrifused at -4 ^{o}C and plasma was stored at -20 ^{o}C till further assay of estradiol.

Radioimmunoassay of estradiol and statistical analysis

The circulating plasma estradiol concentration was measured by using estradiol Direct Radioimmunoassay kit (Biotecx Laboratories, Inc., 6023, South loop East Huston, Tx 77033). The kit was highly specific for 17β-estradiol. The cross reactivity for 17β-estradiol was 100%. The data obtained in present experiment were analysed using an analysis of variance. A two factor mixed design (repeated measures on one factor) was used. Supplemented Newmann—Keuls' multiple range t-tests were employed to ascertain the difference among mean values /4/.

Results

Ovarian weight

Results are summarized in Fig. 1. There was significant variation in ovarian weight ($P < 0.001$) in relation to days i.e. day 0 vs day 30 vs day 60 ($F = 888.071$; $df = 2, 60$) as well as between the photoperiodic groups ($F = 542.187$; $df = 4, 60$). A significant ($P < 0.001$) ovarian growth was marked in the birds of group 3L:27D, 3L:29D and in control group (15L:9D) while group 3L:19D and 3L:21D showed no response. The birds of 3L:27D, 3L:29D and control groups were attained a significant ($P < 0.01$) increase in ovarian weight on day 30. A further significant increase ($P < 0.01$) in ovarian weight was observed on day 60 in respect of day 0 and day 30 in 3L:27D and 3L:29D.

Plasma estradiol

Results are summarized in Table 1. There was a significant variation in plasma estradiol concentration ($P < 0.001$) in relation to days i.e. day 0 vs day 30 vs day 60 ($F = 1490.864$; $df = 1, 20$) as well as between the photoperiodic groups ($F = 406.238$; $df = 4, 20$). A significant increase ($P < 0.001$) in plasma estradiol concentration was marked in group 3L:27D, 3L:29D and in

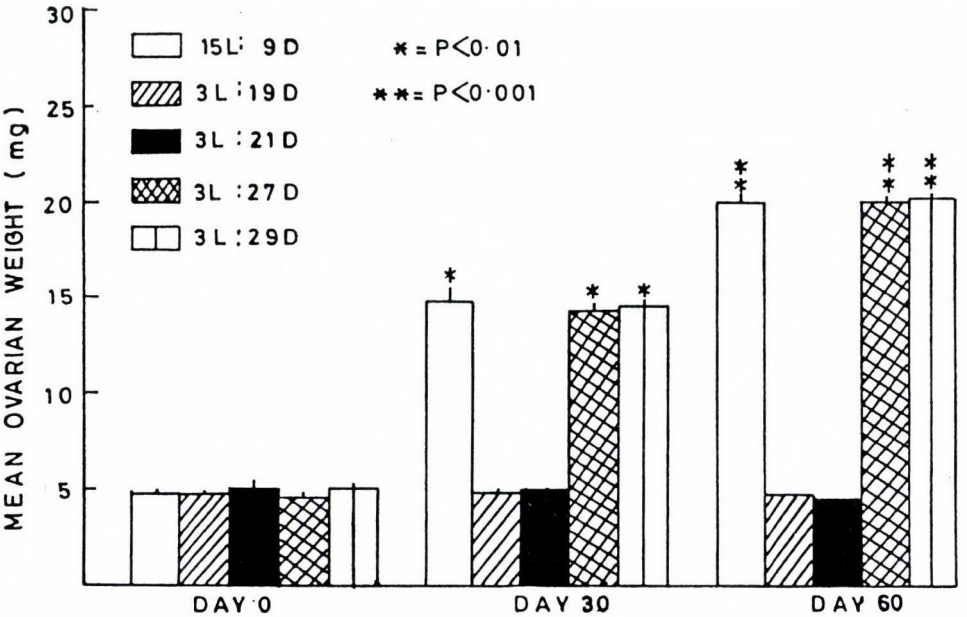


Fig. 1. Response of ultrashort photoperiods upon ovarian weight in female blackheaded bunting. A significant increase in ovarian weight was marked ($P < 0.001$) in 3L:27D, 3L:29D and in control group (15L:9D). 3L:19D and 3L:21D showed no response. Vertical bar means ± SEM, N = 5 birds/group

Table 1

Response to ultrashort days on circulating plasma estradiol concentration in blackheaded bunting

Photoperiodic schedules	T-cycle	Circulating plasma estradiol (pg/ml)		
		day 0	day 30	day 60
15L:9D	Control	<10	51.66 ± 7.57**	161.6 ± 7.63***
3L:19D	T = 22 h	<10	12.6 ± 1.58	<10
3L:21D	T = 24 h	<10	<10	16.3 ± 2.08
3L:27D	T = 30 h	<10	24.3 ± 8.38**	163.3 ± 12.58***
3L:29D	T = 32 h	<10	21.6 ± 4.16**	216.0 ± 8.48***

Birds showed significant increase in plasma estradiol on day 30 and 60 in 15L:9D, 3L:27D and 3L:29D groups. No significant response in 3L:19D and 3L:21D groups. On day 0, plasma estradiol was non-detectable (< 10 pg/ml) in all groups. ± represents SEM, *** -$P < 0.001$ and ** -$P < 0.01$.

control group (15L:9D) while 3L:19D and 3L:21D showed no response. Birds of groups 3L:27D, 3L:29D and control group showed significant increase ($P < 0.01$) on day 30 and a further significant ($P < 0.01$) increase was marked in plasma estradiol on day 60. No significant variation was observed in groups 3L:19D and 3L:21D. On day 0, the plasma estradiol was non-detectable (< 10 pg/ml) in all experimental as well as in control groups.

Discussion

Result obtained from the present investigation clearly indicates the role of an endogenous circadian component in female blackheaded bunting which operates therein and thus it is in agreement with Bünning hypothesis according to which, photoperiodic induction and gonadal growth depends upon repeated coincidence of light with the photoinducible phase (photosensitive phase) of endogenous circadian rhythm. It is interesting to note that the same photoperiod (3 h) was available to all experimental groups, it was effective only in combination with certain dark periods (3L:27D and 3L:29D) while it failed to respond when combined with other dark periods (3L:19D and 3L:21D). A better interpretation about these variations would be possible if the phase advance or delay by the ultrashort day cycle, in a given duration caused the coincidence of the photoinducible phase with external photophase which may result in a photoperiodic response. In the present investigation a significant increase in ovarian weight and circulating plasma estradiol concentration was observed in the bunting only in 3L:27D and 3L:29D cycles indicating that the phase advance or delay by these cycles may ensure that the external photophase coincidence occurs repeatedly with the photoinducible phase of the circadian rhythm. On the other hand no response was marked in 3L:19D and 3L:21D cycles. It can be suggested that the phase advance or delay by these cycles may not be able to provide such coincidence and hence photoinduction of ovarian growth and plasma estradiol fails to occur. In the cycles of 3L:21D the light period always falls into non-photoinducible phase, without any phase advance or delay of the cycle, neglecting the possibility of any response.

The present finding shows consistency with several earlier reports /8, 9, 35, 37/. In Japanese quail 1-3 h of light induction in the cycle of 19 to 36 h were ineffective to induce testicular growth /34/. They suggested that the photoinducible rhythm damps out so rapidly in darkness that there is only very weak gonadal growth in Nanda-Hamner cycle /29/ and T cycle while

free running quails are exposed to a single phase of light the increase in LH secretion is exceedingly small /16, 18, 32/. The ovarian response of female blackheaded bunting under 24 h cycle (3L:21D) is inconsistent with the temperate population in which 3L:21D is photostimulatory. 3L:21D photoschedule also induced the gonadal development in spotted munia (Lonchura punctulata) /6/ and starlings (Sturnus vulgaris) /33/. It was suggested that this could be simply due to population difference.

The higher concentration of plasma estradiol during 3L:27D, 3L:29D and in control group (15L:9D) indicates correlation with ovarian response. It has been shown that Japanese quail (Coturnix coturnix japonica) raised under long photoperiods have higher concentration of estradiol /3/. The plasma estradiol level of bunting which has shown to have increased concentration along with increase in ovarian growth under different photoperiodic regimes supports the earlier finding of Brain et al. /3/ that the time at which ovarian weight increased, yolk filled follicle developed, resulting increased level of plasma estradiol concentration suggesting the view that ovaries are the major source of circulating estradiol hormone. The undetectability of plasma estradiol during photosensitivie phase (treated with 8L:16D) and in some experimental photoperiods shows similarity with white-browed sparrow weaver in which plasma estradiol was only detectable prior to ovulation while rest of the phase it was undetectable /43/. The female blackheaded bunting under short photoperiod showed undetectable level of plasma estradiol and it was detectable only during long days /28/. The undetectability of plasma estradiol during short days in buntings was due to the RIA kit by which we measured the plasma estradiol has lower detectable limit was 10 pg/ml while others /3/ have had the lower detectable range of less than 10 pg/ml and thus the plasma estradiol was detected in short days. Recently it has been reported that long day caused increase in ovarian mass and testicular growth in American tree sparrow /38, 42/ and exposure to long days initially leads to increase in circulating level of testosterone /2/ while short day caused decrease in luteinizing hormone and estrogen. The increase in luteinizing hormone, testicular growth, testosterone concentration and body weight was observed in response to long day in male buntings /22, 24/. Follett et al. /17/ have shown that cycles with short photoperiods are not photoperiodically inductive in Japanese quail but they can be inductive if the daylength is lengthened. In summary, the present finding appears to be consistent with the Bünning hypothesis and indicates the involvement of an endogenous circadian component in the photoperiodic response mechanism of female blackheaded bunting.

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IMMUNOREACTIVE PINOPSIN IN PINEAL AND RETINAL PHOTORECEPTORS OF VARIOUS VERTEBRATES

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Pinopsin is a pineal specific opsin newly identified in the pineal of birds which has an absorption maximum at 470 nm. As the opsin content of photoreceptors in the pineal complex of several species is not yet known, in the present work, we studied their pinopsin immunoreactivity in various vertebrates from cyclostomes to mammals. We also compared the immunoreactivity of pineal photoreceptors to that of retinal cones and rods of each animal. For the immunocytochemistry, we raised antibodies in rabbits against a 14 amino acids containing part of the chicken pinopsin molecule. The immunoreaction was performed at the electron microscopic level.

The pineal organs show a great diversity in vertebrates: there is a pineal organ present from cyclostomes to mammals, in addition, there is a parapineal organ in cyclostomes and fishes, a frontal organ in frogs and a parietal eye in several reptiles. We detected a strong pinopsin immunoreaction on most of the pinealocytes of birds and on the large photoreceptor-type of the pineal of reptiles. Rod-type photoreceptors of the avian retina and a cone of the reptile retina was immunoreactive as well. According to the known absorption maximum of pinopsin, the immunoreactivity may indicate a green-blue light-sensitivity for these photoreceptors.

The immunoreactivity was less pronounced or absent in mammals as well as in less differentiated species. The pineal organ of snakes and the parietal eye of reptiles equally failed to exhibit pinopsin immunoreactive photoreceptors, presumably, due to the absence of green-blue light-sensitive photoreceptors of pinopsin-type in these species.

Keywords: Retina — pineal organs — pinopsin — immunoreaction — electron microscopy — vertebrates

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Introduction

The pineal organs of vertebrates show a great diversity in the individual species: there is a pineal organ present from cyclostomes to mammals, in addition, there is a parapineal organ in cyclostomes and fishes, a frontal organ or frontal eye in frogs and a parietal eye in lacertilians and some other reptiles. All these organs are supposed to differentiate from the paired caudal members of the four eyes of the hypothetical protovertebrate. During evolution, the originally bilateral caudal "proto-eyes" moved to an antero-posterior position, one of the two regressed or both fused to a single organ. The exact homology of the recent organs with the left and right caudal "proto-eyes" is only partially known. From the eighties on, we have been demonstrated the presence of immunoreactive opsins in distinct photoreceptor cell types of the pineal organs of various vertebrates. These findings initiated an extended search in the pineal cells for substances known to play a role in the photochemical transduction cascade of the retina. These studies showed that not only the pineal complex of cyclostomes, fishes and amphibians but also that of higher vertebrates may have a direct photosensitivity /4, 10, 12–14, 17, 18, 24/.

As not all pineal photoreceptor outer segment-membranes were labelled with antibodies to known retinal opsins, the existence of special pineal photopigment(s) were hypothesised. The recently detected chicken pinopsin may represent one of these unknown photopigments. The chicken gene encoding pineal organ-specific opsin -- pinopsin -- has been characterized by Okano et al. /7, 8/ and Max et al. /6/. A similar opsin gene was described by Kawamura and Yokoyama /3/ in the pigeon. The pinopsin was found to be different from known retinal photopigments and to have an absorption maximum at 470 nm (blue-green light). With an antibody specific for pinopsin, intense immunoreactivity was found in the outer segments of pinealocytes of 1 to 2-month-old chickens by Hirunagi et al. /2/.

In the present work, we investigated whether a pinosin-like molecule exists in the photoreceptors of the different pineal organs of various vertebrates from cyclostomes to mammals. We also compared the immunoreactivity of pineal photoreceptors to that of retinal cones and rods of each animal.

Materials and Methods

Pineal organs (pineal organ, parapineal organ, frontal organ, parietal eye) and retinas of a total of 86 animals belonging to various vertebrate classes were compared: Lampetra planeri, Raja clavata, Carassius auratus, Anguilla anguilla, Rana esculenta, Rana arvalis, Bufo viridis, Lacerta viridis, Lacerta agilis, Lacerta muralis, Phelsuma laticauda, Elaphe longissima, Columba livia, Coturnix coturnix japonica, white leghorn chickens, Putorius furo, Meriones unguiculatus, laboratory white rat and cattle. Except the cattles, the animals were kept under normal laboratory light conditions, anesthetized with MS222, urethan or phenobarbital prior to fixation and perfused through the aorta with a mixture of 0.1-2% glutaraldehyde and 2% paraformaldehyde in phosphate buffer. The pineals and retinas of cattles were obtained at a slaughter-house of Budapest.

The excised pineal organs and retinas were washed in buffer, dehydrated in ethanol and embedded in Poly Bed 812 (Polysciences, St. Goar, Germany). Pineal and retina were cut on a Reichert Ultracut S ultramicrotome. Electron microscopic immunoreactions were performed on gold-colored ultrathin sections collected on nickel grids as described earlier /18/.

An antibody against a synthetic peptide of chicken pinopsin was produced in our laboratory. Comparing the C-terminal of the pinopsin molecule to known retinal photopigments, we found a langer sequence specific for pinopsin. By means of a computer epitope-predicting method, we selected a 14 amino acids containing part (QRTGKASPGTPGPH) of this sequence. Mixed breed rabbits were immunized with the synthetized peptide coupled to bovine thyroglobulin with glutaraldehyde as a cross-linking reagent as published previously /1/. The antibodies obtained were used for immunocytochemistry on sections of pineals of various vertebrates and the corresponding retinas.

Specificity of the antibodies was tested by using preimmune serum for immunolabelling or preincubating the sera with the synthetic peptides. No labelling of the pineal structures was found with the preimmune serum or when the sera were incubated with the corresponding peptide (e.g. 2 µg/ml peptide concentration completely blocked labelling with serum No. 3033 at a dilution of 1:3000). The inappropriate peptides were ineffective. In the brain, the labelling was confined to the pineal leaving other brain regions completely unstained.

Results

Pinopsin immunoreactive cells were found in some members of the pineal organs as well as in the retina. In all photoreceptors showing immunopositivity, the reaction was localized to the photoreceptor membranes of the outer segments.

Comparing the presence of pinopsin immunoreactive cells of various pineal organs, the strongest reaction was observed in reptiles and birds and a less pronounced reactivity in both the higher and lower differentiated vertebrates (Table 1). The individual organs exhibited a considerable diversity concerning the presence of immunopositive photoreceptors.

Table 1

Pinopsin immunoreactivity of pineal photoreceptors of the different vertebrate species investigated

	Pineal organ	Parapineal organ	Frontal eye	Parietal eye
Cyclostome				
Lampetra planeri	-	-	0	0
Fishes				
Raja clavata	+	0	0	0
Carassius auratus, Anguilla anguilla	+	-	0	0
Anurans				
Rana esculenta, Rana arvalis, Bufo viridis	++	0	+	0
Reptiles				
Lacertilians	+++	0	0	-
Phelsuma laticauda	+++	0	0	0
Elaphe longissima	-	0	0	0
Birds				
Columba livia, Coturnix coturnix japonica, white leghorn chicken	+++	0	0	0
Mammals				
cattle	+	0	0	0
Putorius furo, Meriones unguiculatus, laboratory white rat	-	0	0	0

+ - +++: level of pinopsin immunoreaction, -: no pinopsin reaction was detected, 0: the organ does not exist in the species in question.

No reaction was seen in the pineal and parapineal organs of the cyclostome lamprey. Only a weak-to-medium immunoreaction was observed on the outer segments of fish and frog (Fig. 1a) pineal but not in the parapineal organ of bony fishes nor in the frontal organ of frogs.

A clear-cut pinopsin immunoreaction was detected on the rhodopsin-negative large reptilian pinealocytes (Fig. 1b). In contrast, we could not find any positivity in the pineal of the snake species investigated. The photoreceptor outer segments of the lacertilian parietal organ also failed to react with pinopsin antisera.

In the birds investigated, most of the pinealocytes were highly immunoreactive (Fig. 1c). No reaction, however, was observed in the mammalian pineals.

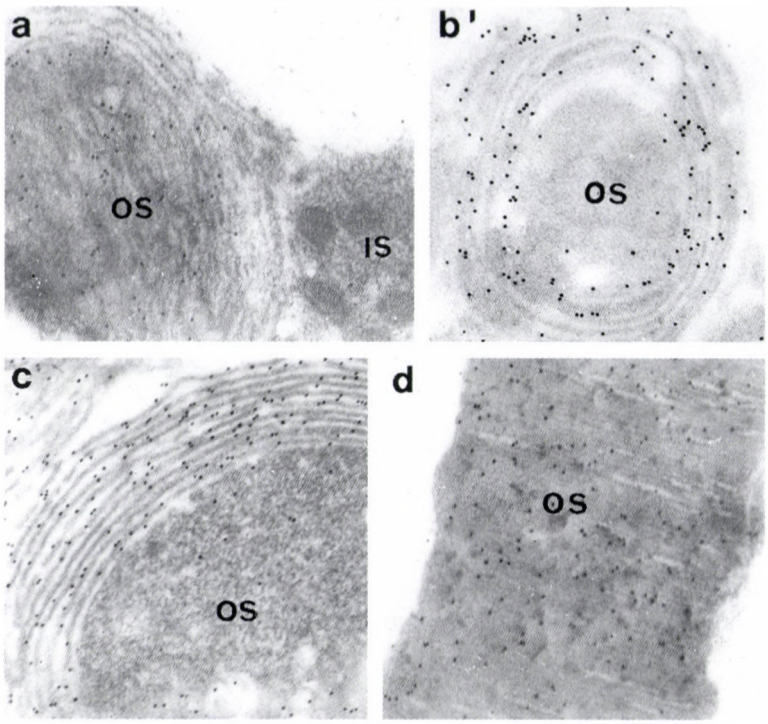


Fig. 1. Pineal and retinal photoreceptors and their pinopsin immunoreactive (black dots of immunogold particles) outer segments. **a**: outer segment (OS) and inner segment (IS) of the rod-type pinealocyte of Bufo viridis, x 44000; **b**: outer segment (OS) of the large-type pinealocyte of Lacerta viridis, x 42600; **c**: pineal outer segment (OS) of Columba livia, x 41600; **d**: outer segment (OS) of retinal rod of Carassius auratus, x 42600

We also detected pinopsin immunoreaction in some retinal photoreceptors from fishes up to mammals. In bony fishes, the outer segments of a few rods were found to be positive (Fig. 1d) but most of them did not immunoreact with pinopsin antibodies. In the anurans investigated, most of the rods were found moderately immunopositive while cones were negative.

In the reptiles, a small, lipid droplet containing cone was immunoreactive with antipinopsin antibodies (Fig. 2a). In the avian retina, the outer segments of rods were positive (Fig. 2b) while cones, including double cones, remained immunonegative. In mammals only the outer segments of the rods of the cattle retina gave moderate positivity.

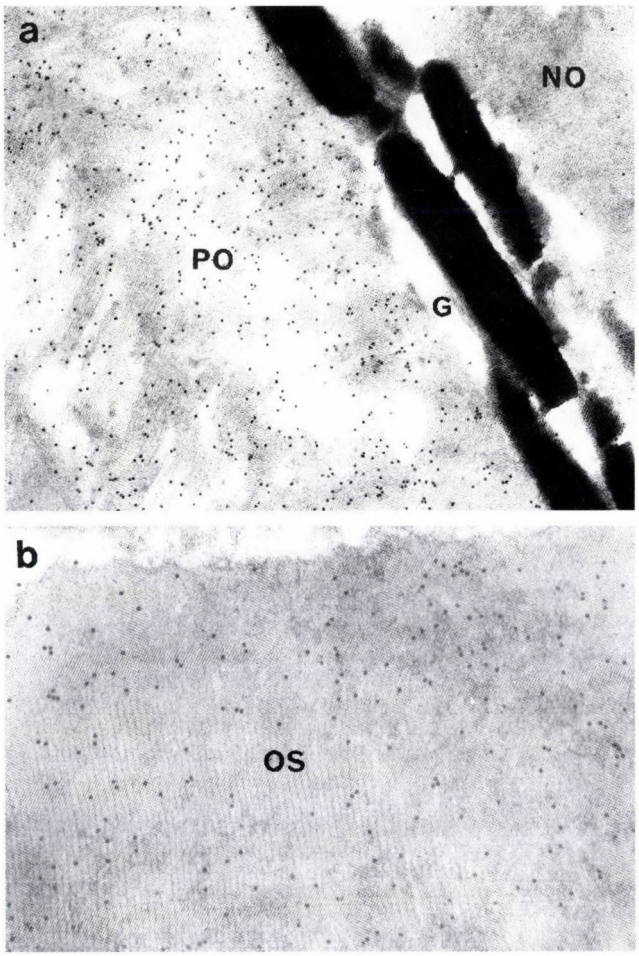


Fig. 2. Pinopsin immunoreactive (black dots of immunogold particles) retinal photoreceptors. **a:** positive outer segment (PO) of gekko, NO: negative outer segment, G: pigment granules, x 36000; **b:** retinal rod outer segment (OS) of the quail (Coturnix coturnix japonica), x 48000

Discussion

Several retinal opsins were already described in different pineal photoreceptor populations. It is known that, in the cyclostome lamprey, the pineal organ contains two types of rhodopsin immunoreactive and one immunonegative cells. Also the parapineal organ contains two types of photoreceptor pinealocytes. Most of them are negative only those of the central portion of the ventral parapineal retina are immunoreactive with all anti-

bodies raised against known retinal opsins /4, 21, 23/. In cartilagenous and bony fishes most of the pineal outer segments immunoreact with rhodopsin antisera. There are four types of pinealocytes and frontal organ photoreceptors in Rana species. The outer segments of large dark pinealocytes immunoreact with rod opsin antisera and OS-2, a photoreceptor specific antibody, whereas the outer segment of the thin dark pinealocytes only immunoreact with OS-2 antibodies. The outer segment of the lipid droplet containing pinealocyte binds the COS-1 antibody detecting green and red pigments /5, 9, 11, 16, 20, 22/, while the small pinealocyte reachts with none of the retinal antibodies.

In lacertilians, one of the pinal photoreceptor types reacts with COS-1 and OS-2, while the other type of outer segment and the photoreceptors of the parietal eye do not react with the antisera raised against retinal opsins. In birds most of the pineal outer segments are labelled with rod-opsin antisera and in mammals the cell membrane of some pinealocytic perikarya immunoreacts with rod-opsin antisera /5, 15, 19, 24/.

By the description of a pineal specific opsin, the pinopsin, a new possibility appeared to detect the opsin specificity of pineal and retinal photoreceptors. As the light-absorption maximum of the pinopsin is known, its immunocytochemical localization helps the better understanding of the light-perceptive function of individual photoreceptors. In the present work, using antibodies raised against pinopsin, we obtained a clear-cut pinopsin immunoreactivity in pineal organs and retina of several species. Concerning the pineal organs, the strongest reaction was observed in outer segments of reptiles and birds representing the recent form of the sauropsidian-line of vertebrates. The result obtained in birds is in accord with the findings of Hirunagi et al. /2/ who also detected pinopsin immunoreactivity in the outer segments of pinealocytes in the chicken.

In lacertilians, the small-type -- OS-2 and rhodopsin-positive -- pinealocytes were immunonegative, in contrast, the large pineal photoreceptors -- not labelled with either rhodopsin or OS-2 antisera -- were stained with pinopsin antibodies. This result has provided us with further information about the hitherto unidentified large reptilian pinealocyte. As pinopsin was found to have an absorption maximum at 470 nm, these reptilian and avian immunoreactive pinealocytes can be considered green-to-blue light-sensitive photoreceptors.

Only weak-to-medium immunoreaction was observed in the rod-type pinealocytes of frogs and fishes, while no reaction was seen in the pinealo-

cytes of cyclostomes i.e. in animals less differentiated than recent reptiles and birds. On the other hand, mammals representing a higher level of differentiation proved to be equally negative. The negativity may indicate the absence of pinopsin-type gree-blue light-sensitive photopigments in these animals. The differences found among the species studied so far show, that the cells of the originally paired pineal organs of the supposed protovertebrate differentiated to a high diversity of photoreceptors, probably, according to the various light conditions of the biotop of each species.

We would like to emphasise that not only pineal photoreceptors but also retinal outer segments of some vertebrates were labelled with pinopsin antisera. Thus, pinopsin resembles the opsins present in some retinal photoreceptors, probably due to partial homologies in the protein structure. Further studies are needed to prove whether these photoreceptors are indeed green-blue light sensitive as predicted from their pinospin molecule content.

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SURFACE SPECIALIZATION OF THE CAPILLARY ENDOTHELIUM IN THE PECTEN OCULI OF THE CHICKEN, AND THEIR OVERT ROLES IN PECTINEAL HAEMODYNAMICS AND NUTRIENT TRANSFER TO THE INNER NEURAL RETINA

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The structure of the capillary endothelium in the pecten oculi of the domestic fowl was investigated by scanning and transmission electron microscopy. Scanning electron microscopy results demonstrated the existence of a vast array of irregular microplicae that projected from the luminal surface of the capillary endothelium. In between these microplicae were numerous crevices. The microplicae were closely packed and showed no preferred orientation regarding either the longitudinal or transverse plane of the capillaries. Transmission electron microscopy revealed the section profiles of the microplicae: their tortuity, branching, interdigitations and the magnitude of the crevices contained. The endothelial cytoplasm exhibited a few mitochondria and micropinocytotic vesicles. The apparent set-up of the luminal plasmalemmal infoldings seemed to be designed for effecting impedance to the pectineal blood flow and thereby facilitating passive permeation of blood-borne nutrients to the inner neural retina. The reasons of such passive transport operation mediated by the endothelial microplicae of the avian pecten oculi are discussed in the perspective of the existing literature.

Keywords: Avian pecten — capillaries — endothelium — surface specializations

Introduction

The avian pecten oculi is a pleated, darkly pigmented and highly vascular organ overlying the linear optic disc /7, 14, 24/. Although the literature on the pectineal structure and function is substantial, the functional role of the pecten has remained poorly understood as is suggested from the 30 functions attributed to this intraocular organ /18, 25/. Apparent consensus exists among contemporary researchers involving its nutritive role /5, 24/. The existence of oxygen gradient from the pecten to the retina has been reported across the vitreous body, that gets disrupted on

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severance of the pectineal supply artery /26/ and the resultant manifestation of the larger robust grafts on the pecten oculi, following neural implants in the vitreous chamber /8/ supports the diffusion of oxygen and nutrients from the pecten to the inner neural retina. However, the inherent mechanism of transport of blood-borne nutrients from the pectineal capillaries to the inner sensory retina is yet to be fully elucidated.

Although the capillaries of the pecten oculi have been investigated by light microscopy /17, 21/, transmission electron microscopy /5, 11, 20/ and vascular casting /1/ the true three-dimensional configurations of their endothelial cell surface specializations have not been unambiguously determined. The chicken, which is the subject of this study is a diurnal bird, with relatively thick avascular retina, measuring about 230 μm /12/. Circumstantial evidence lent support to the pecten oculi as the primary source of nutrients to the avascular retina /18, 24/. In the absence tabgible physiological studies, as understanding of the structure of the capillary endothelium is a prerequisite in elucidating the nutritive role of the pecten.

The present study is an attempt to unravel the nature of the endothelial cell surface in the pectineal capillaries of the chicken by scanning and transmission electron microscope.

Materials and Methods

Five chicken weighing about 1320 grams were anaesthetised using pentobarbitone sodium and perfused via the left and right internal carotid arteries with warm (35 ^{o}C) physiological saline solution. This was followed by perfusion fixation with 2.5% phosphate buffered (pH 7.40) glutaraldehyde solution, before which the right auricle was cut open for drainage. The eyes were enucleated, cut around the equator and the pecten carefully dissected out and immersed in the same fixative for six hours. Then the pecten was cut into small pieces using a sharp blade to expose the luminal surface of the capillaries. These pieces were postfixed in 1% phosphate buffered osmium tetroxide, dehydrated in a graded series of ethanol, critical point dried or dried with the use of Pedri IIR (Ted Pella, inc. USA) and then mounted on aluminium stubs with silver conductive points after which they were sputter coated with gold palladium complex and examined with JEOL JSM 35SF or Zeiss DSM 950 scanning electron microscope at 17 kv.

For transmission electron microscopy, postfixation with osmium tetroxide was followed by dehydration in a graded series of ethanol to propylene oxide and embedding in epoxy resin. Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined with Philips 301 and Zeiss 10 CR transmission electron microscopes.

Results

Scanning electron microscopy

The pecten oculi of the chicken is a highly vascularised intraocular organ situated on the elongated optic disc and projects into the vitreous humour in the posterior chamber of the eye. A scanning electron micrograph of a transversely cut surface of the pecten oculi (Fig. 1) revealed a long pleated lamina varying in thickness from 20 μm to 50 μm and consisting mainly of blood vessels. A more highly magnified view of a transected surface of the pectineal lamina (Fig. 2) demonstrated profiles of thick walled capillaries endowed with ruffled borders. Also evident were the melanocytes loaded with spherical melanosomes. The melanosomes measured roughly about 1.5 μm in diameter (Fig. 2).

Micrographs of a longitudinally transected capillary revealed the contours of the endothelial surface and the existence of a vast multitude of microplicae bulging into the capillary lumen (Fig. 3). At higher magnification, the luminal endothelial surface exhibited presence of closely packed undulating and branching microplicae (Fig. 4), protruding into the capillary lumen that rendered the surface a ruffled appearance at lower magnifications (Fig. 2). This vast array of microplicae showed no preferred orientation regarding either longitudinal or transverse axis of the capillaries and resulted into creation of numerous crevices on the endothelial surface (Figs 4, 5, 8).

Transmission electron microscopy

The capillary wall consisted of a modified endothelium and an extremely thick basal lamina (Figs 6, 7). The endothelium appeared as a thin strip of cytoplasm wedged between an extensively folded apical and basal plasmalemma (Figs 6, 8). A few mitochondria and micropinocytotic vesicles were observed in the endothelial cell cytoplasm (Figs 7, 8). Adjacent endothelial cells were joined together by elaborate occluding cell junctions (Fig. 8). The luminal plasmalemma infoldings projected into the lumen of the capillaries and measured about 1.25 μm in height. A longitudinal section across the luminal plasmalemma infoldings (Fig. 5) demonstrated their close packing, tortuity, branching and interdigitations. The processes measured up to 3.0 μm in length and between 0.2 μm to 0.8 μm in thickness.

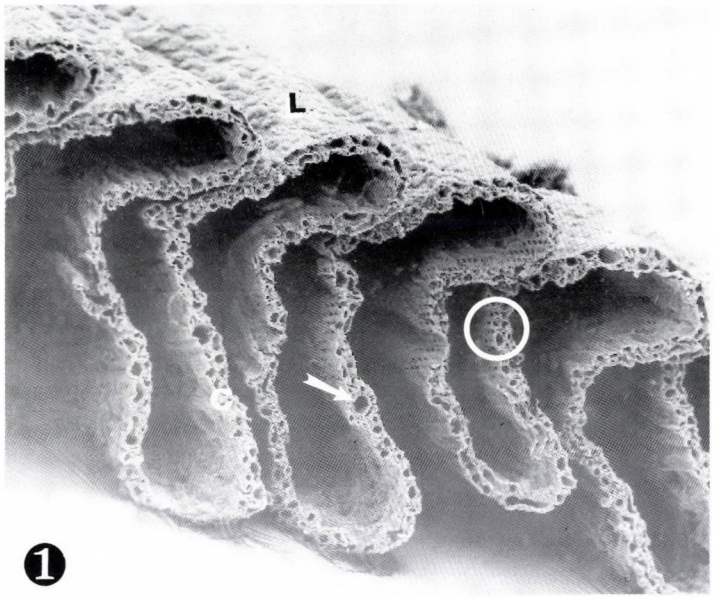


Fig. 1. Scanning electron micrograph of transversely cut surface of the pecten oculi of the chicken, displaying the blood vessels (arrow) and the pleat formation. Lateral aspect of the pectineal lamina (L), x 140



Fig. 2. Scanning electron micrograph of the area shown in the circle in Fig. 1 above. Note the thick-walled capillaries (CP) with ruffled borders (arrow) and the melanosomes (M) appearing in form of spherical bodies between them. Capillary wall (W), x 4800

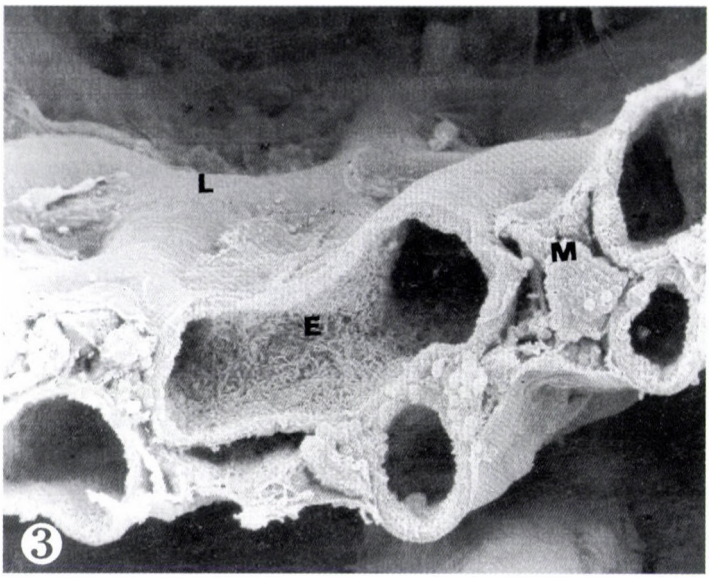


Fig. 3. Scanning electron micrograph of a transversely cut surface of the pecten oculi of the chicken exposing the luminal endothelial surface (E) of the pectineal capillary. Note that the endothelial surface is uniformly ruffled by microprojections. Melanosomes (M) are present. Lateral aspect of the pectineal pleat (L), x 1600

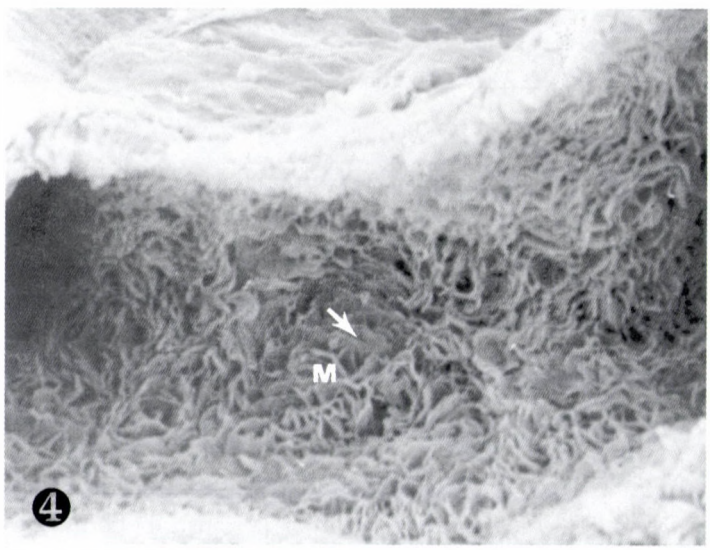


Fig. 4. Scanning electron micrograph of the luminal surface of the endothelium of the pectineal capillary. Note that this surface is highly ruffled by closely packed and branched microplicae (M). Note the crevices (arrow) between the microplicae, x 5610

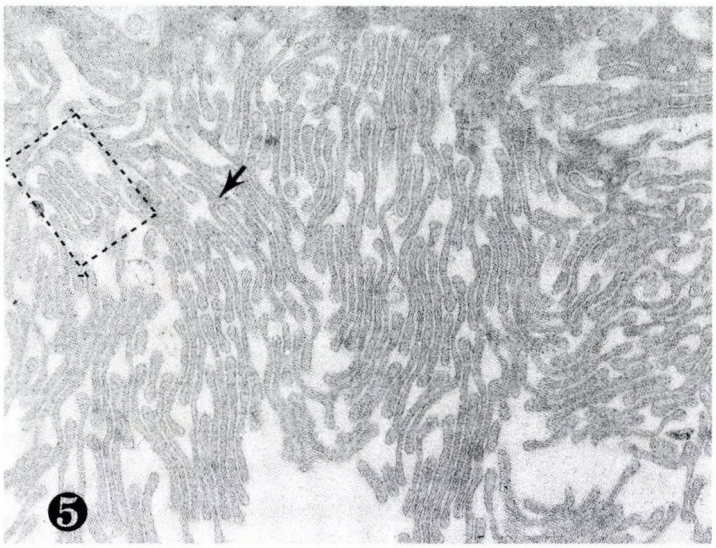


Fig. 5. Transmission electron micrograph of the endothelial microplicae sectioned parallel to the longitudinal axis of the capillary. Note that the closely packed microplicae brach (arrow) and interdigitate (rectangle), x 27800

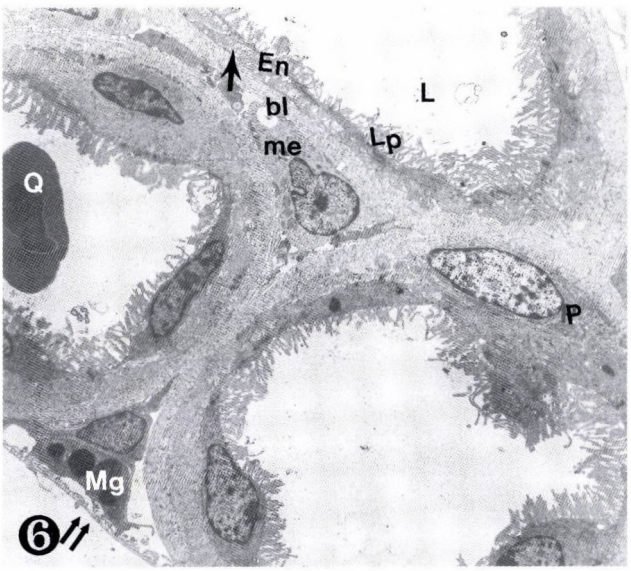


Fig. 6. A transmission electron micrograph showing three capillaries and melanocytes (me) that occupy the intercapillary spaces. Note the capillary endothelium bearing several luminal (Lp) and basal (arrow) microvilli-like projections. Erythrocytes (Q), superficial membrane of the pecten (double arrow), melanosomes (Mg), capillary lumen (L), basal lamina (bl) and pericytes (P) are present, x 4000

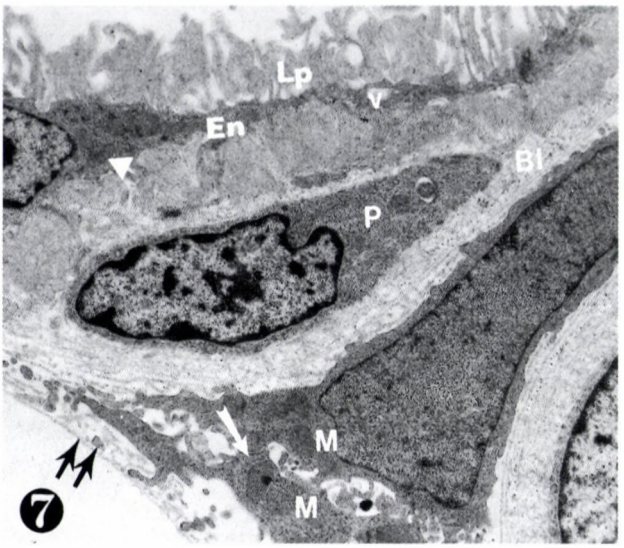


Fig. 7. A transmission electron micrograph displaying a pericyte (P) lodged in the thick capillary basal lamina (BI). Capillary endothelium (En). Superficial membrane (double arrow). Melanocyte cell junction (arrow). Mitochondrion in the capillary endothelium (arrowhead). Melanocytes (M), x 11000

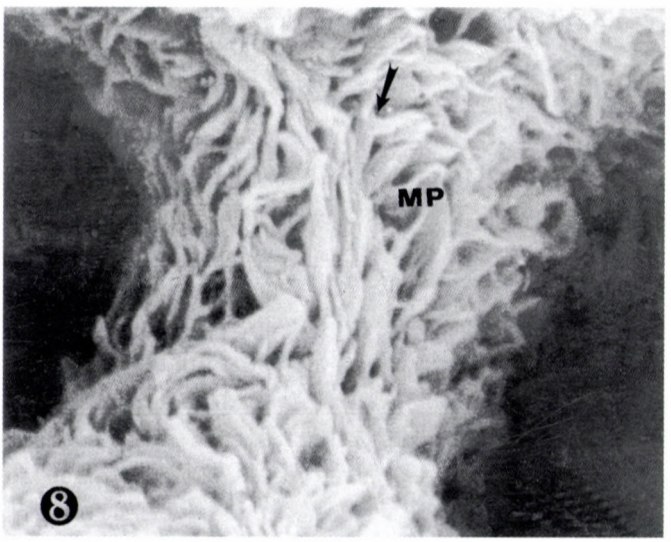


Fig. 8. Scanning electron microscopy view of the luminal surface of the pectineal capillary displaying several microplicae (MP) and crevices (arrow), x 16000

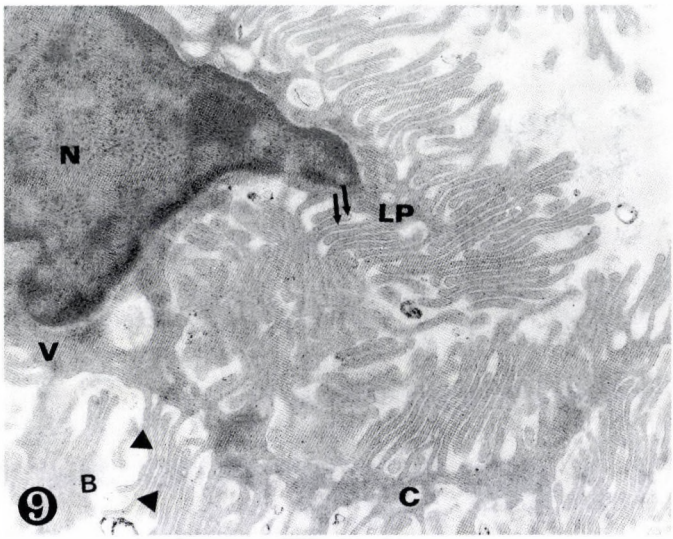


Fig. 9. Transmission electron micrograph of the capillary endothelium shows that the individual microprojections are delineated by a unit membrane which is continuous with the cell membrane between them (double arrow), such that the contents of each projection freely communicates with the cytoplasm. Note the large and small vesicles in the cytoplasm (V). Note also the close packing of the microprojections leaving narrow spaces between them. Cell junctions (arrowhead). Basal microprojections (B), luminal microprojections (LP). Endothelial cells nucleus (N). Cell body (C), x 27000

Discussion

The pecten oculi of the chicken is a highly vacularized and pigmented lamina overlying the linear optic disc /2, 15, 23/. The vascular network consists large drainage and supply vessels and small sized capillaries /21/.

The capillaries of the pecten oculi are reported to possess a highly specialised endothelium endowed with apical and basal specialisation /3, 6/ that are unique to vertebrate angioarchitecture. The apical specializations were shown in this study to be in form of interdigitating and closely packed endothelial microplicae that projected into the capillary lumen. In addition, these luminal microplicae had no specific orientation regarding either longitudinal or transverse plane of the capillary. This observations differs from the conventional view that these microplicae are orientated parallel to the longitudinal axis of the capillaries /1, 6/.

As regards the significance of these endothelial specializations, several investigators have suggested that they offer an increased surface for the transport enzyme systems, like alkaline phosphatase and carbonic anhydrase /3, 18, 25/. Though this could be the principal role, in the context of our observations, conjecture about the overt role these specializations could play in the pectineal haemodynamics seemed logical and tangible. Tailored modulation of blood flow in the pectineal capillaries could be affected by causing impedance to the circulating blood by the projecting microplicae thereby maximising the efficiency of the pecten in diffusion of blood-borne nutrients into the vitreous humour and the inner sensory retina. Impedance of blood flow would result into a de facto compartmentalisation of the capillary cavity into at least two microenvironments: one existing along the crevices between the microplicae where a maximal impediment to blood flow occurs and the other prevailing at the capillary lumen where continuous flow of blood could be effectively maintained. The advantage of such compartmentalisation in nutrient diffusion could be twofold: 1. by increasing the blood cell surface contact time and hence allowing increased time for nutrient extraction, and 2. by acting as sites of local osmotic gradients that facilitate diffusion of blood-borne nutrients. Although no data are available regarding the osmolarity of the fluid contained in the endothelial crevices of the pecten oculi, studies on other epithelia containing plasmalemma infoldings have shown that these infoldings contain fluid that is different from the intracellular fluid and the rest of the extracellular fluid that serve as sites of osmotic gradients facilitating diffusion /19/. The structural complexity, visa a vice, the modality of impedance is likely to vary in different avian species depending on their habits and nutritional demand of the neural retina. Scarce and low-profile endothelial microplicae in the capillaries of the pecten of the nocturnal great horned owl, Bubo virginianus /5/ and in the capillaries of the less specialised conus papillaries of the bobtail goana, Tiliqua rugosa /4/ as opposed to the elaborate endothelial microplicae in the pectineal capillaries of the diurnal Japanese quail /20/, pigeon /21/ and the chicken validates this conjecture.

The occurrance of micropinocytotic vesicles, though scarce coupled with the scarcity of mitochondria in the endothelial cytoplasm of the pectineal capillaries, indicates possibility of transcapillary migration of large molecules by way of passive micropinocytosis /16, 22/. Each vesicle may passively acquire sufficient energy from random collisons of adjacent molecules thereby detaching /22/, sustaining a low rate of turnover as such

without input of metabolic energy by the endothelial cells. Transport by passive diffusion would markedly reduce energy expenditure by the pectineal tissue and hence maximise its efficiency in provision of nutrients to the avascular inner retina. The apparent paucity of mitochondria recorded in this study and earlier studies /11, 21/, and the reported findings by others /9/, that there is little or no Na^+/K^+ ATPase activity in the pectineal capillary endothelium further support energy-independent or low energy-dependent transcapillary transport.

Endowment of a thick basal lamina in the pectineal capillaries appears unconductive for an organ involved in transport. However studies on disulphide passage /11/ and the reported absence of close-packing between the fibrillar layers of the basal lamina of the pectineal capillaries /4/ indicate that the basal lamina does not cause resistance to the movement of molecules. The thickened basal lamina may however be important in controlling the distensibility of the pectineal capillaries and hence in maintenance of plasmalemmal integrity. Such functional attribute appears realistic when one considers the relatively high pectineal blood flow rate of 20.0 ± 6.1 ml/min/g /13/. Moreover the basal lamina may act as a sieve holding back molecules based on size, shape and electrostatic charge /10/.

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NEUROTOXICITY OF PARAQUAT AND TRIPHENYLTIN IN THE EARTHWORM, EISENIA FETIDA SAV. A HISTO- AND CYTOPATHOLOGICAL STUDY

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Neuropathological effects of the pesticides paraquat (PQ, a herbicide) and triphenyltin (TPT, a fungicide) were studied on the postclitellar segmental ganglia of juvenile E. fetida specimens using light and electron microscopic methods. Growth retardation of the worms in standard culture medium was used as quantitative marker of sublethal effect.

There is a distinct neuron group in the segmental ganglia characterised by high sensitivity against PQ- and another one against TPT-toxication. However the marked histo- and neurocytopathological alterations were similar in PQ and TPT treated worms. Both chromatolysis and vacuolation of perikarya were revealed in sensitive neurons. Damaged cells were swollen and possessed degenerated rough endoplasmic reticulum cisternae. They were also characterised by swollen mitochondria, with electronlucent matrix and damaged inner membranes, and vesicular structures of various diameters as well as numerous lysosomes. Necrotic neurons with pyknotic nuclei and highly eosinophilic cytoplasm were also found in affected ganglia.

Several dividing neurons were found in PQ-toxicated worms while no cell division occurred either in control or TPT-toxicated animals. The exact neurochemical and functional identification of PQ- and TPT-sensitive neurons needs further investigations.

Keywords: Earthworm — test species — sublethal effect — paraquat — triphenyltin — segmental ganglia

Introduction

Eisenia fetida is a recommended test species to assess the potential ecotoxicological risks of pollutants /5, 16/. Sublethal effects of paraquat (PQ) /6/ and triphenyltin (TPT) /7/ have been studied on this test species in our laboratory. PQ, a broad-spectrum herbicide, is widely used throughout the agricultural industry and has caused many human deaths through suicidal

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and accidental ingestion /24/. Organotins are widely used fungicides and antifouling agents. Organotins are known to be considerably accumulated in some animals, therefore, their application is hazardous /23/. Both PQ /14/ and organotins /2, 3, 12, 17/ may result in heavy neurotoxic effects in mammals.

It is known that 1-methyl-4-phenyl-1,2,3,6-tetrahidro-pyridine (MPTP) induces destruction of dopaminergic neurons because MPTP is metabolised in the brain by monoamine oxidase-B primarily to the toxic metabolite N-methyl-4-phenyl-pyridinium (MPP^+) /13/. When the structure of the toxic product of MPTP was identified as MPP^+, which had been previously used as a potential herbicide (Cyperquat), attention was focussed on the known mechanisms of toxicity of a related herbicide, PQ (1,1'-dimethyl-4-4'-bipyridinium). The toxicity of PQ is associated with its NADPH-dependent metabolism to the free radical. This paraquat radical undergoes cyclic reduction and autoxidation, resulting in the production of reactive oxygen species, the superoxide anion, hydroxyl radical and hydrogen peroxide /1, 24/.

Trimethyltin (TMT) is known to produce neuronal necrosis in the mammalian central nervous system (CNS). The neurotoxic effect of TMT /4, 12/ and triphenyltin (TPT) /17, 18/ promoted the induced release of excitatory amino acids (EAAs) resulting in excitotoxication. The contribution of N-methyl-D-aspartate receptors (NMDA-receptors) to excitotoxicity has also been suggested /12/.

Both PQ /21, 22/ and TPT /20/ may depress mitochondrial respiration in vertebrates. Oxidative damage is frequently recognised in neurodegenerative diseases /11/. Neurons in invertebrate CNS are also vulnerable to anoxia and hyperoxia /19/, respectively.

This paper describes the histo- and cytopathological effects of sublethal concentrations of PQ and TPT on the segmental ganglia of E. fetida. Though earthworms are widely used reference species not only in ecotoxicological /16/ but also in neurobiological /10/ experiments no data are available so far on the neurotoxic effects of PQ and TPT in this species. Our observations prove that these environmentally introduced toxicants enter the central nervous system (CNS) of E. fetida resulting in neuronal lesions similar to was found in mammals.

Materials and Methods

Animals and culture medium

Specimens of Eisenia fetida Sav. (Annelida, Oligochaeta) were obtained from stock culture in our laboratory. The culture medium consists of peaty marshland soil and horse manure in proportions 1:1 (w:w), and was wetted to attain a moisture-content approximately 60%. Plastic bags containing 500 g wet-weight substratum were used and 10 juvenile worms were introduced in each bag at the start of the experiments. The average initial live weight was 200-230 mg. The experiments were carried out at 20 $\pm$ 2 ^{o}C temperature.

Chemicals and exposure

Stock solutions of the chemicals were mixed into the culture medium to give the desired concentrations. The applied concentrations for PQ were 200, 300 and 400, for TPT 3, 6 and 9 $mgkg^{-1}$.

Two kinds of controls were used. For control 1, the worms were kept in uncontaminated culture medium with added food. For control 2, food-deprived worms were used which were kept in moistened peaty marshland soil without added horse manure. The second control group was deemed necessary because at a certain concentration of pesticides worms failed to grow; indeed they lost weight. Despite the high food-content of the standard culture medium, worms exposed to PQ and TPT experienced physiological starvation status.

For light microscopic investigations (see below) worms kept in control or contaminated substates for 4 weeks.

Tissue preparation

For light microscopy, 40 individual worms were used in each experimental category. Worms were kept on moist filter paper for one day before dissection in order to minimise gut content. After anesthesia in 30% ethanol, postclitellar segments (30-34 segments) were dissected and fixed in Carnoy's solution and embedded in paraffin wax. The 10 μm thick sections were stained with Gomori's chromhaematoxylin and eosin.

For electron microscopy, 5 individual worms were used in each experimental category. The dissected ganglia from postclitellar segments were fixed in modified Karnovsky's solution (5% glutaraldehyde and 4% paraformaldehyde, buffered with 0.2 M phosphate buffer, pH 7.4). The ganglia were then postfixed with 1% osmium tetroxide in 0.2 M phosphate buffer, pH 7.4. The ultrathin sections were contrasted with uranyl acetate and lead citrate.

Results

Growth rate as sublethal marker

Both TPT and PQ resulted in a dose and exposure-time dependent reduction of the body mass of experimental animals. TPT at 3 $mgkg^{-1}$ had, slight retarding effect on the growth, but did not cause mortality. In contrast,

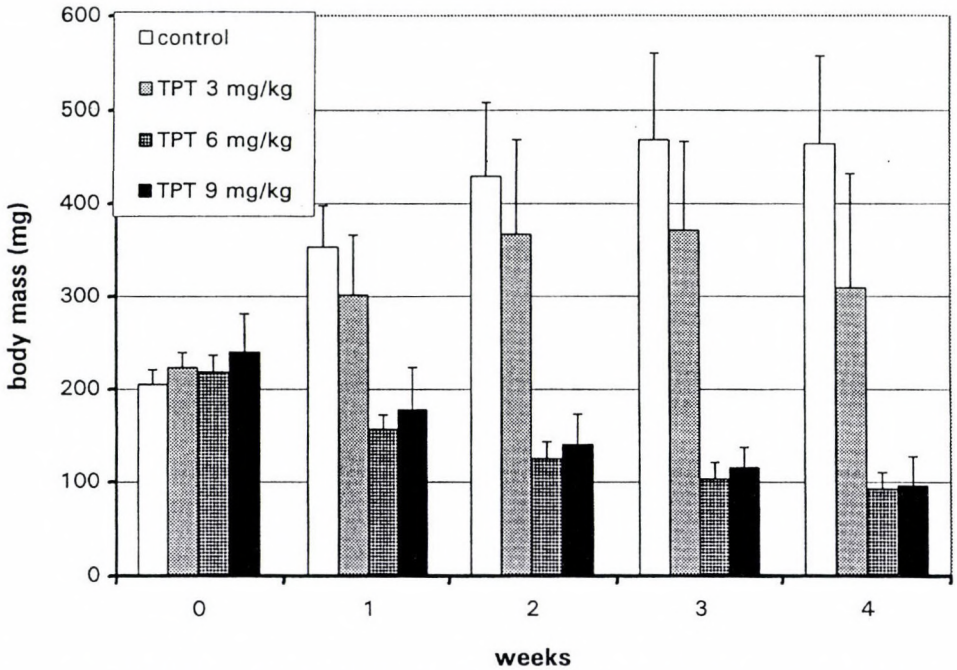


Fig. 1. The effect of triphenyltin (TPT) on the growth of E. fetida during a 4-week treatment. Bars indicate the standard error

only 50% of worms, kept in 9 $mgkg^{-1}$ TPT-containing medium survived the 4 week long toxication. Though 6 $mgkg^{-1}$ TPT retarded the body mass gain of worms (Fig. 1), most of them (87%) survived the experiments. Therefore these animals were used for histological investigations.

All of the PQ concentrations investigated yielded high toxicity. Only 5% and 12.5% of worms kept in substrata containing 300 and 400 $mgkg^{-1}$ PQ, respectively, survived the 4 week toxication period (Fig. 2). Since 42% of worms exposed to 200 $mgkg^{-1}$ PQ survived the toxication, their segmental ganglia were also morphologically investigated.

Histological observations

The light and electron microscopic structure of segmental ganglia of intact E. fetida is well documented /10/. Neurons are characterised by a highly basophilic perikaryon and a well developed nucleus containing a prominent nucleolus. Neither degenerating nor dividing cells occurred in the ganglia of control groups (Fig. 3).

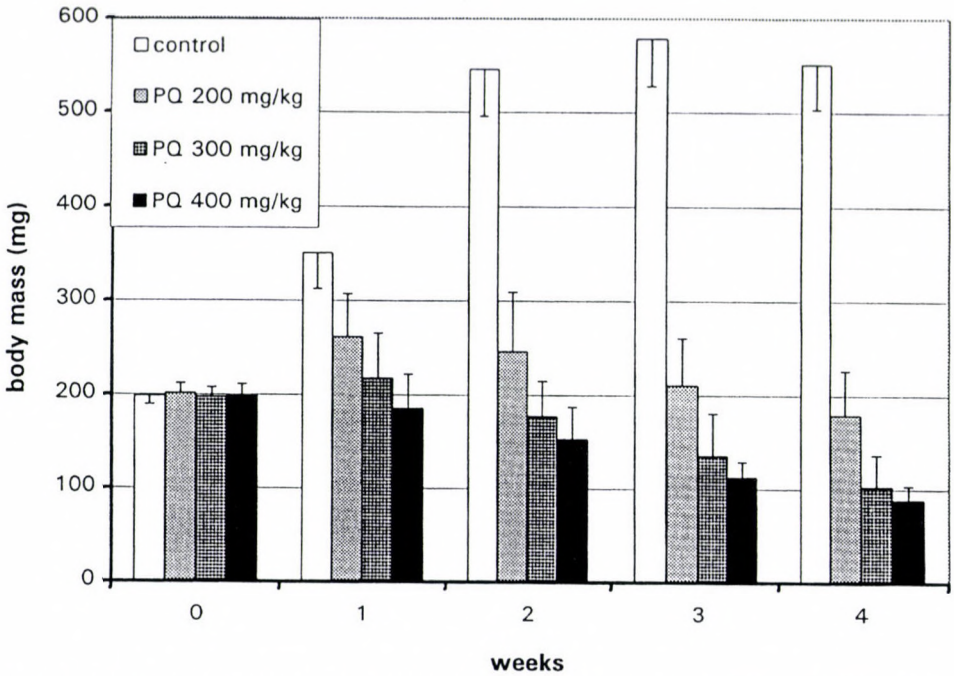
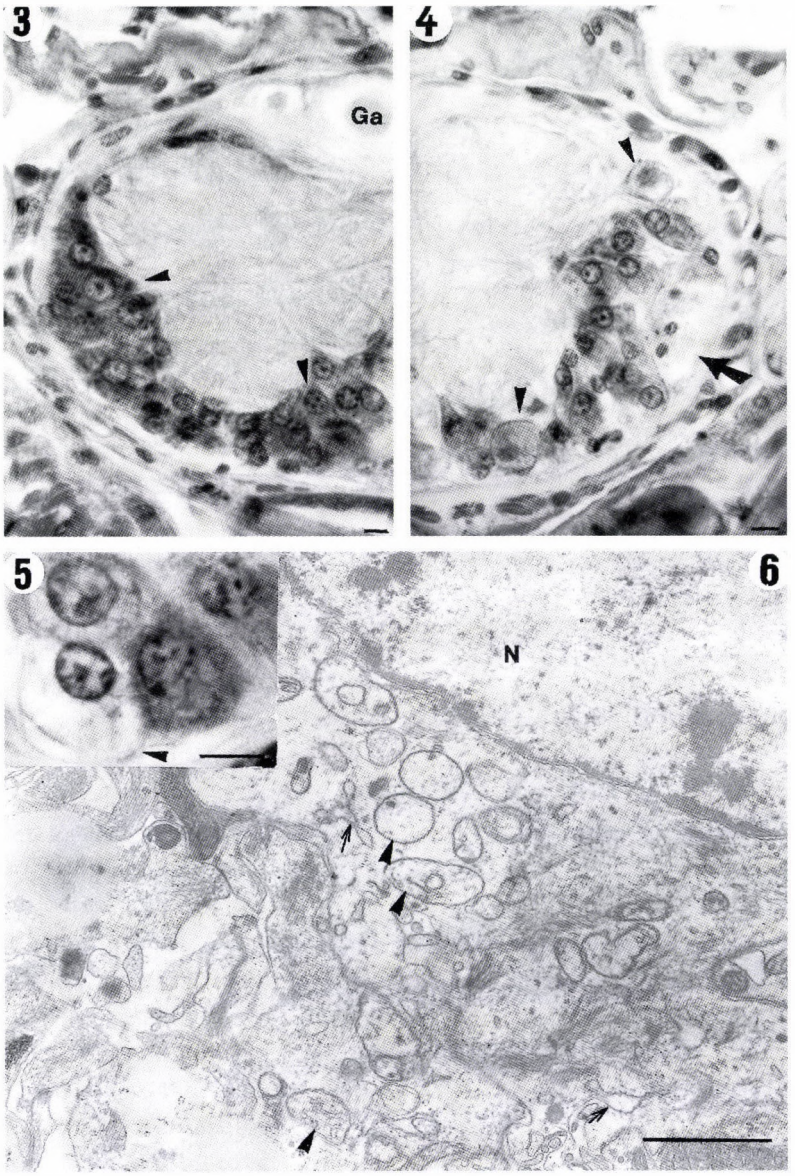


Fig. 2. The effect of paraquat on the growth of E. fetida during a 4-week treatment. Bars indicate the standard error

Effects of TPT on the structure of segmental ganglia

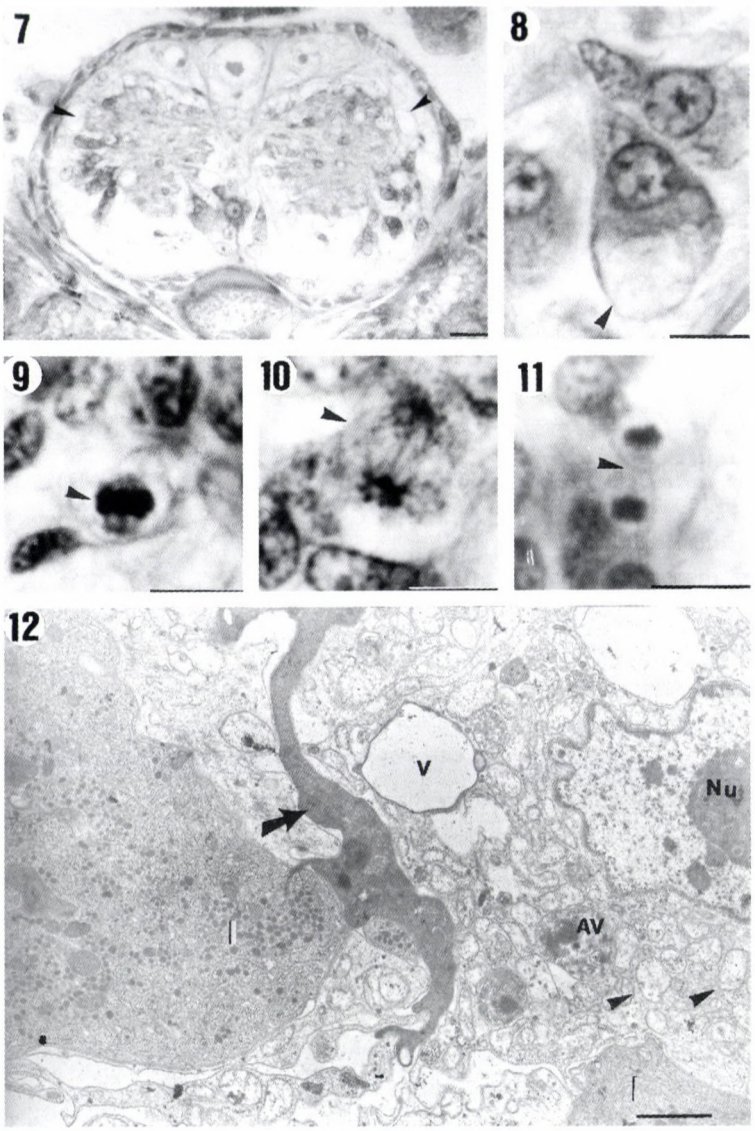
Some neurons were structurally unaffected while others appeared to be highly susceptible to TPT-toxication. Sensitive neurons were mainly located in the lateral regions of the ganglia. After 2 weeks of TPT exposure, significant chromatolysis was observed in the damaged lateral cells. The ganglia of animals toxicated for 4 weeks revealed not only necrotic neurons with pyknotic nuclei and highly eosinophilic cytoplasm (not shown) but also the swelling and vacuolisation of perykaria (Fig. 4).

Several neurons showed total loss of Nissl-substance resulting from TPT exposure (Fig. 5). Ultrastructural investigations of these cells indicated that the rough endoplasmic reticulum was degranulated, and that the mitochondria were disrupted to the extent that their matrices were electron-lucent and inner membranes were lost completely (Fig. 6). Furthermore, the accumulation of autophagic vacuoles and lysosomal structures were characteristic features (not shown) of damaged neurons.



Figs 3—6.

3. Low power light micrograph of a segmental ganglion of control E. fetida. Note the neurons with highly basophilic perikaryon (arrowheads). Ga: giant axon. Scale bar: 10 μm. 4. The histopathological effect of 6 $mgkg^{-1}$ TPT (4 weeks) on the segmental ganglia of E. fetida. Note the neuron lesion (arrow) and moderate chromatolysis (arrowhead). Scale bar: 10 μm. 5. High-power light micrograph of normal and vacuolised (arrowhead) neurons of a 6 $mgkg^{-1}$ TPT-treated (4 weeks) E. fetida. Scale bar: 10 μm. 6. Electronmicrograph of a vacuolised neuron. Damage to mitochondria (arrowheads) and endoplasmic reticulum cisternae (arrows) are characteristic. N: nucleus. Scale bar: 1 μm



Figs 7—12.

7. The histopathological effect of 400 $mgkg^{-1}$ PQ (4 weeks) on the segmental ganglia of E. fetida. Note the total loss of neurons (arrowheads) at the periphery of ganglion. Scale bar: 10 µm. 8. Normal and damaged neurons in a segmental ganglion of a 200 $mgkg^{-1}$ PQ-treated (4 weeks) E. fetida. Arrowhead shows a marked chromatolysis in the perikaryon. Scale bar: 10 µm. 9—11. Mitotic cells (arrowheads) in the segmental ganglia of 200 $mgkg^{-1}$ paraquat-treated (4 weeks) E. fetida. Fig. 9 = metaphase; Fig. 10 = anaphase; Fig. 11 = telophase. Scale bars: 10 µm. 12. Electronmicrograph of normal and damaged neurons in a 200 $mgkg^{-1}$ PQ-treated (4 weeks) E. fetida. Note the PQ-resistant neurons (I) with normal cytoplasm, pyknotic neural process (arrow) and a part of a vacuolised perikaryon. AV: autophagic vacuole, V: vacuole, Nu: nucleolus, swollen mitochondria (arrowheads). Scale bar: 1 µm

Effects of PQ on the structure of segmental ganglia

The position of the PQ-sensitive neuron population was not identical to that of the TPT-sensitive cells. PQ-damaged cells were situated mainly in the dorsomedian and dorsolateral part of the ganglia (not shown); however, in severely toxicated animals exposed to 400 $mgkg^{-1}$ PQ for 4 weeks, most neurons at the periphery of the ganglia were degenerated (Fig. 7). The pathological alterations in PQ-sensitive and TPT-sensitive neurons were similar. Both chromatolysis (Fig. 8), neuronal necrosis (not shown), as well as vacuolation of perikarya were observed. However the most striking histological alteration was the occurrance of dividing cells in the PQ-affected ganglia (Figs 9--11), a feature not observed in TPT-exposed or control ganglia.

At the ultrastructural level, the accumulation of vacuolar structures and lysosomes with various diameters, swollen endoplasmic reticulum cisternae, and damaged mitochondria were evident after PQ-exposure (Fig. 12).

Discussion

Recent data prove that the growth of juvenile E. fetida may be markedly reduced by a great variety of heavy metals and organic pollutants /16/. Retarded growth of E. fetida is a dose- and exposure time-dependent, susceptible for the sublethal effects of both PQ /6/ and TPT /7/. Beside the possible metabolic reasons for retarded growth, direct toxicant effects on neuronal or neuromuscular functions resulting in diminished feeding ability should not be excluded.

It is known that PQ is strongly adsorbed by negatively charged clay surfaces; therefore, it has been suggested that the availability and, therefore, the potential toxicity of the molecule to soil organisms is often limited under field conditions /23/. Our study proves that, despite incorporating PQ into organic soil containing a low amount of clay minerals, it may be heavily toxic to E. fetida /6/. Consequently, despite living in organic soil, E. fetida may be a target organism for PQ-toxication.

In some regions of Canada and Hong Kong a close relationship between the historical herbicidal use of PQ and the incidence of Parkinson's disease has been reported /14/. Other data indicate that PQ can cause marked neurotoxicity /13, 24/. In mammals PQ primarily induces degeneration in the nigrostriatal dopaminergic system /13/. Our histological examinations on the

segmental ganglia of the PQ treated E. fetida prove that environmentally administrated PQ may enter the CNS of the earthworms resulting in histopathological alterations in the dorsomedian and dorsolateral neuron populations. However other neuron groups in strongly toxicated animals have also shown these pathologic changes indicating that there are neurons with various sensitivities against PQ toxication. Damage to the most sensitive neurons could be induced by 200 $mgkg^{-1}$ PQ, while damage to the less sensitive occur only exposure to 300 or 400 $mgkg^{-1}$ PQ.

PQ-induced damage in neurons of E. fetida shows similar histological features to those previously described in the neurons of the ganglia of hyperbaric oxygen-treated leeches /19/, thus suggesting that the PQ-induced neuron degeneration is mediated via oxyradicals.

The occurrance of mitotic figures in damaged ganglia of PQ-toxicated worms raises two questions. First, are these dividing cells neurons or do they represent another type of ganglion (glial cell or stem cell)? We cannot fully exclude non-neuronal mitosis, but the size and position of dividing cells strongly suggest a neuronal origin. The second question is whether PQ has a direct stimulating effect on cell division, or is this feature a consequence of the disturbance of a neuronal metabolic pathway? Our results indicate that PQ has a stimulating effect on cell division. Induced mitotic activity in the segmental ganglia of PQ-treated worms may relate to the high regeneration capacity of earthworms. During the high body growth phase of juveniles or starved worms introduced into fresh food-amended substrate, mitotic figures were never found in segmental ganglia. The growth of the ganglia during postembryonic life is achieved by the enlargement of the perikarya and the neuronal processes (Molnár, unpublished). In spite of the PQ induced mitotic activity, we established a very low level of neuronal recovery after PQ toxication.

Organotin compounds, including TPT, are well-known neurotoxicants /16, 17/ in mammals. Although TMT was recognized first as a hippocampal neurotoxin, later studies indicated that its central effects are more widespread /11/. The exact molecular target and mechanism of neurotoxicity for triorganotins in not known, but strong evidence supports a link to the metabolism and release of the excitatory amino acid glutamate /4/. Several ecotoxicological effects of organotins on different invertebrates groups /8, 22/ are well known. Though pathological effects on the reproductive system in gastropods /9/ and muscular lesions in earthworms (Fischer and Zsombok, unpublished) have already been noted, data on organotin-induced neurotoxicity in invertebrates are still sparse /14/.

The present work proves that sublethal concentrations of TPT result in neuronal damage in the segmental ganglia. Lateral neurons are the primary targets of this intoxication. Ultrastructural alterations resembled those of PQ-evoked ones. However, it is noteworthy that mitotic figures were not found in the ganglia of TPT treated worms, and that postexposure recovery was also moderate.

In conclusion, these results prove that PQ and TPT applied in sublethal concentrations may enter into the segmental ganglia of earthworms thus causing marked neuronal lesions. Consequently, earthworm may be used for testing the neurotoxic effect of these and similar neurotoxicants. The precise neurochemical and functional identification of the PQ and TPT affected neurons require further studies.

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BIOTOXICITY OF MERCURY TO CHLORELLA VULGARIS AS INFLUENCED BY AMINO ACIDS

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The toxicity of mercury ion, on Chlorella vulgaris, is largely influenced by amino acids. Five amino acids, namely alanine, asparagine, glutamic acid, cysteine and histidine, were added separately to the medium containing static dose of mercury. Survival (%) of the alga was reduced with the increasing concentrations of mercury. Of these five amino acids, cysteine was found to be the most effective while alanine and glutamic acid were the least effective on reducing the toxic effect of mercury on the alga measured in terms of growth, chlorophyll and protein content.

The order of detoxification was Alanine < Glutamate < Asparargine < Histidine < Cysteine. Amino acids form ligands with Hg^{2+} making it less toxic to the alga and produce an additional source of energy for growth and development.

Keywords: Amino acids — Chlorella — mercury — static dose

Introduction

Mercury, a non-essential heavy metal, is toxic in metallic, ionic and organic forms. In addition to simple salts such as chloride, nitrate and sulfate, mercury has the ability to form an important class of organometallic compounds. These are characterised by the attachment of mercury to either one or two carbon atoms /20/ of the organic compound to form organic complex. This leads to the assumption that mercury is capable of forming ligands with the surrounding organic substances in the environment, thereby being unavailable to the aquatic organisms. The influence of a number of organic compounds including amino acids, on the toxicity of various heavy metals has been tested /3, 8, 11/. However, study to evaluate amino acid ligands on microorganisms of freshwater environment in a situation when mer-

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cury just stops their growth, though under laboratory conditions, is interesting. In this paper the effects of selected amino acids on the toxicity of Hg^{2+} to green alga Chlorella vulgaris has been presented.

Materials and Methods

The axenic stock cultures of Chlorella vulgaris Beij. were grown at 27 ± 2 °C in 100 ml borosilicate conical flasks containing 50 ml Chu No. 10+ medium /18/ with all six micronutrients /9/. An aliquot of 0.4 ml mid-log phase (7-days-old) culture per 10 ml (initial density 1×10^5 cells/ml) was added as the initial inoculum. The cultures were maintained in a condition similar to that described by Mohapatra and Mohanty /15/. The initial absorbance (at 678 nm) of the cultures after algal inoculation ranged between 0.015 and 0.019. The amount of Chlorophyll, and protein contents of the experimental cultures at the beginning of incubation was 0.102 ± 0.016 mg/l and 4.79 ± 0.68 mg/l, respectively.

Survival test was made by inoculating the alga in nutrient agar plates as done by Mohanty et al. /14/. The colonies were counted after 10 days with the help of a binocular microscope. Alanine, asparagine, glutamic acid, histidine and cysteine were added separately at different concentrations (0.0 to 20.0 mg/l) to the culture tubes containing the static dose of mercury (0.200 ± 0.004 mg/l at $p = 0.05$) at the beginning of log phase of growth to study the mercury speciation and effect of ligand formation with amino acids on the alga. Measurement of growth (O.D. at 678 nm), chlorophyll content /1/ and protein content /13/ were made at 4 days interval till the 12th day. Results were statistically tested with calculation of least significant difference (LSD), confidence limit (at $p = 0.05$), correlation coefficients and standard errors /19/. The LC_{50} and static concentration of the metal were calculated as per the procedure described by Mohanty et al. /14/.

Results

Reduction of survival of the alga was noticed at all the selected concentrations of Hg^{2+} (Table 1). Considering the survival of the alga in control as 100% it was observed that it gradually decreased with increase of Hg^{2+} concentrations. Significant reduction of the survival of the alga was observed with $\geq$ 0.1 mg/l of Hg^{2+}. In the test alga, the LC_{50} of the heavy metal was 0.128 ± 0.009 mg/l while the population remained static at 0.20 ± 0.004 mg/l. Complete death of the cells was reported at 0.3 mg/l of the metal.

Analysis of growth showed that addition of cysteine to the culture containing static dose of mercury produced significant increase in growth to the extent that mercury toxicity seemed to be virtually eliminated at 15 mg/l on the 12th day. For example, the growth of the culture in medium with-

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 ± 0.009 mg/l, $p \leq 0.05$
Static concentration = 0.20 ± 0.004 mg/l, $p \leq 0.05$

out Hg^{2+} after 12 days of incubation was nearly equal to that observed in the Hg^{2+} containing medium supplemented with $\geq$ 10 mg/l of cysteine (Table 2). However growth enhancement by cysteine on the 4th day was not remarkable at concentrations $\leq$ 5 mg/l (Table 2). Significant increase in growth by cysteine was seen at all its tested concentrations on the 8th and 12th day (LSD = 0.017 and 0.021, respectively). Histidine-enriched cultures were next to cysteine in enhancing growth of Chlorella. On the 4th day there was no significant increase of growth at any of the tested concentrations of histidine. Significant acceleration of growth could be observed after 8 days when the culture were supplemented with $\geq$ 5 mg/l of histidine but the difference among the cultures containing 5-15 mg/l of histidine was insignificant. Growth was significant between 5 mg/l and 15 mg/l with histidine in the medium on the 12th day (LSD = 0.02). On adding asparagine, there was an increase in growth at 5 mg/l though the rise in growth remained insignificant thereafter (LSD = 0.001 and 0.004, respectively). This indicated that increase in concentration of asparagine from 5 mg/l up to 20 mg/l did not cause progressive and significant decrease of Hg^{2+} toxicity to the alga. on the 12th day there was significant increase of growth at 10 mg/l and 15 mg/l of the amino acid but the correlation between its concentration and growth was not significant ($r = 0.67$; $n = 5$). Addition of glutamic acid and alanine had no significant effect on Hg^{2+} toxicity as the acceleration in growth, whatsoever, was minimal. In glutamic acid-supplemented media none of the se-

Table 2

The effect of amino acids on growth, chlorophyll and protein content of C. vulgaris at static dose of mercury

Amino acid (mg/l)		Growth (O.D.)			Chlorophyll (mg/l)			Protein (mg/l)		
		4th day	8th day	12th day	4th day	8th day	12th day	4th day	8th day	12th day
Control ($-Hg^{2+}$) (Without Hg^{2+})		0.045	0.233	0.528	0.252	4.386	6.863	12.174	30.364	46.892
ALA	0	0.022^{a}	0.022^{a}	0.022^{a}	0.091^{a}	0.091^{a}	0.091^{a}	4.380^{a}	4.380^{a}	4.380^{a}
	5	0.022^{a}	0.022^{a}	0.022^{a}	0.091^{a}	0.091^{a}	0.091^{a}	5.480^{b}	6.210^{b}	6.210^{b}
	10	0.022^{a}	0.022^{a}	0.060^{b}	0.091^{a}	0.091^{a}	0.532^{c}	8.520^{c}	7.540^{c}	7.790^{c}
	15	0.022^{a}	0.022^{a}	0.060^{b}	0.091^{a}	0.091^{a}	0.622^{c}	9.800^{c}	7.910^{c}	7.790^{c}
	20	0.022^{a}	0.022^{a}	0.033^{a}	0.091^{a}	0.091^{a}	0.182^{b}	7.544^{b}	7.030^{c}	6.080^{b}
ASP	0	0.018^{a}	0.018^{a}	0.020^{a}	0.152^{a}	0.152^{a}	0.152^{a}	5.480^{a}	5.600^{a}	5.720^{a}
	5	0.022^{b}	0.025^{b}	0.052^{b}	0.182^{a}	0.323^{c}	0.619^{b}	5.480^{a}	6.691^{b}	12.050^{b}
	10	0.022^{b}	0.025^{b}	0.125^{d}	0.182^{a}	0.289^{b}	2.160^{d}	5.480^{a}	9.130^{c}	21.051^{d}
	15	0.022^{b}	0.028^{b}	0.123^{d}	0.182^{a}	0.289^{b}	1.676^{c}	5.480^{a}	6.691^{b}	20.561^{d}
	20	0.022^{b}	0.030^{b}	0.080^{c}	0.182^{a}	0.289^{b}	1.399^{c}	5.480^{a}	6.822^{b}	17.280^{c}
GLU	0	0.018^{a}	0.018^{a}	0.013^{a}	0.092^{a}	0.058^{a}	0.056^{a}	4.343^{a}	1.338^{a}	1.137^{a}
	5	0.023^{a}	0.023^{a}	0.055^{b}	0.098^{a}	0.250^{b}	0.285^{b}	4.583^{a}	1.582^{a}	5.110^{b}
	10	0.025^{a}	0.025^{a}	0.058^{b}	0.101^{a}	0.255^{b}	0.419^{c}	4.337^{a}	5.840^{b}	7.057^{b}
	15	0.028^{a}	0.037^{b}	0.062^{b}	0.251^{b}	0.521^{c}	0.802^{d}	5.503^{a}	5.840^{b}	10.220^{c}
	20	0.032^{a}	0.043^{b}	0.072^{c}	0.256^{b}	0.731^{d}	0.889^{d}	6.623^{a}	6.205^{b}	12.045^{d}
HIS	0	0.015^{a}	0.015^{a}	0.015^{a}	0.091^{a}	0.091^{a}	0.091^{a}	4.750^{a}	4.750^{a}	4.750^{a}
	5	0.015^{a}	0.045^{b}	0.060^{b}	0.091^{a}	1.224^{b}	1.500^{b}	5.480^{b}	6.570^{c}	10.330^{b}
	10	0.015^{a}	0.055^{b}	0.165^{c}	0.091^{a}	1.279^{b}	2.241^{b}	5.480^{b}	6.450^{c}	21.300^{c}
	15	0.015^{a}	0.065^{b}	0.290^{d}	0.091^{a}	1.332^{b}	3.337^{c}	5.600^{b}	7.720^{d}	29.330^{d}
	20	0.015^{a}	0.070^{b}	0.155^{c}	0.091^{a}	1.197^{b}	1.023^{b}	4.750^{a}	5.110^{b}	10.350^{b}
CYS	0	0.020^{a}	0.020^{a}	0.020^{a}	0.161^{a}	0.162^{a}	0.182^{a}	5.252^{a}	5.136^{a}	5.252^{a}
	5	0.038^{b}	0.170^{b}	0.453^{c}	0.174^{a}	3.595^{b}	5.988^{c}	8.170^{b}	22.993^{b}	38.517^{b}
	10	0.043^{c}	0.218^{d}	0.487^{d}	0.229^{b}	4.016^{d}	6.513^{d}	8.754^{b}	25.328^{d}	42.368^{c}
	15	0.043^{c}	0.226^{d}	0.503^{e}	0.249^{b}	4.239^{d}	7.018^{e}	9.221^{b}	29.646^{e}	44.287^{c}
	20	0.045^{c}	0.200^{c}	0.400^{b}	0.168^{a}	3.263^{c}	5.499^{b}	8.637^{b}	24.744^{c}	40.851^{b}

*Values represented by same letter are not significantly different from each other at $p \leq 0.05$ tested through LSD.
Alanine (ALA), Asparagine (ASP), Glutamic acid (GLU), Histidine (HIS), Cysteine (CYS)

lected concentrations of the amino acid were effective to reduce toxicity of Hg^{2+} within first 4 days of incubation but after 8 days the concentrations $\geq$ 15 mg/l of glutamic acid caused a significant growth acceleration compared to that recorded in culture containing Hg^{2+} only. After 12 days, however, each concentration of the amino acid significantly reduced Hg^{2+} toxicity to the alga.

Like growth, chlorophyll content of Chlorella was found to be influenced by addition of cysteine in Hg^{2+} amended medium. Remarkable detoxifica-

tion on the 8th and 12th day by cysteine resulted in significant increase in chlorophyll content even at its lowest tested concentration (LSD = 0.046 and 0.366, respectively). There existed a significant positive correlation between the concentration of cysteine and increase in chlorophyll-content on the 8th day as well as 12th day ($r = 0.84$ and 0.92, respectively; $n = 5$). For histidine, the significant increase in chlorophyll-content was observed on the 8th day for all tested concentrations and on the 12th day (LSD = 0.773) for 15 mg/l. With addition of asparagine, the effect of mercury was reduced but to a lesser extent in comparison to cysteine and histidine. Increase in chlorophyll-content at 5-10 mg/l of the amino acid was found on the 8th day (Table 2) and maximum chlorophyll content at 10 mg/l on the 12th day, though reduction occurred at higher concentrations. There was no significant increase of the pigment till the 8th day with alanine addition. However, on the 12th day, significant increase of chlorophyll content with alanine addition between 10 and 15 mg/l was noticed but this was not remarkable (LSD = = 0.093). In glutamic acid-enriched cultures the pigment-content varied significantly at all the tested concentrations except with 10 mg/l on the 4th day but the correlation between glutamic acid concentrations and chlorophyll-contents was insignificant ($r = 0.07$, $n = 5$). Significant increase in the pigment content was seen at 15-20 mg/l on the 8th day and at 20 mg/l on the 12th day (Table 2).

Cysteine and histidine were both effective as they almost showed a same trend in influencing the protein content of Chlorella at the static dose of mercury. On the 4th day, significant rise in protein content at 5 mg/l of cysteine (LSD = 1.425) as well as histidine (LSD = 0.450) was noticed and the increase remained insignificant at the higher concentrations. There was no increase in protein-content with asparagine on the 4th day (Table 2). However, at 10 mg/l of the amino acid maximum protein-content was observed on the 8th day and 12th day. Addition of alanine caused significant increase in protein-content from 5 mg/l up to 15 mg/l on the 4th day (LSD = = 0.239) while from 5 mg/l to 10 mg/l on the 8th day (LSD = 0.106) and 12th day (LSD = 0.108). Addition of glutamic acid did not increase the protein-content on the 4th day but showed significant increase at 10 mg/l on the 8th day (LSD = 0.274) and from 10 mg/l up to 20 mg/l on the 12th day (LSD = 1.249).

Discussion

The present results indicate that amino acids affect the process of detoxification of heavy metals and these are in agreement with the observations of Kosakowska et al. /12/. Formation of complexes of amino acids with ions of heavy metals has been postulated by Jones /11/ and this has been regarded as the mechanism of extracellular regulation in the concentrations of free heavy metal ions.

The amino acids can decrease the toxicity of mercury at a particular range of concentration. The biological activity of Hg^{2+} can be dramatically changed in the presence of cysteine, manifested by enhancement in growth at the lowest tested concentration. Studies with copper have demonstrated that addition of inorganic (ammonia and hydroxyl) and low molecular weight organic (alanine, glycine, glutamate and citrate) ligands cause Cu^{2+} ions less toxic /4, 5/. Quite possibly the amino acid Hg^{2+} ligand results in acceleration of growth of Chlorella. Cowan et al. /5/ reported that Cu^{2+} formed less toxic complexes with organic carbon sources like hexoses, organic acids and amino acids and Farrell et al. /7/ have observed that Hg^{2+} formed less toxic (low charged) or non-toxic (uncharged) complexes with different amino acids.

Mercury toxicity is dependent on the nature of the co-ion present in the medium. Cationic metal ligands are generally considered to be toxic whereas anionic ligands and neutral species are not toxic because they cannot compete for sites on the cell-surfaces /2/. Mercury forms cationic ligands with histidine /Hg (H-HIS) HIS^{+}/ and alanine ($HgAla^{+}$). These ligands are unable to reduce mercury toxicity to the extent as cysteine, though are non-toxic to Chlorella. Asparagine and glutamic acid form neutral complexes ($HgGlu^{O}$ and $HgAsp^{O}$). Their inability to reduce toxicity is probably a consequence of a polar nature of the molecules i.e. the Hg^{2+} ion is bound through α-amino and α-carboxyl groups and carries a charge of +1 while the terminal carboxyl group is negatively charged resulting in a net charge of zero /7/. Such detoxifying effects of the amino acids are concentration dependent. The amino acids are quite effective between a range of 5 mg/l to 15 mg/l. Higher concentrations of amino acids such as $\geq$ 20 mg/l perhaps augment mercury toxicity as the uptake of mercury-amino acid ligand becomes saturated.

Amino acid dissolved in Hg^{2+}-amended medium cause a substantial increase in chlorophyll-content. Rai et al. /17/ have suggested that the inhibition of the photosynthetic electron transport coupled with ATP generation is the major cause for inhibition of biological activities. Mercury, in the

free and uncomplexed forms, (Hg^{+} and Hg^{2+}) affects the chlorophyll molecules in the antenna side of the pigment system /21/. The reduction of photosynthesis and chlorophyll content is resulted when Hg^{+} is added with humic acid instead of organic nutrients due to the acidic nature of the carbon source /10/. The low ameliorative action of glutamic acid, alanine and asparagine probably occurs similarly in the present experiment.

Mercury affects the protein-content of algae by influencing both synthesis and degradation /6/. The metal ion has a greater affinity towards sulphur containing amino acids than other amino acids. The binding preference of Hg^{2+} for sulfhydryl, thioether and imidazole groups at catalytically active centers in enzymes provides the biochemical basis for much of its toxicity /16/. Consequently, molecular arrangements which inhibit the ability of Hg^{2+} to combine with these enzymes result in reduced toxicity. The ameliorative effect of amino acids bears a direct relation with the protein content up to a certain concentration and this may be due to molecular arrangement of the complexes.

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BOOK REVIEW

CEPHALOPOD BEHAVIOUR

by

Roger T. Hanlon and John B. Messenger

Cambridge University Press, Cambridge, 1996, pp. 232, 50,- GBP
ISBN 0 521 42083 0

Cephalopods are considered as being among the most mysterious and phantastic living creatures of our planet. The explanation seems to be simple and plausible: they have a rather secret life and, at least for our -- anthropomorphically trained — eyes, a really extremely peculiar physical/morphological appearance, which shows a curious "segmentation" of their body with long arms on the head, human-like eyes, a continuously changing form, and perhaps the most important, a capability of performing the phantastic camouflages which includes the combination of the different elements of the body pattern. Behind all of these characteristics, a very complex behavioural repertoir is to be found. The authors, both of them outstanding representants of the cephalopod neuroscience and behaviour research, have presented a beautiful and, at the same time, scientifically high standard book. In 10 chapters, the book contains all important informations on cephalopod behaviour, from the neural, sensory and efferent aspects to the different forms of behaviour. The 10 chapters are the following: 1. Introduction, including the classification, phylogeny and some historical aspects of the study of cephalopod behaviour; 2. Senses, effectors and the brain; 3. Colour change and body patterning; 4. Feeding and foraging; 5. Defence; 6. Reproductive behaviour; 7. Communication; 8. Learning and the development of behaviour; 9. Ecological aspects of behaviour; 10. Nautilus. The book also contains a list of more than 1000 references, and a Subject Index, as well as 115 Figures, including both drawings and photographs. The great majority of the photographs were taken masterly by Roger Hanlon, introducing the reader into really intimate events of cephalopod life. The book can be recommended to neuroscientists dealing with comparative neurobiology and/or neuroethology as well as to readers lecturing neuroscience at both lower and higher university levels.

K. Elekes
(Tihany)

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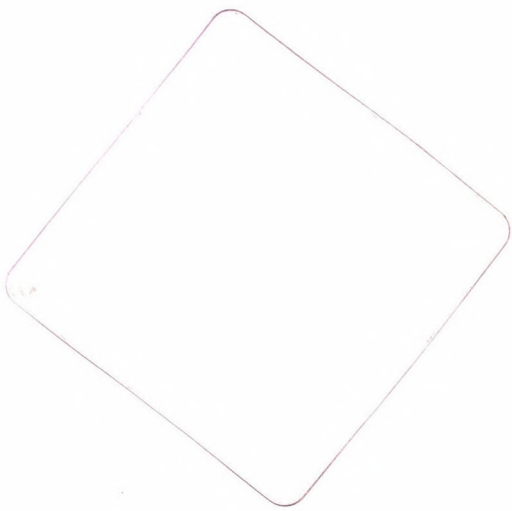
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