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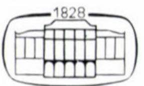

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ROLE, MECHANISM OF ACTION AND APPLICATION OF GONADOLIBERINS IN REPRODUCTIVE PROCESSES

I. TEPLÁN

1st Institute of Biochemistry, Semmelweis University Medical School, Budapest, Hungary

(Received 1988--10--28)

Gonadoliberin (gonadotropin releasing hormone, GnRH) plays a central role in the regulation of reproductive functions as it regulates the release of both luteinizing hormone (LH) and follicle stimulating hormone (FSH). The isolation and structure determination of GnRH opened the possibility of its use for influencing reproductive processes. This possibility initiated a rapid development in the design of potent and long-acting GnRH agonists and antagonists. The most important structural modifications of GnRH leading to superagonists are the D-amino acid substitutions in position 6 combined with Pro^9-ethylamide or $azaGly^{10}$ at the C-terminus. We have synthesized several superagonists of GnRH according to these substitution principles. Furthermore, our L-isoaspartyl modification in position 6, as a new approach to GnRH agonist design, also resulted in superactive analogs. The recently discovered sequences of non-mammalian GnRH-s opened new routes for us to synthesize species specific GnRH agonists. All three groups of the above mentioned GnRH analogs have been successfully used for the treatment of sexual disorders of different animals (cattle, pigs, rabbits, etc.). Ovulation synchronization and a 30% increase in the fertility rate could be achieved by using GnRH agonists in cattle breeding. Analogs derived from species specific sequences could be applied for the induced artificial propagation of fish even out of the spawning season. It is known that superactive GnRH analogs can suppress the growth of certain hormone-dependent tumours. In vitro and in vivo tests of our analogs showed promising antitumour activity in breast cancer which might be explained by the mechanism of desensitization. Almost a hundred antagonist analogs of GnRH have been developed in our laboratory. The most effective ones contain 4 or 5 D-amino acids, and one of them is even orally active. The inhibition of ovulation can also be achieved by the administration of GnRH superagonists. This phenomenon might also be explained by the desensitization of LH-release. Radioactive analogs specifically labeled with tritium in different amino acid residues have been synthesized and used for studying tissue distribution and biodegradation of gonadoliberins. Analogs containing a photoreactive group have been prepared and applied for the trials of GnRH receptor isolation.

Send offprint requests to: Prof. I. Teplán, 1st Institute of Biochemistry, Semmelweis University, Medical School, H–1088 Budapest, Puskin u. 9, Hungary

Akadémiai Kiadó, Budapest

Keywords: GnRH — Agonist analogs — Antagonist analogs — Structure-activity relationship — Desensitization of LH-release — Antitumour activity — Tritium labeling — Biodegradation — Veterinary applications — Clinical applications — Photoaffinity labeling

The role of GnRH in the regulation of sexual functions

People, especially scientists, have always been interested in the fundamental feature of living beings that they can reproduce themselves. The explanation of regulation of the reproductive processes and of the mechanism of regulation is especially exciting in the case of vertebrates, primarily in the case of mammals including humans. The sexual functions and processes of sexual behavior which cannot be separated from the reproductive functions are controlled by a complicated hormonal and neuro-regulatory mechanism in which, beside the effects of the nervous system, hormonal interactions of the hypothalamus, hypophysis and gonads have an important role.

The sexual functions are regulated by an extremely complicated system containing various feed-back circuits influenced by a lot of parameters. The complexity of the regulation, counter- and interactions are presented in Fig. 1 /3, 9, 10, 11, 36/. If the regulation of sexual functions is investigated through the secretion of gonadotropins (FSH and LH), it is obvious that GnRH plays a central role in the regulation.

The postulation and also the experimental evidence for the existence of hypothalamic factors releasing luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were presented by G. Harris /30/.

In the sixties, several research groups were in a competition to isolate and determine the sequence of LH, FSH releasing factors as well as other factors of hypothalamic origin. Finally in 1971 Andrew W. Schally isolated the homogeneous hormone from porcine hypothalami and determined the primary structure of GnRH /2, 48, 49/. This factor was revealed to be a peptide of ten amino acids with pyroglutamic acid at the N-terminus and amide at the C-terminus (Fig. 2).

At the same time independently from A.V. Schally, R. Guillemin isolated a releasing factor from ovine hypothalami /8/ and found the sequence identical with that of porcine GnRH; later the same releasing hormone was also found in human placental tissue /91/. After the identification of this peptide it became generally accepted that this decapeptide

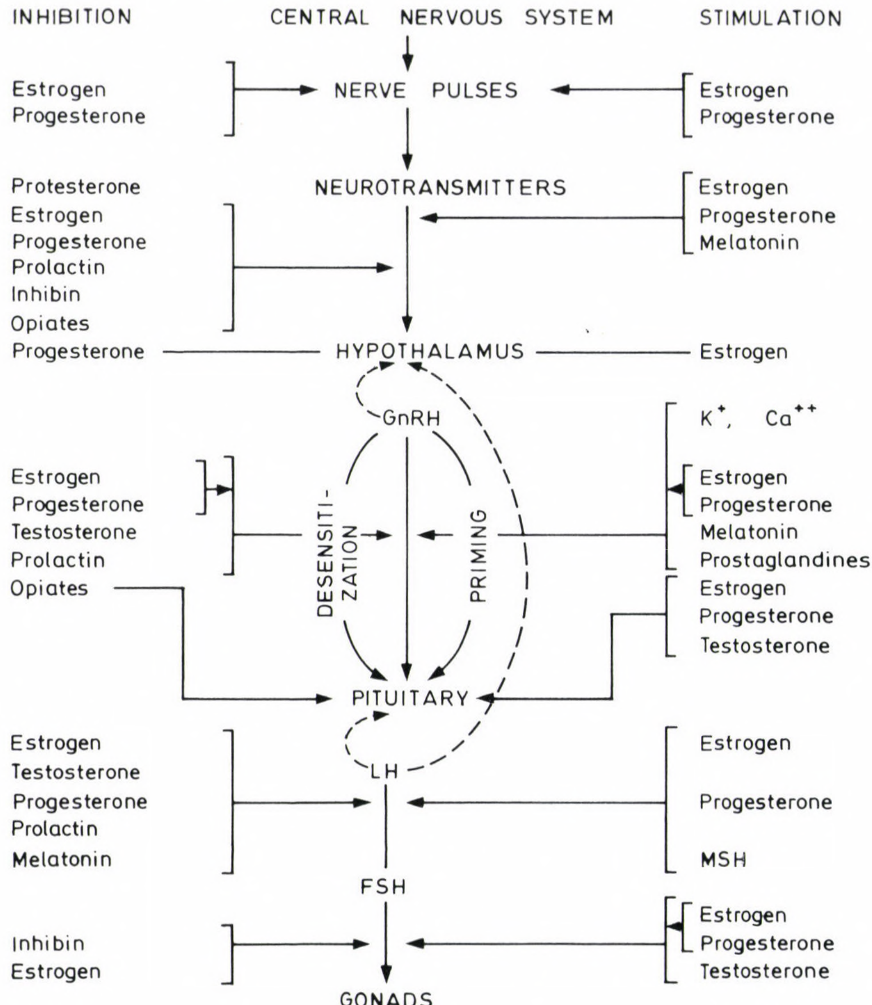


Fig. 1. Simplified representation of various hormonal interactions in the regulation of the reproductive system

was a single molecular form to release both LH and FSH /75/.

Following the isolation and structure determination of GnRH, the possibility arose that this hormone or its analogs could be suitable for influencing reproductive functions. The discovery was followed by extremely intensive efforts all over the world to design different potent and long-acting GnRH analogs. Our research group was also among the laboratories embarking in producing new GnRH analogs. The research aimed at the synthesis of two different types of GnRH derivatives:

1 Glp — 2 His — 3 Trp — 4 Ser — 5 Tyr — 6 Gly — 7 Leu — 8 Arg — 9 Pro — 10 Gly – NH_2

Fig. 2. Structure of GnRH isolated from porcine hypothalami

— agonists which are significantly more potent than the native GnRH and show prolonged activity;

— antagonists, or inhibitory analogs, which can block the ovulation and thus prevent fertilization.

From the first group of GnRH derivatives, the so-called "superactive" analogs, we expected that they will be suitable for treating certain forms of human infertility as well as for increasing the fertility rate in animal husbandry (Fig. 3).

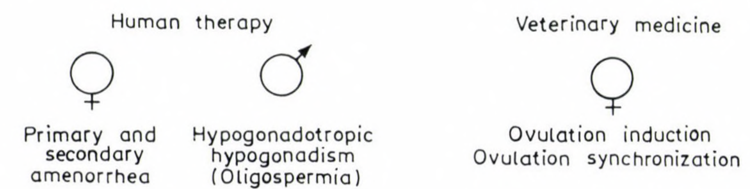


Fig. 3. Application areas of GnRH and its "superactive" analogs

GnRH agonists; structure – activity relationships

Our intensive research to synthesize GnRH superagonists and examine structure-activity relationships resulted in a great number of superactive analogs and allowed us to recognize general principles of substitution which are in agreement with the results of other researchers (Fig. 4) /55/.

An important structural modification of GnRH is the replacement of the Gly^6 residue with bulky apolar, aromatic D-amino acids. This substitution has a stabilizing effect on the conformation which is favourable for binding to the receptor and makes the analog resistant against enzymatic degradation. The $N\text{-}Me\text{-}Leu^7$ modification seems to play a similar role in increasing the potency of the parent peptide /47/. The replacement of the glycine amide in position 10 with alkyl amides /57/ or azaglycine also resulted in more potent agonists /20, 21/. The combinations of these structural modifications yielded superactive and long-acting analogs exhibiting very high biological potency /13, 26/.

Presently, the above mentioned combined substitutions in position 6 and 10 result in the most potent GnRH agonists which are approximately 50—200 times more potent than native GnRH (Fig. 5).

One of the analogs synthesized by our group /1/ — which was named OVURELIN and was introduced in Hungary by Reanal — can also be seen in Fig. 5. The stimulation of LH and FSH release by this peptide is shown on

Fig. 6. This analog exhibits more potent and longer lasting LH and FSH releasing activity than GnRH.

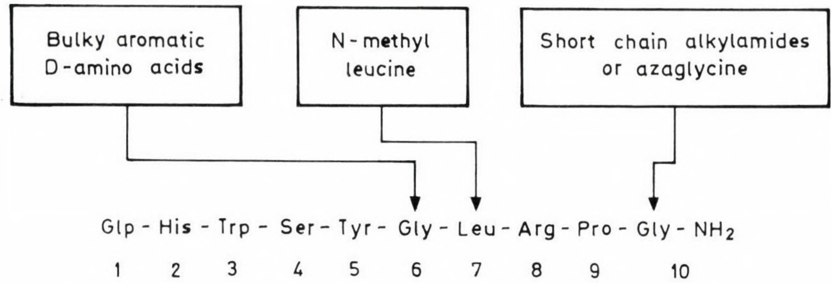


Fig. 4. Principal substitutions in the GnRH molecule leading to long-acting "superactive" analogs of the hormone. Combination of these substitutions are additive and result in "superactive" analogs

	COMPOUND 1 2 3 4 5 6 7 8 9 10	MANUFACTURER / PRODUCT CODE AND/OR BRAND NAME	RELATIVE LH-RELEASING POTENCY IN VIVO (GnRH=1)
	____________D-Ala_______NHEt	Ayerst / AY-25205 Wyeth / WY-18481 American Home Products	50 - 70
	___________D-Leu________NHEt	Takeda / TAP-144 Serous / Leuprolide	70 - 100
	___________D-Trp________NHEt	Ayerst / AY-25650	70 - 100
	___________D-Trp________NHEt	Syntex	120 - 150
+	___________D-Trp-Nml____NHEt	Wyeth / WY-40972	120 - 150
++	___________D-Nal_____________	Syntex / Nafarelin	180 - 220
	___________D-Ser(tBu)____NHEt	Hoechst / HOE-766 / Buserelin	140 - 170
+++	___________D-Ser(tBu)___AzGly	ICI / 118630	70 - 100
	___________D-Phe_______NHEt	Reanal / Ovurelin	100 - 130

Fig. 5. Most potent agonist analogs of GnRH under clinical testing for reproductive disorders
+Nml=N-methyl=leucine; ++D-Nal=D-(2-naphtyl) - alanine; +++AzGly=azaglycine

Generally, the incorporation of any bulky, hydrophobic D-amino acid in position 6 results in a superactive GnRH analog /57, 58, 59/. Two reasons prompted us to synthesize and test the D-4-chloro-phenylalanine^{6}, D-thyronine^{6} and D-thyroxine^{6} substituted GnRH-derivatives:

— these amino acids have large, bulky and lipophilic side chains, so we expected a large increase in the biological activity. Table 1 shows both the in vitro and in vivo biological potency data of these compounds. It can be observed that the LH-releasing capacity of these analogs is much higher than that of the native hormone,

— a direct halogen — tritium exchange can be achieved by catalytical tritiation of these halogenated aromatic derivatives /92/. The radiolabeled hormone analogs obtained by this technique are valuable tools in the investigation of their mechanism of action and metabolism.

The high activity of the D-amino acid[6] substituted superactive GnRH analogs and the observation that the L-amino acid substitutions in this position resulted in completely inactive analogs led to the hypothesis that the D-configuration stabilizes a β-turn conformation in this part of the molecule /55/ which is required for the biological activity, while L-amino acids prevent the formation of this β-turn. This suggested us the conclusion that certain special amino acids of L-configuration e.g. iso-aspartic acid or β-aspartyl residue — because of their considerable structural similarity — could promote the formation of a conformation

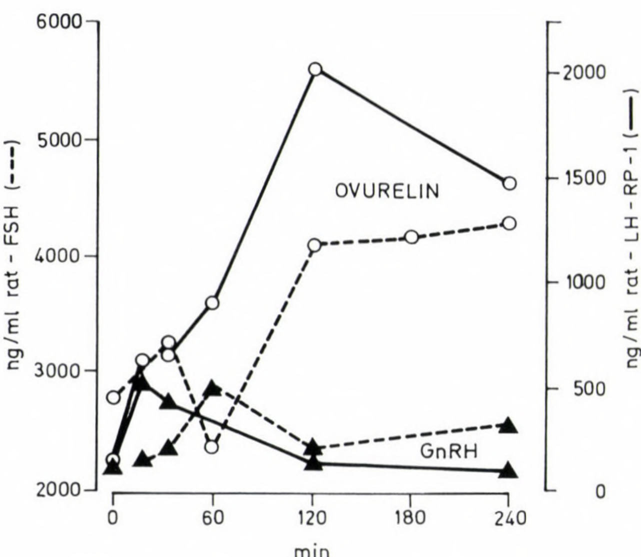


Fig. 6. Effect of OVURELIN (/D-Phe^{6}/-GnRH-(1-9)-EA) (o) and native GnRH (▲) on LH- (———) and FSH-release (- - -) in male mice. 1 μg hormone/animal, s.c.

Table 1

Relative LH-releasing potency of synthetic analogs of GnRH in rats

Analog	$ED_{50} \times 10^6$ (M)	Relative LH-releasing potency in vitro	in vivo
GnRH	343.6	1.0	1.0
/D-Thx^6/GnRH-(1-9)-EA	28.9	11.9	0.3
/D-Thy^6/GnRH-(1-9)-EA	24.3	14.1	14.2
/D-Cpa^6/GnRH-(1-9)-EA	20.6	16.6	15.3

Abbreviations: D-Thx = D-thyroxine, D-Thy = D-thyronine, D-Cpa = D-4-chloro-phenylalanine

Fig. 7. Structural and conformational similarity between a D-amino acid residue and an L-isoaspartyl (L-β-aspartyl) residue in a peptide chain

stabilized usually by D-amino acids, which is absolutely necessary for the superactivity (Fig. 7). On the basis of this theory, different GnRH analogs containing isoaspartyl residue have been synthesized. In these analogs, groups of different sizes and different lipophilicity were attached to the α-carboxylic group of aspartic acid /80, 81, 83/. Biological activities of these analogs and LH-release caused by one of these analogs plotted against time are shown in Figs 8 and 9, respectively.

An accidental discovery led us to the third group of superactive analogs different from the previously mentioned ones. For a long time the opinion was insisted on that GnRH was not species specific and its amino acid sequence was identical for all species. In 1982—83 it was clarified — mainly by Millar's and Rivier's work — that the GnRH of chicken and of salmon differ from mammalian GnRH in 1—2 amino acid residues (Fig. 10). The basic amino acid sequence of GnRH is rather conservative. Those few changes during the evolution, however, caused a significant alteration of biological activity especially in the case of lower vertebrates, so these specific GnRH analogs cannot replace each other /43, 44, 53, 54, 70, 84, 85/.

Analog	ED_{50} (at 15. min) ng/animal	Total LH in 6 hrs. relative activity
GnRH	22.5	1.0
[L-isoAsp^6-NH_2] GnRH	593.5	0.4
[L-isoAsp^6-NH-◇] GnRH	346.4	0.4
[L-isoAsp^6-N⬡] GnRH	321.4	0.4
[L-isoAsp^6-NH-⌬] GnRH	257.5	1.2
[L-isoAsp^6-NH-C(Me)(Me)-CH ⌬] GnRH	73.5	1.7
[L-isoAsp^6-N(Et)(Et)] GnRH-(1-9)-EA	708.3	0.8
[L-isoAsp^6-OtBu] GnRH-(1-9)-EA	1070.0	1.1
[L-isoAsp^6-OH] GnRH-(1-9)-EA	35.1	1.0
[L-isoAsp^6-OBzl] GnRH-(1-9)-EA	30.2	12.0
[L-isoAsp^6-OMe] GnRH-(1-9)-EA	39.4	20.0

Fig. 8. Relative LH-releasing potency of GnRH-analogs containing L-iso-aspartyl residue determined in mice

After the publication of the amino acid sequence of chicken and salmon GnRH, we immediately synthesized their analogs modified in the 6, 7, 8th and 10th positions according to the previously mentioned principles. One of these analogs (analog No. 8 on Fig. 10) proved to be very potent in inducing ovulation in certain species of fish /28, 29, 34, 35/.

Our research resulted in patented analogs of all three groups and we have several patents also for the biological or medical applications of these compounds /1, 27, 28, 29, 69, 83/. One of these analogs, OVURELIN® is already commercially available, two others are good candidates for human medicines.

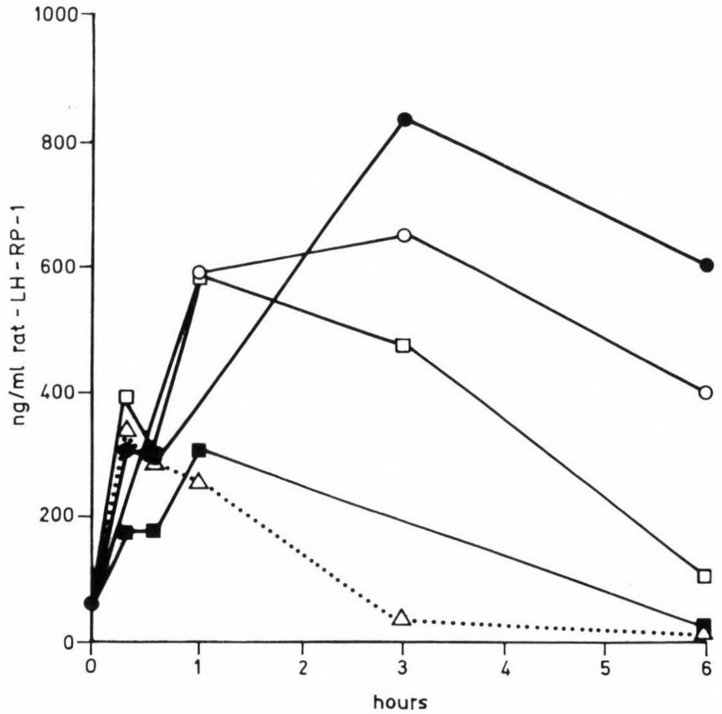


Fig. 9. Time course of LH-release induced by 0.08 μg (■), 0.16 μg (□), 0.8 μg (o) and 4 μg (●) /L-isoAsp^6-OMe/-GnRH-(1-9)-EA analog or 1 μg native GnRH (Δ) in mice

NATIVE GnRH-s	1	2	3	4	5	6	7	8	9	10	
MAMMALS	Glp	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2	(1971)
CHICKEN I	-	-	-	-	-	-	-	Gln	-	-	(1982)
CHICKEN II	-	-	-	-	His	-	Trp	Tyr	-	-	(1984)
SALMON	-	-	-	-	-	-	Trp	Leu	-	-	(1983)
LAMPREY	-	-	Tyr	-	Leu	Glu	Trp	Lys	-	-	(1986)
SYNTHETIC ANALOGS											
1.	-	-	-	-	-	D-Phe	-	Gln	-	-	
2.	-	-	-	-	-	D-Phe	Trp	Leu	-	-	
3.	-	-	-	-	-	-	Trp	Gln	-	-	
4.	-	-	-	-	-	-	Phe	Gln	-	-	
5.	-	-	-	-	-	-	Phe	Leu	-	-	
6.	-	-	-	-	-	-	Trp	Leu	-	NH-C_2H_5	
7.	-	-	-	-	-	-	Trp	Gln	-	NH-C_2H_5	
8.	-	-	-	-	-	D-Phe	-	Gln	-	NH-C_2H_5	

Fig. 10. Structure of known GnRH-s isolated from different species (upper panel) and some of their analogs synthesized in our laboratory (lower panel)

Application of GnRH analogs

The question may arise: what may be the practical significance of these products and their applications? In order to answer this question, studies in animal breeding and in veterinary medicine was initiated by our group more than 10 years ago in collaboration with different stock-farms and veterinary colleges and clinics /4, 68, 71, 87, 88, 89/.

Disfunction of sexual cycle -- as a consequence of abnormal functioning of the hypothalamo-hypophyseal system or the shortcomings in stock breeding and feeding -- may cause reversible and even irreversible lesion of the ovary. Exogen gonadotropins or GnRH-s causing endogen gonadotropin release can invert a great part of these processes which would otherwise prevent propagation.

Although gonadotropins isolated from natural sources had earlier been applied for healing these lesions, the high prices and the limited supply of these natural products as well as the fact that these gonadotropins are species specific -- and therefore they can induce antibody production -- can restrain them from general application in veterinary medicine.

Synthetic, superactive analogs of GnRH do not have any of these disadvantages, therefore they may have wide application in veterinary medicine. Some fields of application will be listed below, without the aim of completeness.

Results obtained during the experiments with one of our GnRH analogs (OVURELIN^R) are summarized in Table 2. Experiments were carried out in about 20 cooperative stock-farms and inseminating stations with a considerable number of cows. This analog stimulates follicular maturation, luteolysis and ovulation, whereupon it can induce a new cycle in an animal with ovarial disfunction as well as in the case of anoestrus and cystic ovary. Dysgenesia of cattle, especially of highly milk-productive ones, can attribute to disfunction of uterus reversion after parturition — a symptom called involution. In this case this product is useful for the preventive therapy of infertility because it increases the ratio of fertilization. It seems to be very important that the ratio of fertilization increases approximately by 30 per cent, when healthy animals are treated with our preparation simultaneously with insemination.

Experiments with pigs are promising as well. These were done in cooperation with a pig breeding farm and they resulted in the induction of

Table 2

Collected data on application of OVURELIN in various reproductive disorders and in insemination of cattle[+]

Application	Conception/Total	%	Control %
1. Ovarian cysts	53/74	71	-
2. Anoestrus	39/56	70	-
3. "Repeat breeder" syndrome	48/80	60	-
4. Involution	31/42	78	63
5. Treatment for inducing ovulation simultaneous to insemination	351/730	48	35

[+]Application fields 1—3 are self-controlled, with pretreatment values of 0%, while controls for applications 4—5 are indicated

ovulation with high efficiency when using OVURELIN. On the basis of the experimental data it was possible to determine the optimal period of reproductive cycle of pigs, in which the superactive GnRH analogs not only increased the fertility of animals but also gave good opportunity to synchronize the oestrus and to increase the amount of progeny with 20—30 per cent.

There have been successful attempts to use different GnRH analogs in other animal species. So for example, in the case of rabbits the applica-

tion of these compounds became part of the fertilization technology in several places, and today they seem to be essential.

The species specific GnRH analogs together with a special application procedure proved to be suitable for the artificial propagation of economically important fish species (e.g. sturgeon), which has not been able to propagate in an artificial environment before. In this topic we have got several patents, the practical introduction of which is in progress /28, 29, 34, 35/. We have achieved excellent results with one of these compounds in the induced, artificial propagation of different fish. The analog is suitable for the artificial propagation of different fish even out of the spawning season (Table 3).

We recognized the possibility of increasing spermatogenesis and libido of birds with GnRH analogs in 1978, when we found that after administering classical GnRH superactive analogs several times in small quantity, the spermatogenesis, — i.e. the number, motility and viability

Table 3

Application of /D-Phe^{6}, Gln^{8} /GnRH-(1-9)-EA for the induced artificial propagation of fishes

I. In spawning season:

Fish species	Repetitions	Number of treated fish	Ovulation %	Fertilization %	Number of raised offspring
Pike	30-40	appr. 1100	50- 90	90	8-11 x 10^{6}
Perch	100	1500	95-100	30-90	20-30 "
Pike perch	6	153	60-80	85-95	1-2 "
Sterlet	50-70	2000	75-80	75-90	1 "
Carp	10-20	100	60-80	90-100	5-6 "
Grass carp	5	15	60-80	80-90	0-5 "
Silver carp	5	15	60-80	80-90	0-5 "
Wels	25-30	150	60-70	90-95	4-5 "

II. Out of spawning season:

Fish species	Repetitions	Number of treated fish	Ovulation %	Fertilization %	Number of raised offspring
Sterlet	15-20	200	40-60	40-70	0.4 x 10^{6}
Wels	10-15	60	30-60	80-95	1.2 "
Trout	5	60	All fishes ovulated		
Carp	10-15	100	50-70	80-95	-
Koi	5	38	50-70	80-90	1.3 "
Golden carp sp		57	65-70	70-90	0.08 "

of sperms, -- and the sexual activity of animals considerably increased and it remained constant for months after the treatment. On the basis of in vivo experiments carried out in cocks then expanded to rabbits and cattle, we found together with biologists, that these analogs are suitable for correcting sexual abnormalities and increasing the fertility in male animals for breeding /69/.

Summing up the above, we can conclude that in Hungary the use of superactive or species specific derivatives of GnRH has begun, though quite slowly, by the middle of the 80's.

The expectations concerning their application are partly fulfilled and the inclusion of new biological procedures based on GnRH analogs into the process of production is under way.

At the same time it is difficult to find any initiatives in the case of human applications in Hungary which are worth mentioning in spite of the wide range of possible applications (Table 4) and our great efforts. Recently it has been found that superactive hormone derivatives may play an important role in the treatment of hormone-dependent tumours such as prostate and breast cancer /12, 22, 76/. That is why we also initiated -- in a suitable cooperation -- some investigations connected with this topic.

Table 4

Fields of clinical applications of GnRH and its analogs

1. DIAGNOSTIC APPLICATIONS

   Diagnosing the functional integrity of the hyothalamus-pituitary-gonads system

2. THERAPEUTIC APPLICATIONS

   Stimulation of reproductive functions in amenorrhea, anovulation, hypogonadism, oligospermia, etc.
   Treatment of pubertas praecox, gonadal steroid-dependent tumours, endometriosis, ovarian cysts, hirsutism, etc.

3. CONTRACEPTION

   Inhibition of ovulation
   Inhibition of spermatogenesis

It is known that prostate and breast cancers are the most frequent cancer diseases -- primarily in old age -- in men and women, respectively. The presence of estrogens and prolactin in human breast cancer cells has clearly been demonstrated as an essential proof of hormone dependence.

An anticancer activity of superactive GnRH derivatives can be supposed on the basis of the mechanism of hormonal desensitization which we also examined in detail. This is why we thought it was important to begin in vivo experiments in this field, for which we used the analog found the most active in in vitro desensitization experiments. In the case of breast cancer induced in mice, the D-Cpa^{6}-GnRH-(1-9)-ethylamide decreased the volume of the tumours by 60% in comparison with the untreated control group on the 30th day after the transplantation which we measured according to criteria accepted in the literature (Fig. 11). The weight of the tumour decreased by 40—45% after a 100 or 200 µg peptide/day treatment. On the other hand, due to the treatment the incidence of induced tumours decreased by 25%

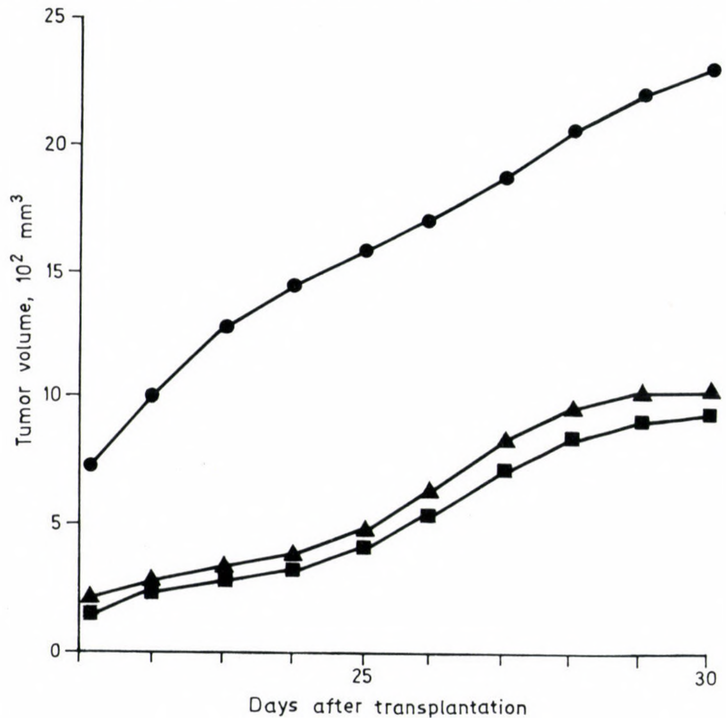


Fig. 11. Effect of /D-Cpa^{6}/-GnRH-(1-9)-EA on the growth of MXT mammary carcinoma in mice
(--●-- = control; --▲-- = 100 µg/day; --■-- = 200 µg/day)

— on the basis of an examination on the 30th day after the transplantation (Table 5).

These data clearly show that this material has a well-defined tumour inhibiting effect in breast cancer. Further investigation is needed before

starting tests and application in humans, and we would also like to extend the investigation to other — mainly prostate and ovarial — tumours.

Table 5

Effect of /D-Cpa6/GnRH-(1-9)-EA on the growth of MXT mammary carcinoma induced in BDR-1 mice

Treatment	Incidence of tumours %	Tumourweight mg ± S.D.	Inhibition of tumour growth %
None (Control)	92	3663 ± 978	0
100 µg/day	75	2201 ± 895	39.9
200 µg/day	67	2020 ± 836	44.85

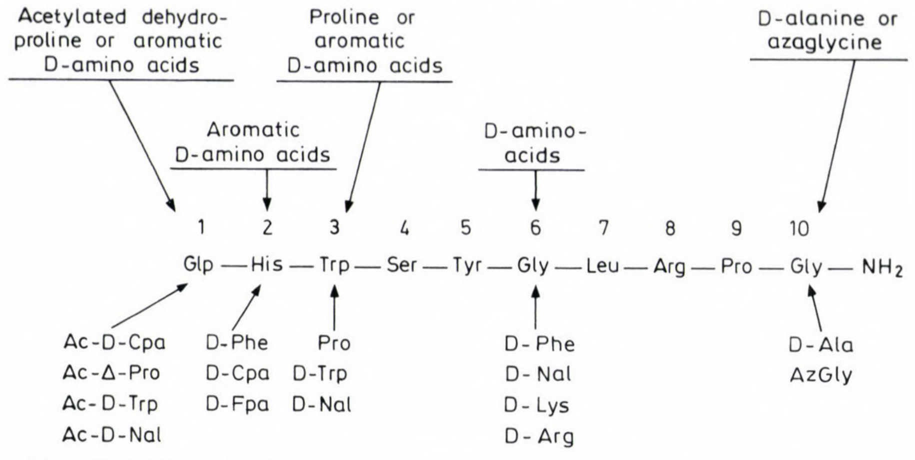
GnRH antagonists; structure-activity relationships

Regarding the regulation of ovulation, not only ovulation-induction, but also its inhibition must be considered. It could be assumed that GnRH analogs modified in certain amino acid moieties would be appropriate for inhibition of ovulation as competitive inhibitors. In 1973, an American group succeeded in synthesizing the first GnRH antagonist capable of inhibiting ovulation. With the deletion of His in position 2, a completely inactive molecule was obtained which proved to be a competitive inhibitor when applied in large concentration.

From the results of research in the last 15 years, it is possible to deduce the major principles of substitutions which lead to potent antagonist analogs. The appropriate substitutions in position 1, 2, 3, 6 and 10 of the hormone resulted in the most potent GnRH inhibitors (Fig. 12) /14, 33, 45, 72/. Unfortunately, the potency-increasing effect of the combinations of single substitutions was not found to be generally additive, and this made the design of highly potent inhibitory analogs difficult /50/.

More than 1000 GnRH analogs have been synthesized world-wide and approximately 100 in our laboratory, the most important of which are listed in Fig. 13 /15, 18, 19, 23, 33, 45, 50, 78/. The dates of publications and the corresponding doses show the development of our research.

It is worth mentioning that two of these inhibitory analogs were especially important. The D-Lys-containing analog was the first compound capable of inhibiting ovulation in a very low dose during long-term application /45/, while the D-Arg^{6} analog /56/ was the first orally active inhibitor.



MOST POTENT GnRH INHIBITORS PUBLISHED IN 1982

Compound (1 2 3 4 5 6 7 8 9 10)	Authors	ID*100
Ac-D-Cpa-D-Cpa-D-Trp ----D-Arg----D-AlaNH2	Coy et al.	3.0 µg
Ac-Δ-Pro-D-Fpa-D-Nal----D-Nal ----------	Rivier et al.	2.5 µg
Ac-D-Trp-D-Cpa-D-Trp----D-Lys----D-AlaNH2	Mező et al.	2.0 µg
Ac-D-Nal-D-Cpa-D-Trp----D-Arg----D-AlaNH2	Horváth et al.	1.0 µg

Fig. 12. Upper panel: Principal substitutions in the GnRH molecule leading to competitive inhibitors of the hormone. Lower panel: Most potent GnRH-antagonists published in 1982. * Dose required for 100% inhibition of ovulation. Abbreviations: D-Cpa = D-4-chloro-phenylalanine; D-Fpa = D-4-fluoro-phenylalanine; D-Nal = D-(2-naphtyl)-alanine

The most potent GnRH inhibitors contain 4-5 D-amino acids, therefore even if they are active in very low doses, they are still to expensive for general use in contraception; though the encouraging tendency described above justifies expectations.

Any substitution in the N-terminal region but especially in position 2 of the molecule, results in the total loss of LH and FSH releasing activity. For this reason, it seems that this part of GnRH might be responsible for the biological activity. On the other hand appropriate substitutions in the C-terminal part of the molecule, — by increasing the binding to the receptor and the resistance against enzymatic degradation, — can increase the biological activity similarly in the series of both agonist and antagonist analogs (Fig. 14).

	COMPOUND	Dose required for 100 % inhibition of ovulation, mg*
	1 2 3 4 5 6 7 8 9 10	
1975	Glp-D-Phe-Phe-Ser-Tyr-D-Phe-Leu-Arg-Pro-Gly-NH_2	3.0
1976	---D-Phe-D-Trp----D-Phe------------	1.0
1977	---D-Phe-D-Trp----D-Lys------------ \ isophtaloyl dimer ---D-Phe-D-Trp----D-Lys / ------------	0.25
1978	D-Glp-D-Cpa-D-Trp----D-Phe------------	0.12
1979	Ac-D-Cpa-D-Cpa-D-Trp----D-Phe------------	0.05
1980	Ac-D-Cpa-D-Cpa-D-Trp----D-Phe--------D-Ala-NH_2	0.01
1981	Ac-D-Cpa-D-Cpa-D-Trp----D-Lys--------D-Ala-NH_2	0.002**
1982	Ac-D-Nal-D-Cpa-D-Trp----D-Arg--------D-Ala-NH_2	0.001***

Fig. 13. Development of GnRH antagonists in our laboratory * Single s.c. dose/rat determined on proestrus day; ** Also inhibits ovulation over 14 days in a dose of 5 μg/rat s.c.; *** Also effective orally, 100% inhibition of ovulation in a dose of 2 mg/rat. For abbreviations see Fig. 12

Effector region | Receptor-binding region

Glp - His - Trp - Ser - Tyr - Gly - Leu - Arg - Pro - Gly - NH_2

1 2 3 4 5 6 7 8 9 10

Fig. 14. Effector and receptor-binding region of GnRH as deduced from structure-activity relationships

Sensitization and desensitization induced by GnRH and its analogs

In parallel with the encouraging development in the research of GnRH antagonists, experiments aimed at the increase of fertility with the help of the most potent superagonists applied by chronic administration had a surprising outcome. More and more papers were published on the "paradoxical antifertility effect" of GnRH agonists /73, 74, 77/. In many cases the experiments resulted in the decrease of fertility in both sexes. Our results supporting these findings can be seen in Table 6. First, these findings were interpreted as a consequence of the direct action of GnRH at the gonads. A direct action like this, however, could be demonstrated only in some species as for example in rat, but not in human. As a further possibility, an inhibitory mechanism at the pituitary level was postulated which would occur in the case of a chronic treatment with superactive GnRH analogs.

Table 6

Ovulation-inhibitory effect of prolonged treatment with GnRH analogs in female rats

Analog	Dose/day	Inhibition of oestrus cycle over 21 days
$/D\text{-}Phe^{6}/$ GnRH-(1-9)-EA	0.25 µg	100 %
	0.1 µg	50 %
$/D\text{-}Thy^{6}/$ GnRH-(1-9)-EA	0.25 µg	100 %
	0.1 µg	25 %
$/D\text{-}Cpa^{6}/$ GnRH-(1-9)-EA	0.25 µg	100 %
	0.1 µg	0 %
$/L\text{-}isoAsp^{6}\text{-}OMe/$ GnRH-(1-9)-EA	0.5 µg	75 %
	0.35 µg	50 %

Abbreviations: D-Thy = D-thyronine,
D-Cpa = D-4-chloro-phenylalanine

In order to clarify this possibility, simultaneously with other research groups but independently from them, we investigated the possibility of the desensitization of the pituitary /37, 38, 40/. We found that in pituitary cell culture, GnRH and its superagonistic analogs desensitize the gonadotrop cells of the pituitary toward further stimuli (see Fig. 15). The antagonists do not produce such an effect. In the course of our investi-

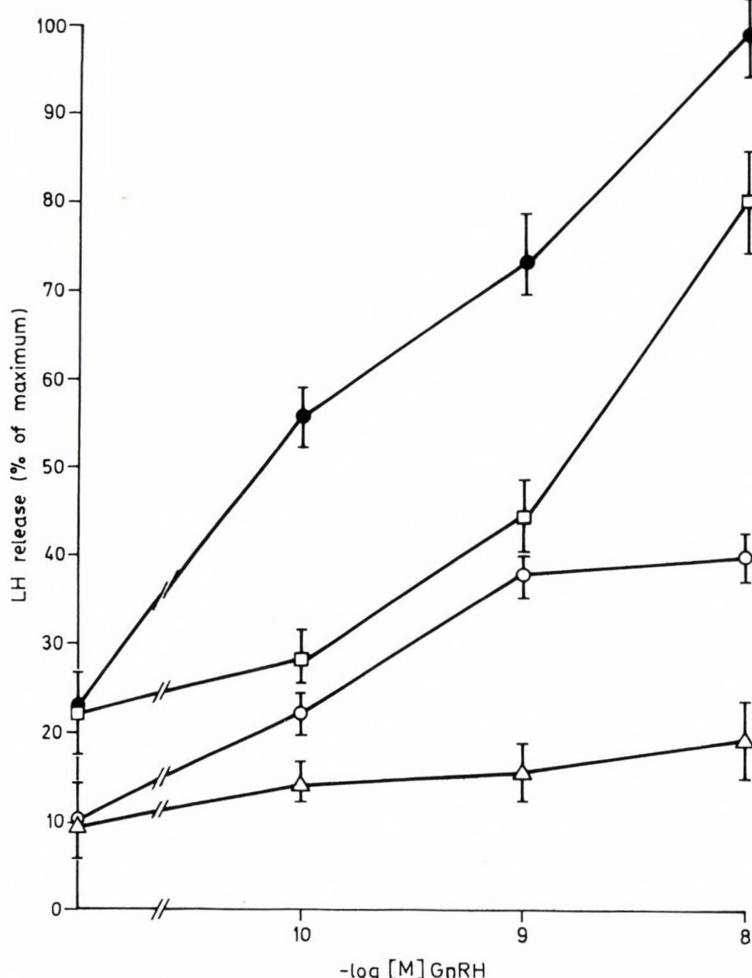


Fig. 15. Desensitization of pituitary gonadotroph cells by different GnRH analogs in vitro. Pituitary cell culture was preincubated with 10^{-8} M GnRH (o), 10^{-8} M inhibitory analog (□) or 10^{-8} M superactive analog (△) for 4 hours (● = control, no hormone added), then medium was removed, cells were washed and reincubated with different concentrations of GnRH for 1 hour. LH released into the medium during reincubation was determined by RIA

gations we demonstrated that GnRH and superactive GnRH analogs are capable not only of desensitizing but also sensitizing the gonadotroph cells of the pituitary, depending on the dose and the time of preincubation (Fig. 16) /39/. This effect seems to be partly the consequence of intracellular processes and partly of processes at the receptor level /62/. The protocol worked out in vitro to achieve sensitization was used later in our in vivo experiments with various kinds of animals. The release of LH and FSH was measured as a function of the preincubation time followed by the addition of a GnRH pulse (See Fig. 16) /36/. It can be seen that the increasing of the time and the preincubating concentration results in desensitization which explains the "paradoxical antifertility effect" (Table 6). The desensitization mechanism of pituitary gained considerable significance, to such an extent, that with the most potent superagonistic compounds clinical trials for contraception have been started /26/. In the light of these encouraging results, the introduction of a superagonistic GnRH analog for contraception in some years would not be a surprise.

As it can be seen, the mechanism of desensitization involves that GnRH superagonists decrease the levels of luteinizing and follicle-stimulating hormones, and as a consequence of this, they also decrease the concentration of steroid hormones. The promising results obtained in the treatment of certain hormone-dependent prostate and mammary tumours can be attributed to this phenomenon.

Because not only GnRH antagonists but also GnRH superagonists can have inhibitory effect, a question arises: how can the fertility be increased, how can the hypogonadotrophic hypogonadism and amenorrhea be treated with GnRH or its analogs.

The answer came from the recognition of the more subtle details of the mechanisms. The key was given to this by the discovery of a group of American scientists who found that GnRH is released from the hypothalamus not continuously but in a pulsatile manner /96/. The experiments which demonstrated the desensitization proved that the hypophysis will be desensitized only by long-lasting stimuli (Fig. 17) and not by pulses with 30-60 min frequency. With the aid of therapies planned according to this, the hypothalamic hypogonadism became treatable recently. There are already commercially available special portable pumps which can release GnRH at certain intervals into the circulation of the patient for weeks or months, and thus, stimulate the release of gonadotropins.

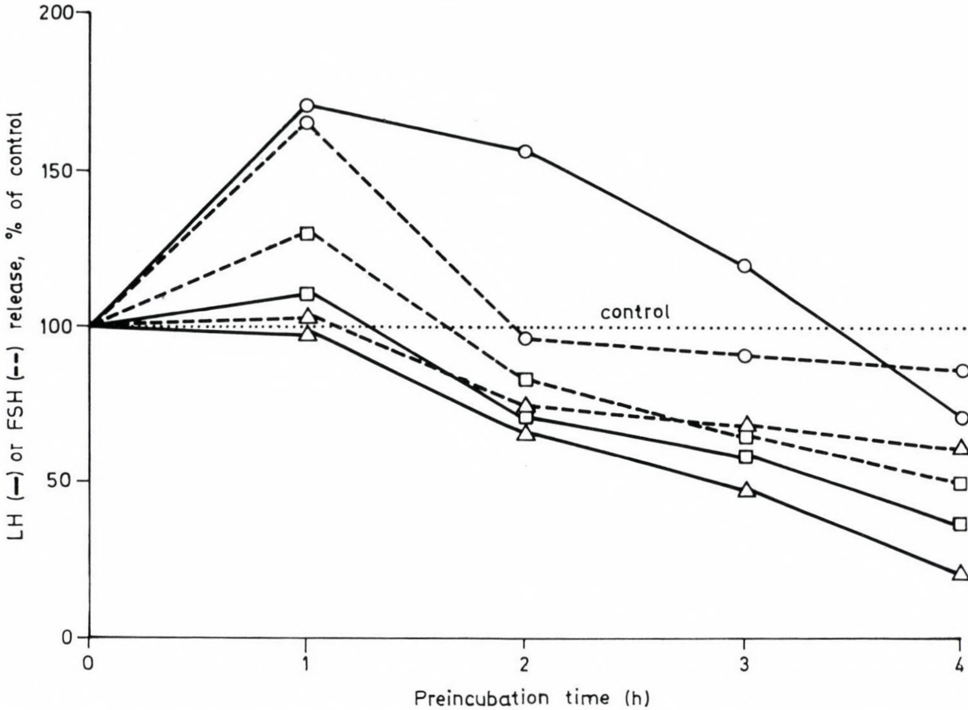


Fig. 16. Sensitization and desensitization of LH (———) and FSH (- - -) release of pituitary cells as a function of the duration and GnRH-concentration of the preincubation. Cells were preincubated with 10^{-9} M (○), 10^{-8} M (□) or 10^{-7} M (△) GnRH for various time intervals and then washed (control=no GnRH added). Reincubation was performed with the same concentrations of GnRH as used for preincubation and lasted for 1 hour. LH released into the medium during reincubation are shown as per cent of control

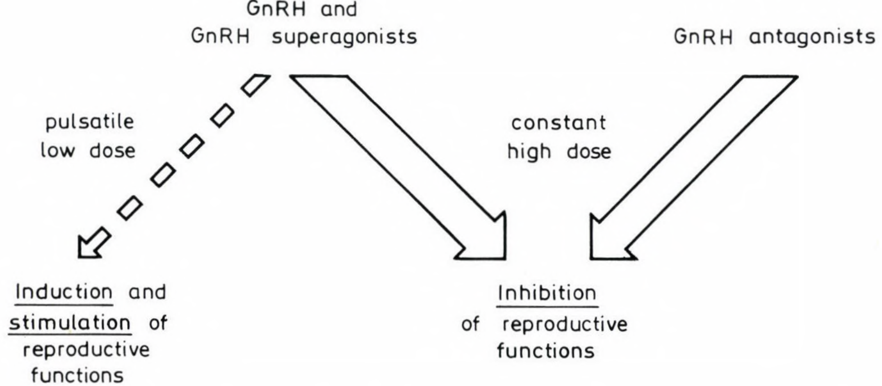


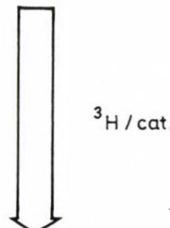
Fig. 17. Potential methods for influencing reproductive function by GnRH analogs

Studies on the biodegradation of GnRH

In the hypothalamo-hypophyseal portal circulation, GnRH could be detected at a concentration of 20-800 pM, while in the peripheral circulation it was not detectable. Therefore, it was assumed that the pituitary degraded GnRH or at least the major part of it. This theory was further supported by the finding that very high GnRH-degrading activities were found to be present in pituitary tissue homogenates. The relevancy of these results remained questionable, however, since it was reasonable to assume that under physiological conditions, the hormone did not come into contact with enzymes originating from the disrupted cells. In order to clarify this contradiction and also to study the finer details of the metabolism, we chose an experimental system which seemed to be the best possible model of the physiological state: the degradation of the hormone was studied in the medium of cell and tissue cultures /5, 63, 64, 65, 66, 90/. In these experiments, a highly sensitive and specific detection method and, — in order to assure perfect structural identity, — tritium-labeled hormone were used /41, 42, 90/. This analog was specifically tritiated in its Pro residue in position 9. In the course of the synthesis, a dehydroproline-containing precursor was prepared first, which was then catalytically hydrogenated with tritium gas /7/.

For other experiments, tritium-labeled GnRH and GnRH-analogs were prepared by a method which was developed by our group earlier /92/, and which has since widely been used. This method involves the incorporation of a halogen-substituted amino acid (derivatives of Phe or Tyr) into the peptide to be labeled, followed by the catalytic exchange of the halogen with tritium. For instance, the D-4-chloro-phenylalanine-containing synthetic GnRH derivative — as a precursor — was catalytically hydrogenated by carrier-free tritium gas in a special equipment to yield ^{3}H-Ovurelin (Fig. 18). It follows from the nature of the process that peptides can be specifically labeled in different amino acid residues applying this technique. Using this method, compounds obtained with high specific activity can be advantageously used for metabolic studies. Returning to metabolic investigations of GnRH, the hormone labeled with tritium in position 9 was added to pituitary tissue or cultured pituitary cells. Following incubation, high performance liquid chromatography was used for the separation and on-line liquid scintillation counting for the quantitation of possible degradation products in the appropriately pretreated samples. The results clearly

[4—Cl—D—Phe^6] GnRH—(1—9)—EA



[4—^{3}H—D—Phe^6] GnRH—(1—9)—EA

Specific activity: 225 GBq/mmol

(6 Ci/mmol)

Fig. 18. Preparation of ^{3}H-OVURELIN specifically labeled in position 6 from a precursor compound

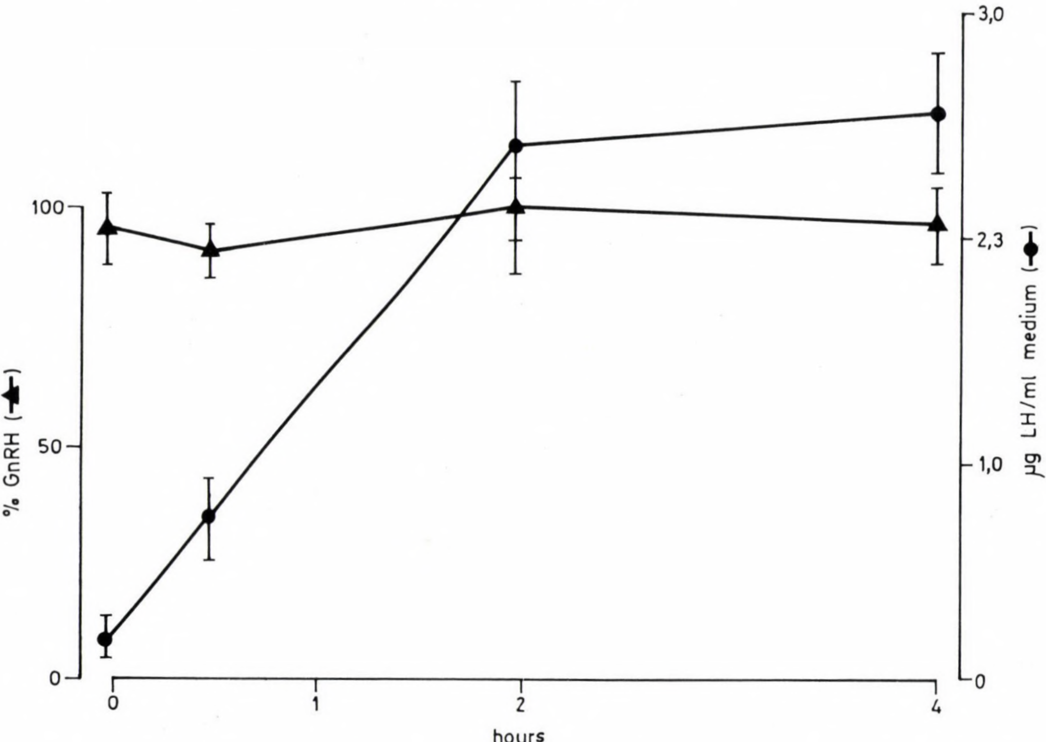


Fig. 19. GnRH (△) and LH (●) content of the medium during incubation of 10^{-8}M ^{3}H-GnRH with intact pituitary cells in culture. GnRH content is expressed as per cent of control incubated identically but without cells

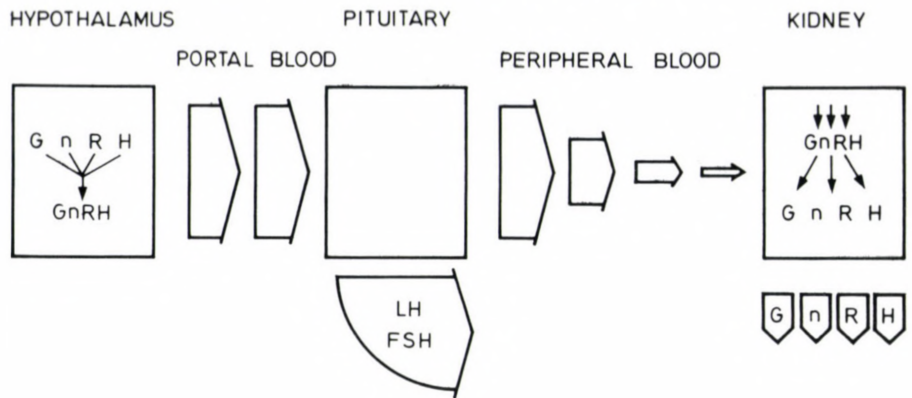


Fig. 20. Schematic representation of the "fate" of endogeneous hypophysiotrop GnRH in the body from the biosynthesis to the excretion of the degradation products

showed that intact pituitary cells did not degrade GnRH (Fig. 19), consequently it was transported from the pituitary to the peripheral blood in unchanged form. Similar results were obtained with intact hypothalamic cells (fetal hypothalamic cell culture) as well.

Our results refuted the earlier widely accepted concept according to which GnRH degradation in the hypothalamus or pituitary could play an important role in the regulation of the hormone level, and thus, in the regulation of sexual functions.

Simultaneously, using an exceptionally sensitive radioimmunoassay, Japanese researchers demonstrated the presence of the preovulatory GnRH "peak" also in the peripheral circulation. Thus, according to our present view, GnRH is not degraded in the pituitary, it only becomes diluted in peripheral blood and this is why it was undetectable earlier. Finally, based on results published later by an American group /86/, GnRH undergoes proteolytic degradation in renal tubules, then it is excreted in form of its fragments and/or amino acids (Fig. 20).

Studies on tissue distribution of Ovurelin were carried out with a GnRH analog containing tritium-labeled phenylalanine in position six, which is structurally identical to Ovurelin (Table 7). When it was administered i.v., highest radioactivity values were found in the kidney, lung and small intestine.

The ratio of pituitary/brain radioactivities was found to be between 1.8—2.0 within the first two hours after administration. In 72 hours

Table 7

Distribution of radioactivity in different organs of rabbits after i.v. administration of ^{3}H-Ovurelin

Dose: 4 uCi/animal

Organs	Radioactivity ($x10^{3}$ dpm/g tissue) 15 min	60 min	240 min
Liver	17.7±4	26.3±6	6.2±2
Lung	11.5±3	15.0±5	7.9±0.5
Kidney	12.4±4	13.5±5	8.4±3
Spleen	11.8±2	12.0±1	10.2±4
Brain[+]	2.7±2	3.0±3	2.0±1
Pituitary	6.8±2	4.0±1	2.9±1
Colon	3.5±2	3.3±2	4.2±2
Small intestine	8.4±3	13.5±4	5.6±2
Skeletal muscle	0.9±1	2.2±2	1.8±1
Ovary	1.8±1	2.1±1	1.6±1

[+]without pituitary

more than 50% of labeled hormone, supposedly in the form of its metabolites, was excreted (Table 8). Conclusions regarding the structure of excreted degradation products can be drawn from experiments with GnRH-analogs labeled in different amino acid residues.

Table 8

Passage of radioactivity in rabbits treated with ^{3}H-Ovurelin

Dose: 8 uCi/animal, i.v.

Time (hours)	Cumulative passage in percentage of dose Urine	Feces	Cage
6	24.0 ± 12		
24	36.0 ± 17	0.7 ± 0.4	
48	43.3 ± 18	7.2 ± 3.4	
72	45.4 ± 18	9.0 ± 3.8	1.6

Photoaffinity labeling for receptor studies

The GnRH-receptor available in pure, isolated form could be of great value for the better understanding of the mechanism of action of GnRH. Detailed knowledge about the characteristics of hormone-receptor binding can be extremely useful for the design and synthesis of novel, more effective analogs. The photoaffinity labeling method seemed to be a promising step in receptor isolation. According to this method, an analog containing a photoreactive moiety is attached to the receptor by a covalent bond as a consequence of exposure to intense light. For this purpose, a few photoreactive derivatives of both agonist and antagonist analogs were synthesized (Fig. 21) /61, 67/. These photoreactive derivatives were incubated in pituitary cell culture and irradiated then with UV light. Control cells were treated identically without irradiation (dark control). After washing the cells, the amount of LH released into the fresh medium was measured by radioimmunoassay (Fig. 22). No LH-release was found in case of dark control

NO_2
N_3—C₆H₃—S—Cl + $H_2N-(CH_2)_3-CH_2$ ---------- PEPTIDE

2. 4—NAPS—Cl (D—Lys[6])—GnRH—(1—9)—ethylamide

pH 8. indole
90 min. in darkness

NO_2
N_3—C₆H₃—S—NH—$(CH_2)_3-CH_2$ --------- PEPTIDE

(D—Lys/NAPS/[6])—GnRH—(1—9)—ethylamide

hν. 360 nm
20 min. 30 °C

NO_2
RECEPTOR ←--NH—C₆H₃—S—NH—$(CH_2)_3-CH_2$ -------- PEPTIDE

Fig. 21. Synthesis and photolysis of the photoreactive derivative of an agonist analog of GnRH

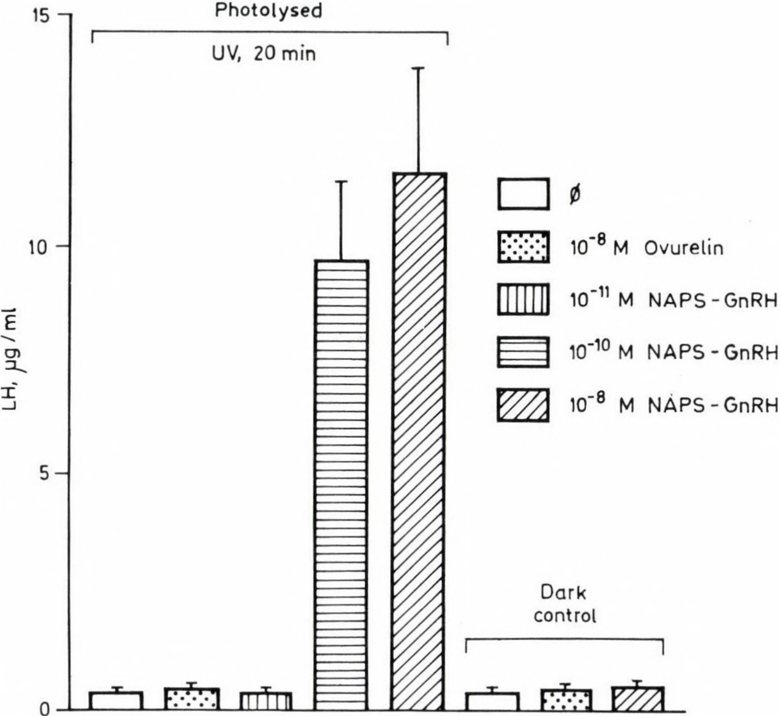


Fig. 22. Photoaffinity labeling of pituitary cells with the photoreactive derivative /D-Lys(NAPS)^6/-GnRH-(1-9)-EA. Cells were incubated for 2 h in the dark with no additive (Ø), 10^{-8} M D-Phe^6-GnRH-(1-9)-EA (Ovurelin), or different concentrations of /D-Lys(NAPS)^6/-GnRH-(1-9)-EA (NAPS-GnRH). Then cells were exposed to UV light, washed, and incubated with fresh medium for 4 hours. "Dark controls" were treated identically but covered during irradiation. LH released into medium during reincubation was measured by RIA

incubated with 10^{-8} M /D-Lys(NAPS)^6-GnRH-(1-9)-ethylamide (a photoreactive derivative of a GnRH-agonist) since the hormone was completely removed by the washing procedure. On the contrary, maximal LH-release was observed when the same analog was photolysed in presence of pituitary cells, indicating that the GnRH analog became covalently attached to the receptor. Our experiments also showed that the effect of the covalently-bound hormone is dose-dependent (Fig. 22). Based on the above, it was reasonable to assume that photoaffinity labeling can be favourably utilized in the isolation of the receptor. This has in fact been accomplished since; a member of our group presently working for Genentech, Inc., USA, succeeded in isolating a receptor protein partly as a continuation of these results, partly with the help of other approaches (K. Nikolics, personal communication).

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CELL-MEDIATED TRANSMISSION OF HORMONAL IMPRINTING TO VIRGIN MAMMALIAN CELLS IN CULTURE AND FAILURE OF TRANSMISSION WITH THE NUTRIENT MEDIUM

P. KOVÁCS, G. CSABA and OTTÍLIA TÖRÖK

Department of Biology, Semmelweis University of Medicine, Budapest

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Chinese hamster ovary (CHO) cells and Chang liver cells which had already interacted with a hormone (gonadotropin, TSH, insulin) in culture, transmitted hormonal imprinting to virgin cells not previously involved in the interaction. The information associated with imprinting was not mediated by the nutrient medium, because the nutrient medium of the hormone-treated cells did not induce imprinting in virgin cells and even reduced rather than enhanced the hormone binding capacity thereof. Thus the transmission of information is in all probability associated with a direct cell-cell contact.

Keywords: Hormonal imprinting — Cell-cell contact — Information transfer — Cell cultures

Introduction

Hormonal imprinting occurs at the primary interaction of the hormone with the target cell, which usually takes place in the perinatal period /4/ and accounts for receptor maturation and for the establishment of normal cellular binding capacity and response /5/. These events take a different turn if hormonal imprinting is lacking /6/. In the perinatal period the receptors are not always able to differentiate hormone-like molecules (related hormones) from the adequate hormone and may therefore become imprinted by these /8/. This usually alters the normal course of imprinting, but does

Send offprint requests to: Dr. P. Kovács, Department of Biology, Semmelweis University of Medicine, H–1445 Budapest, P.O.Box 370, Hungary

Akadémiai Kiadó, Budapest

not prevent, only quantitatively change, the binding relations of the adequate hormone /10, 11/. Such binding differences are demonstrable, among others, by functional changes /9/.

In cell cultures or cell lines, interaction with the hormone alters the later binding capacity of the receptros by a long-term effect /7/. Since culture (mammalian) cells had interacted with the hormone long ago in vivo, primary interaction in culture cannot induce to a genuine imprinting. Yet its events are so similar to the latter that cell lines can be used with success for modeling hormonal imprinting.

Evidence has been accumulated /16, 17/ that hormonal stimulation is transmitted from interacting to not interacting cells of the same culture. It has been suggested that the gap junctions are involved in the transmission of the stimulus. Immunocytochemical, autoradiographic and biochemical (protein kinase detection) studies demonstrated that the cells responding to the hormone had considerably outnumbered those which had effectively bound it. Earlier studies in this department /13/ have shown that cell-to-cell transmission also applies to hormonal imprinting, although in that instance cell-cell contact and coated pits rather than gap junctions seem to play the leading role in information transfer.

Apart from cell cultures, the unicellular Tetrahymena is a valuable model organism for studies into hormonal imprinting /8/, because the imprinted Tetrahymena cells transmit imprinting not only vertically to many progeny generations, but also horizontally to other cells not previously involved in interaction with the hormone /12/. Since information transfer by direct cell-cell contact is practically impossible in the ciliated Tetrahymena, we presented experimental evidence that the information transfer factor was present in the culture medium, which was in itself able to induce imprinting in virgin cells. In view of this we investigated whether or not hormonal imprinting was transmissible with the nutrient medium also in mammalian cell cultures.

Materials and Methods

The CHO K1 and the Chang liver cell lines were used. The CHO cells were maintained in F12+10% neonatal colostrum deprived calf serum, the Chang liver cells in Parker medium+10% calf serum (Phylaxia, Budapest). The cells were trypsinized (0.25%) for subcultivation. For experimental treatment — two days after subcultivation — the cell monolayers were detached from the walls of the Kolle flasks with trypsin, the nutrient medium was

withdrawn with a pipette and centrifuged. The supernatant was decanted, the sediment was re-suspended in nutrient medium, and the cells were counted in a Buerker counting chamber. The cell suspension was then distributed to culture tubes which were rotated for 4 h, and part of them were treated with hormone during rotation, as follows: 10^{-6}M thyrotropin (TSH, Ambinon, Organon, Oss) or 10^{-5}M gonadotropin (FSH+Lh, 1:1, Pergonal, Serono Rome — Human, Budapest) was added to part of the CHO cells, and 10^{-6}M insulin (Semilente, Novo, Copenhagen) was added to the experimental series of the Chang liver cells (high doses were chosen for avoiding — and compensating — the possible adverse effect of hormones present in the serum). The control cultures were not treated with hormone in either series. After 4 h the cells were centrifuged, washed in two changes of the adequate nutrient medium, resuspended in nutrient medium, and distributed to H-tubes for obtaining coverslip cultures. The following experimental groups were established:

CHO cells: (1) untreated control cells; (2) gonadotropin or thyrotropin-treated cells; (3) equal counts of control + gonadotropin or thyrotropin-treated cells. Chang liver cells. (1) control cells; (2) insulin-treated cells; (3) equal counts of control cells + insulin-treated cells. Cell density was 30 000 cells/ml in each culture.

After 24 h the nutrient medium was decanted from each tube, passed through a Millipore filter, combined with one third part fresh medium, and added to an adequate (CHO or Chang liver) "virgin" culture not previously treated with hormone. The cultures from which the medium was decanted were fixed immediately in 4% formaline (pH 7.2, in PBS) for 5 min, those, to which the decanted media were added were similarly fixed after culturing for 24 h. The fixed cells were washed in two changes of PBS and were incubated in presence of FITC-labeled hormone for 1 h at room temperature (the FITC/protein ratio was 0.42 for insulin, 1.78 for gonadotropin and 1.39 for TSH; the protein concentration was 0.04 mg/ml in all labeled preparations).

After incubation the cells were washed in two changes of PBS, rinsed in distilled water, dried and examined for intensity of fluorescence in a Zeiss Fluoval cytofluorimeter. The analogous signals of a photomultiplier, connected with the microscope, were transformed to digital signals by a digital processor, and were recorded by a Hewlett Packard 41 CX calculator programmed for the statistical evaluation of mean values, standard deviation and significance of inter-group differences. In each group, 20 cells were assayed at random for the intensity of fluorescence above the nucleus and above the cytoplasm (since the nuclear membrane also contains receptors for polypeptide hormones — 3). Three replica assays were performed in each case, thus each column shown in the Fig. 1 represents the mean for 60 cells.

Results and Discussion

The results shown in Fig. 1 have substantiated our earlier experimental observations along this line. All three hormones gave rise to imprinting, as indicated by a significant increase in hormone binding at the second exposure relative to the first. Transmission of imprinting from imprinted to virgin cells (in culture) was shown by the fact that the binding values of the mixed (control+hormone-treated) cultures were either equiva-

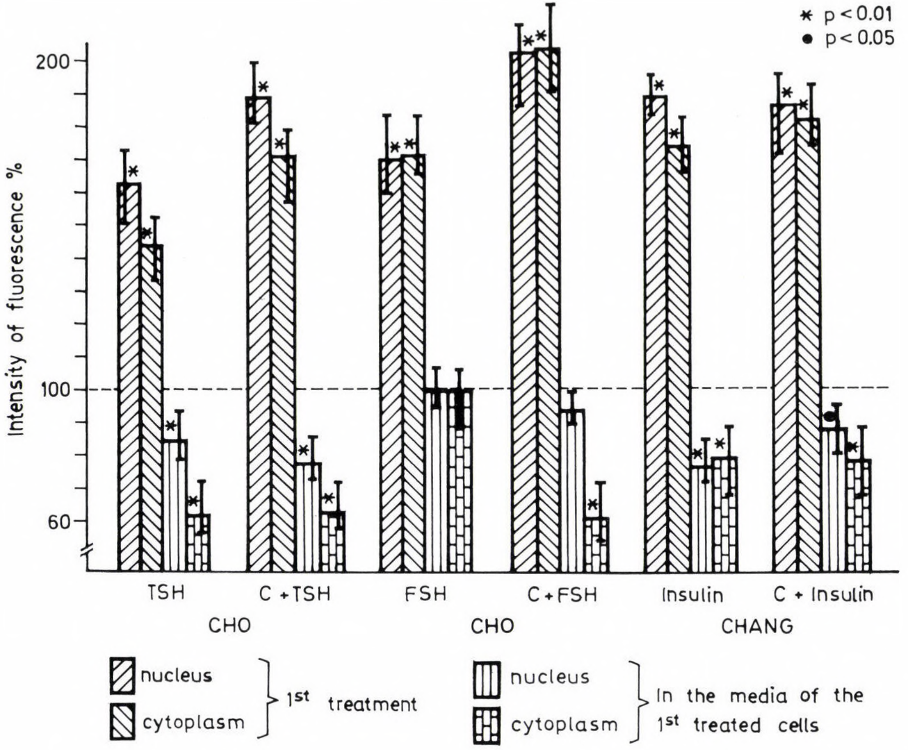

Fig. 1. Binding of FITC-labeled hormones (TSH, FSH, insulin) to hormone-treated (1st treatment) and mixed (hormone-treated + untreated; = C+) cell cultures and to cells which were treated with the media of hormone treated (1st treated) cells, relative to the control as 100. Significance values are related to the prevailing control

lent to (insulin), or even superior to (gonadotropin or TSH), those demonstrated in the 100% hormone-treated cultures. Additional evidence was obtained that gonadotropin and thyrotropin, which have a common alpha subunit and a slightly different beta subunit accounting for the specificity of their actions /18/, are able to bind to one another's receptors /1, 2/, and the binding of TSH to the FSH-receptors may be equivalent, or even superior to the binding of FSH /10, 11/, not only perinatally /9/, but also in cell culture /14, 15/.

The nutrient medium of the hormone-treated cells failed to transmit the information associated with hormonal imprinting to virgin cells, and accounted for a decrease rather than increase in binding capacity, especially for TSH and insulin. It follows that the transmission of hormonal imprinting differs between Tetrahymena and mammalian cell cultures. Whereas

in Tetrahymena cultures the lack of a cell-cell contact due to the presence of cilia causes the information transfer factor to appear in the nutrient medium, in mammalian cell cultures the direct contact of cells in the monolayer provides a means of transmission without involvement of the nutrient medium.

The negative results of transmission with the culture medium cannot be attributed to a negative imprinting, for the cells were assayed immediately after removal from the medium and not later for a delayed effect as in hormonal imprinting studies. It is, therefore, difficult to identify the true cause of the negative effect. Probably the "used" two thirds of the medium was responsible for reduced binding, but then the control cells, to which the binding values were related, were themselves maintained in a similarly "used" medium. An alternative explanation may be based on supposing that the greater proliferation of the cells in presence than in absence of the hormone may have caused a greater "exhaustion" of the medium which, however, may well have been compensated by addition of one third part of fresh medium in an incubation time as short as 24 h. Thus the true cause of the binding decrease remains obscure for the time being. At the same time the fact that the nutrient medium of imprinted cells did not give rise to a binding increase in not imprinted (virgin) cells, presents an unequivocal proof that the factor responsible for imprinting in mammalian cell cultures in not released into the nutrient medium but is mediated via cell-cell contact.

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BEHAVIOR-GENETIC ANALYSIS OF THE PARADISE FISH, MACROPODUS OPERCULARIS III. GENETIC ANALYSIS OF THE RESPONSE TO NOVELTY USING RECOMBINANT INBRED STRAINS

JUDIT GERVAI[1] and V. CSÁNYI

Department of Behavior Genetics, Eötvös Loránd University, Göd, Hungary

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Fifteen behavioral measurements were taken on paradise fish of two inbred progenitor strains and of 16 recombinant lines derived from their cross and maintained under inbreeding with gynogenesis and sib-mating. Univariate and multivariate analyses showed significant differences among the RI means on all measures. Four combined variables extracted by principal component analysis showed that there were common sources of a large part of the behavioral variability measured in the arbitrarily designed test situations. There were no separate subgroups of the RI strain means, and overlapping ranges point to a polygenic genetic determination of the studied behavioral phenotypes. A biometrical analysis of the distribution pattern of recombinant lines and the progenitor strains showed that in several characters non-allelic genic interactions made a significant contribution to the variation. Additive and interaction components of the mean, the heritabilities and the minimum number of effective factors were estimated for all studied behavioral phenotypes, and the combined variables as well.

Keywords: Defensive behavior — Response to novelty — Recombinant inbred strains — Polygenes — Factor analysis — Biometrical genetics

Introduction

In the recent 10—15 years the introduction of the recombinant inbred strain methodology by Bailey /1, 2/ and Taylor /21/ has provided a

[1]Present address and send offprint requests to: Dr. Judit Gervai, Institute for Psychology of the Hungarian Academy of Sciences, H—1394 Budapest, P.O.Box 398, Hungary

Akadémiai Kiadó, Budapest

new tool for genetic analysis. It has also been used in the analysis of the behavioral phenotype (for reviews see 14, 22).

In a previous paper /7/ behavior of inbred strains of paradise fish, Macropodus opercularis, was characterized in novel situations. A considerable genetic variation was found among the strains in various behavioral measurements.

In the present article we introduce RI strains of this new species, produced by gynogenesis and sib-mating, and attempt to analyse the genetic determination of their responses in various novel situations. A number of behavior test measurements were made, and followed by univariate and multivariate analyses.

Materials and Methods

Subjects

In the present experiments individuals of two inbred paradise fish strains, namely U and S and 16 recombinant inbred (RI) strains derived from their cross were used. These strains are the product of a G-S inbreeding program using gynogenesis (G) and sib-mating (S) /17/. The initial population of recombinant females was obtained by artificial gynogenesis of a single F_1 hybrid female /6/. These females were subsequently propagated by gynogenesis, thus providing a highly homozygous offspring carrying exclusively maternal genome. The presence of males among gynogenetic offspring made sib-mating possible. Of the initial 37 females 22 strains were started of which only 16 strains were extant after six generations of G-S inbreeding at the time of th'present behavioral measurements. These were named according to generally accepted convention /20/ as UXS2, UXS3, ... UXS37, where U and S stood for the progenitor strains and 2, 3 ..., 37 designated the individual G_2 females of which the RI strains had been derived. Detailed description of breeding system, following the decrease of heterozygosis etc. have been described separately /8/.

The animals used in the present behavioral experiments come from the sixth generation. The strains at this stage cannot be regarded as strictly inbred. Estimation of the inbreeding coefficient was possible according to Nagy and Csányi /17/. Based on the four known crossing over frequencies the estimated F was 0.91.

Procedures

The 130--145-day-old adult female subjects began the test sequence following one-week habituation period in isolated tanks. The general procedure of maintenance, observations, apparatuses and measures have been described in detail in the first article of this series /7/. The following is a summary of tests and measures in the sequence in which they were given. 1) Home activity: number of air gulps (HAG); 2) Jump off: latency (JUL); 3) Restricted space: number of air gulps (RAG), number of jumps (JUN), fre-

quency of freezing (FRF), cumulated duration of freezing (FRD); 4) Novel object approach: latency (APL), proportion of time spent near the stimulus object (APD), number of air gulps (AAG); 5) Emergence from shelter: latency (EML); 6) Open-field: activity in inner rings (OFI) and the wall ring (OFW) of the circular tank; 7) Unfamiliar place: air gulping latency (AGL), number of air gulps (UAG).

Results and Discussion

A total of 360 animals were measured in nine week-long blocks. Care was taken to randomize subjects belonging to various strains, and crude comparison by Kruskal-Wallis test did not show significant differences across the blocks.

Fifteen behavior measurements were taken from each subject. Only progenitor means of raw scores are shown on Table 1, because tabulating all means of all RI strain measures would require too much space. Within-strain variances were not equal in most cases, but logarithmic and square root transformations supplied satisfying solutions for scaling problems. Progenitor and RI strain means were compared by one-way ANOVAs and where subjects were classified into classes, as "jumpers" and "non-jumpers" or "approachers" and "non-approachers" k-sample χ^2 test was used. The respective F or χ^2 statistics and corresponding df-s are also shown in Table 1.

Figure 1 shows the distribution of parental and RI strain means of 12 variables, obtained after respective transformations. Subgroups (not shown) were established by Duncan multiple range test.

The distributions of RI means show 4—10 overlapping subgroup ranges, SDPs cannot be established, which points to a polygenic determination of all studied phenotypes. However there are peculiarities in several distributions. Certain characteristics, such as activity in the inner rings of the open-field, or the air gulping frequencies in various new situations (see Fig. 1), in which the progenitor strains do not differ, show a considerable unidirectional variation in the recombinants. There are other characteristics, such as novelty approach latency, emergence and air gulping latencies, on the basis of which one of the parental strains, strain U is separated from all others. On the other hand all recombinants differ from parental strain S in their home air gulping frequency and quite a few of them are completely outside the parental range.

Table 1

Behavior test measurements (Mean+SE) of the parental strains and results of comparisons of the parental and 16 RI strains

No.	Measurements	Strain U n = 20	Strain S n = 20	Scale transformation	Applied analysis for all strains	df	F or ^{2}
	Home activity						
1	Air gulping	31.8 $\pm$ 7.1	51.8 $\pm$ 4.7	ln (x)	ANOVA	17,342	8.96^{xxx}
	Restricted space						
2	Air gulping	74.0 $\pm$ 5.5	85.4 $\pm$ 8.1	$\sqrt{x}$	ANOVA	17,342	8.77^{xxx}
3	Jumpers	10	0	-	χ^2	17	59.29^{xxx}
4	Freezers	4	12	-	χ^2	17	83.46^{xxx}
5	Freezing duration	13.5 $\pm$ 2.3	132.3 $\pm$ 31.3	ln (x)	ANOVA	17,211	3.10^{xxx}
	Novel object approach						
6	Air gulping	35.6 $\pm$ 3.2	33.9 $\pm$ 5.9	$\sqrt{x}$	ANOVA	17,342	9.80^{xxx}
7	Approachers	19	15	-	χ^2	17	76.04^{xxx}
8	Approach latency	62.4 $\pm$ 18.0	264.2 $\pm$ 45.3	$\sqrt{x}$	ANOVA	17,216	3.74^{xxx}
9	Approach duration	65.6 $\pm$ 5.5	46.6 $\pm$ 4.5	$\sqrt{x}$	ANOVA	17,216	4.03^{xxx}
	Open field activity						
10	Inner rings	177.7 $\pm$ 11.7	205.4 $\pm$ 21.2	ln (x)	ANOVA	17,342	8.36^{xxx}
11	Wall ring	872.4 $\pm$ 56.7	393.9 $\pm$ 54.0	$\sqrt{x}$	ANOVA	17,342	7.61^{xxx}
12	Emergency latency	14.8 $\pm$ 1.9	64.1 $\pm$ 7.6	ln (x)	ANOVA	17,342	14.40^{xxx}
13	Jump of latency	16.3 $\pm$ 1.4	24.8 $\pm$ 6.3	ln (x)	ANOVA	17,342	3.57^{xxx}
	Unfamiliar place						
14	Air gulping latency	16.5 $\pm$ 1.5	86.2 $\pm$ 35.0	ln (x)	ANOVA	17,342	11.48^{xxx}
15	Air gulping	47.9 $\pm$ 2.8	43.9 $\pm$ 4.0	$\sqrt{x}$	ANOVA	17,342	8.78^{xxx}

$^{xxx}P < 0.001$

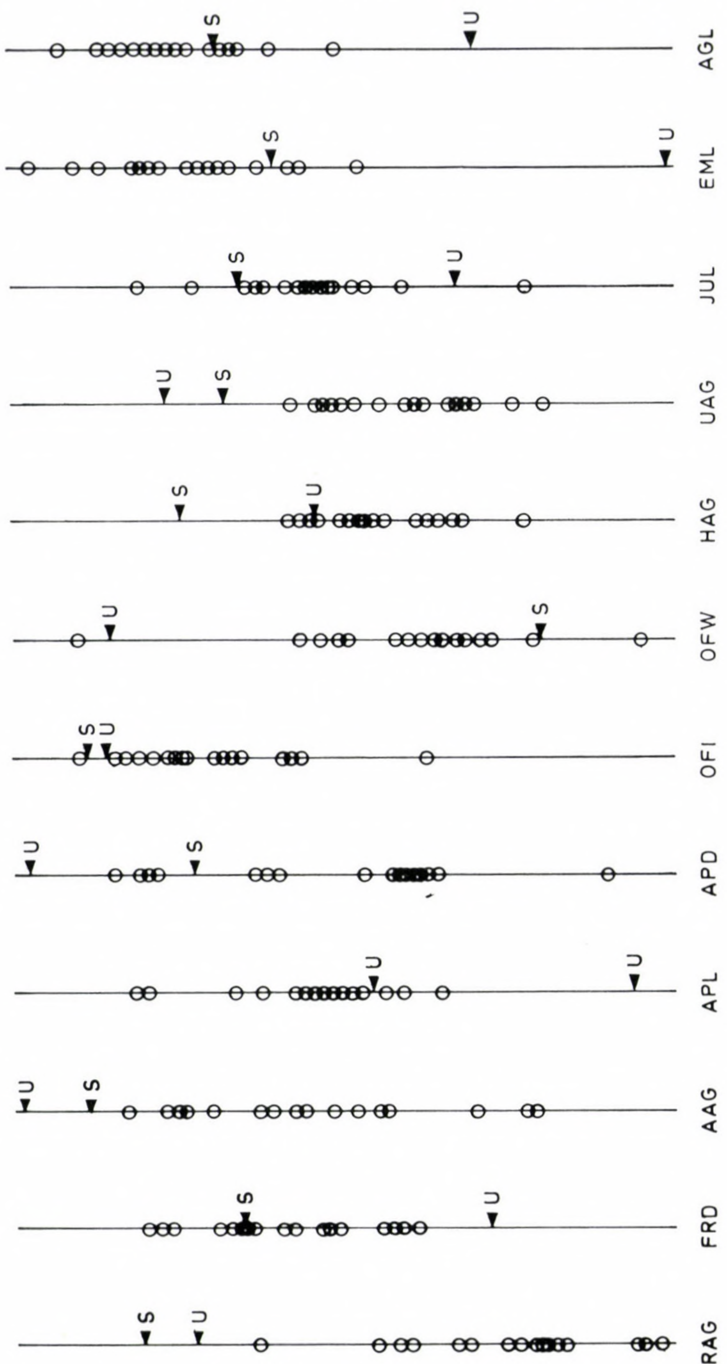

Fig. 1. Distribution of progenitor and UXS RI strain means of test measurements after data transformations

Biometrical genetic analysis of RI data

In a very different context the RI analysis approach has been developed in plant genetics, more precisely in the biometrical genetic study of the variation in Nicotiana rustica. RI lines were initiated by selfing F_2 plants in 1968, and characteristics of the 82 RI lines were described by Eaves and Brumpton /5/. Later, a number of theoretical articles were published on the prediction of the properties of these lines /9–11, 18, 19/. In these papers the effect of allelic and non-allelic interactions on the range and the distribution of RI means are investigated theoretically and tested on experimental data.

Using the biometrical genetic approach we can test various models describing the continuous variation of RI lines. In a simple case, when an additive-dominance model is adequate, the means of all recombinant inbred lines are normally distributed and their expected mean is $\underline{m}$, the mid-parent value, and the variance is $\underline{D}$, the additive genetic variance[2]. The probability that a recombinant inbred strain falls outside the parental range will approximate to the two-tail normal probability integral corresponding with the value: $[\underline{d}]/\sqrt{\underline{D}}$, where $[\underline{d}]$ is the sum of the additive effects. In the presence of complications, such as epistasis, the mean of recombinant inbreds is still $\underline{m}$, while the mean of the progenitor strains is $\underline{m}+[\underline{i}]$, where $[\underline{i}]$ represents the sum of interaction effects. The consequence of a significant $[\underline{i}]$ is the skewness of the distribution of RI means, i.e. an asymmetry in the expected proportion of inbred lines falling outside of parental range.

Observed characteristics, the mean and variance of RI means, and the number of strains, which scored significantly higher and lower than the higher and lower scoring parents, are shown in Table 2, along with the estimates of heritabilities, the interaction and additive components of the parental strain means. Interaction, $[\underline{i}]$ was estimated from the difference of mid-parent values and the corresponding means of RI lines. The additive component, $r_d\,[\underline{d}]$ was estimated from the difference between parental means, r_d is the association coefficient of genes of like effect. From these parameters the proportion of RI lines falling below and above the parental range can be predicted. These predicted values are shown in parentheses besides the observed ones in Table 2. In four measurements, freezing dura-

[2] Notations conform with Mather and Jinks /12/.

Table 2

Observed characteristics of the distribution of means of inbred RI lines and estimates of h^2, i , d , h , k and RI lines falling outside the parental range

Measurements	Mid-parent value m+[i]	UXS RI lines: Mean m	SE^a	$>\bar{P}_1$	$<\bar{P}_2$	[i]	r_d [d]	[d]	$[h]^b$	h^{2c}	k
Restricted space											
Air gulping	8.83	6.30	0.81	0(0)	15(16)	2.53^{xx}	0.21^{ns}			0.83	4
Freezing duration	3.33	3.93	0.52	1(1)	0(1)	-0.60^{ns}	0.94^{xxx}	0.91^{xxx}	-0.44^{xxx}	0.68	3
Novel object											
Air gulping	5.58	3.74	0.98	0(0)	11(15)	1.84^{xx}	0.26^{ns}			0.86	4
Approach latency	11.40	16.91	1.58	4(12)	0(0)	-5.51^{xx}	3.95^{xxx}			0.44	4
Approach duration	7.25	5.63	0.98	0(0)	9(13)	1.62^{x}	0.64			0.75	4
Open field ambulation											
Inner rings	5.17	4.75	0.33	0(1)	11(14)	0.42^{ns}	0.04^{ns}			0.89	4
Wall ring	23.90	21.71	3.10	0(0)	0(0)	2.19^{ns}	5.45^{xxx}	7.10^{xxx}	1.84^{ns}	0.87	6
Air gulping frequency											
Home	3.63	3.11	0.25	0(0)	6(3)	0.51^{x}	0.25^{xx}			0.82	4
Novel place	6.63	5.03	0.56	0(0)	15(16)	1.60^{xx}	0.23^{ns}			0.84	3
Jump off latency	2.47	2.78	0.65	1(2)	0(2)	-0.31^{ns}	0.80^{xx}	1.47^{xxx}	-0.54^{ns}	0.79	5
Emergence latency	3.25	4.33	0.33	9(13)	0(0)	-1.08^{xx}	0.77^{xxx}			0.80	4
Air gulping latency	3.25	3.87	0.26	5(11)	0(0)	-0.63^{xx}	0.49^{xxx}			0.76	3

[a] In additive-dominance model SE equals D, the additive genetic variance, in case of epistases D+I; [b] The dominance effect $h = F_1 - \frac{1}{2}(\bar{P}_1+\bar{P}_2)$ according to the simple model; [c] h^2 is the narrow heritability in absence of significant interaction, otherwise it contains interaction component of in the numerator. It was estimated from the components of variances as $h^2 = \sigma_b^2/(\sigma_b^2+\sigma_w^2)$

$^{x}P<0.05$; $^{xx}P<0.01$; $^{xxx}P<0.001$; ^{ns}non-significant

tion, open field activity scores and jump off latency, $[\underline{i}]$ was not significant, and accordingly distributions of RI means are symmetric along the parental range, with the exception of activity scores in inner rings of the open field. In these cases $[\underline{d}]$ can be estimated as half of the difference of the highest and lowest RI means. According to the simple model we expect the equality in the proportion RI lines that fall outside of the parental range. In the above three characteristics the observations do not differ significantly from prediction.

In all other measurements significant non-allelic interactions were found, which explained the peculiar distributions seen on Fig. 1. Taking significant $[\underline{i}]$ into account expected asymmetry of distribution of RI means was generally in good agreement with observations.

The number of effective factors, $\underline{k}$, is estimated from the range of the inbreds (see Eaves and Brumpton, /7/: $(\overline{P}_H - \overline{P}_L)^2/4\underline{D}$, where $\overline{P}_H$ and $\overline{P}_L$ are the highest and lowest RI means respectively and $\underline{D}$ is the variance of all RI means. This however is a minimum estimation since the probability of extremes not being represented is dependent on the number of RI lines, and increases with the number of effective factors. With a sample of 16 lines extremes are likely to be represented in the case of fewer than four effective factors involved. Estimates of $\underline{k}$ should also be considered carefully when non-allelic interactions are significant since variance of RI means then equals $\underline{D} + \underline{I}$.

Multivariate analysis

Similarity of distributions of RI means and their peculiarities in some test measurements, such as the emergence latency in the open field test and air gulping latency in a relatively small novel place confirms the correlated nature of these measurements, found earlier in the strains /7/ and points to the possible common source of variances of these characters. Testing the possibility of finding a more simple genetic model to explain the observed behavioral variation intercorrelations of 12 measurements were subjected to principal component analysis. Four significant factors were extracted after an oblique rotation (Fig. 2) which together accounted for 61.3% of the total standardized variance. Communalities ranged form 0.26 for OFI to 0.85 for APD indicating that for some measures only a part of the variation is shared with one or more factors.

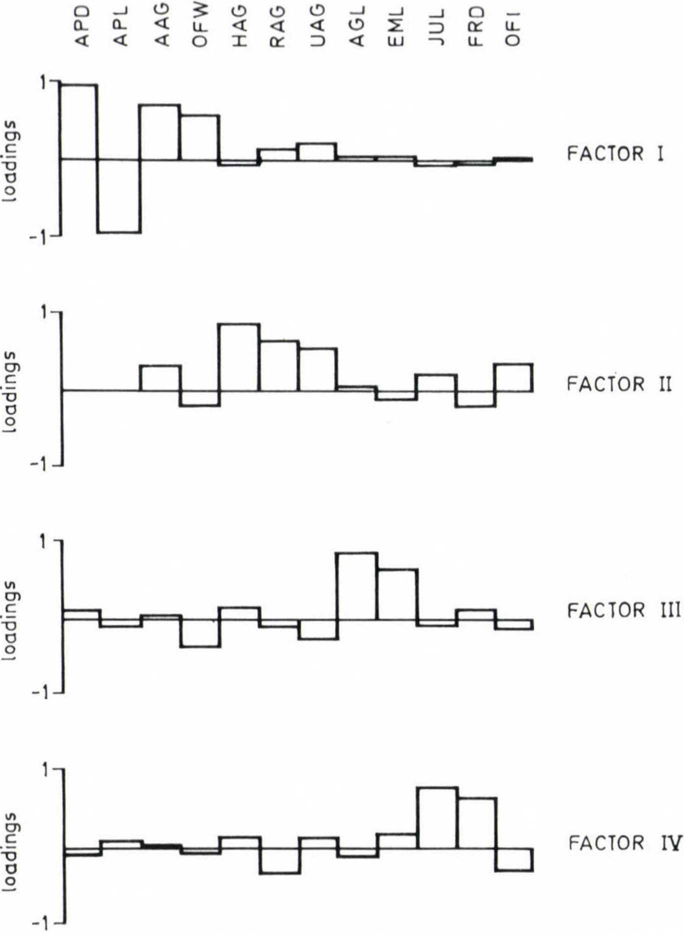

Fig. 2. Structure of four extracted factors following oblique rotation. Abbreviations of behavior test variables are listed in the Method section

The resulting factors showed a remarkable consistence with those found in the analysis of four inbred strains /7/. Factor I, Exploration, obtained previously corresponds Factor I of this study, while Factor II, Defense, corresponds to the present Factor IV. Since there is only a moderate difference between the progenitor strains in this latter feature, the factor explains a relatively smaller fraction of the total variation. Factor II obtained from RI data is connected with air gulping frequences, which were represented in various factors earlier, and Factor III reflecting the initial fear reaction can be related to the Timidity factor found in the study comparing the various inbred strains.

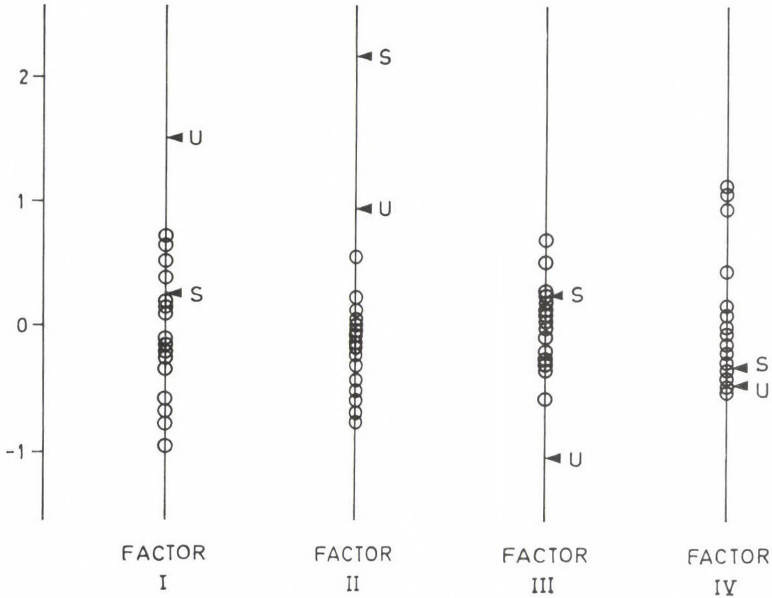

Fig. 3. Distribution of progenitor and UXS RI strain means of factor scores

There are small but significant correlations between the factors. Factors I and II reflecting activity are positively correlated, and both of them are negatively correlated with Factors III and IV reflecting different passive behaviors. Figure 3 shows the distribution of the strain means of individual factor scores obtained from the rotated factor solution. Their comparison by Duncan test resulted in several overlapping ranges again pointing to the polygenic determination of these underlying composite variables. There is a marked asymmetry in the distribution of strain means of Factors I and II where one or both parents significantly differ from all or most of the recombinant inbreds. Accordingly the biometrical analysis (Table 3) indicates significant non-allelic interaction components. Estimates of effective factors are somewhat lower than those obtained in the univariate analysis. Factor I, for example, gives a value for $\underline{k}$ of 3 and loads mainly on measurements obtained in the novel object approach test and the open-field. The estimated $\underline{k}$ for the complex variates reflect the number of effective factors thought to be common for particular groups of variables.

Table 3

Characteristics of the distribution of factor means of RI lines and estimates of h^2, i , d , k and number of RI lines falling out of the parental range

Phenotype	Mid-parent value $m + \underline{i}$	UXS RI lines				$[\underline{i}]$	$r_d [\underline{d}]$	$[\underline{d}]$	h^2	$\underline{k}$
		Mean m	SE	$> \bar{P}_1$	$< \bar{P}_2$					
Factor I	1.37	-0.113	0.523	0(0)	9(15)	1.48^{xx}	0.62^{xxx}		0.89	3
Factor II	1.56	-0.196	0.368	0(0)	15(16)	1.76^{xx}	0.61^{xxx}		0.93	3
Factor III	-0.45	0.022	0.318	1(0)	0(0)	0.47^{ns}	0.49^{xxx}	0.74^{xxx}	0.79	6
Factor IV	-0.41	0.055	0.538	6(1)	0(1)	-0.46^{ns}	0.05^{ns}	0.79^{xxx}	0.88	3

$^{xx}P < 0.01$; $^{xxx}P < 0.001$; ^{ns}non-significant

Conclusion

Recombinant inbred strain analysis of behavioral phenotypes was applied because of its suitability to detect major gene effects, by simultaneously exploiting the advantages of genetic recombination and of inbreeding. The latter has been especially important, since behavioral phenotypes are usually very sensitive to microenvironmental variation, so estimation of phenotypic values is much more reliable if made by measuring a large number of subjects of the same genotype.

Though the major gene effects on behavior reported by others /3, 13—15/ raised our expectations, we were unable to detect simple genetic systems underlying the studied behavioral variation.

In spite of the fact that the progenitor strains were extremely different in 8 out of 15 behavioral measurements SDPs could not be established. Although we think that the lack of definite subgroups are caused by the polygenic determination of the studied behavioral phenotypes, it should be considered that the UXS strains were not strictly inbred. Estimated inbreeding coefficient was 0.91 and genotypic identity, as a consequence of gynogenetic propagation was 0.92 /17/. The remaining heterozygosity can distort the RI means, but its effect is significant only in the presence of great dominant-recessive allelic interactions. Considering the extreme case of complete dominance and 91% of recessive homozygotes, the population mean deviates 0.2 $\underline{d}$ from the mean of the 100% homozygous population. Thus in the case of 1 or 2 major genes some heterozygosity of RI strains does not exclude the identification of major gene effects. This was clearly shown by Cazala and Guenet /4/, who used the eighth generation of CXD2 mice (F=0.867) and showed a single gene effect determining difference in self-stimulation frequency. We think however, that most behavior patterns — especially in vertebrates — are the product of the complicated nervous system and thus the polygenic control of their variation is conceivable.

While there are various strategies and devices to study polygenic variation /12/, RI strain analysis combined with biometrical approach can still be informative especially in detecting non-allelic interactions and linkages.

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BEHAVIOR-GENETIC ANALYSIS OF THE PARADISE FISH (MACROPODUS OPERCULARIS) IV. BEHAVIORAL UNIT ANALYSIS OF THE RESPONSE TO NOVELTY USING RECOMBINANT INBRED STRAINS

JUDIT GERVAI[1] and V. CSÁNYI

Department of Behavioral Genetics, Eötvös Loránd University, Göd, Hungary

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Frequencies and relative durations of eleven ethologically defined behavioral units were measured on paradise fish of two inbred strains and 9 recombinant inbred lines derived from their cross and then maintained under inbreeding. Significant genotype-dependent differences were found in all but one behavior units. Strain distribution patterns showed polygenic genetic determination of all units, and in several cases non-allelic genic interactions seem to contribute to the variation. Additive and interaction components of the progenitor means and minimal number of effective factors were estimated for each behavior unit.

Keywords: Behavioral elements — Recombinant inbred (RI) strains — Response to novelty — Behavioral genetics

Introduction

Changes of environment have a strong effect on animal behavior. Exploration of novel features, defensive actions against real or supposed dangers are of immense adaptive significance. Understanding of behavior in various novel situations is one of the major concerns of this laboratory's work on the paradise fish (Macropodus opercularis). In this we have made use of the great experience of animal psychology, and devised several tests for measuring emotionality, fear, curiosity, etc. (for review, see /2/).

[1]Present address and send offprint requests to: Dr. Judit Gervai, Institute for Psychology of the Hungarian Academy of Sciences, H–1394 Budapest, P.O.Box 398, Hungary

Akadémiai Kiadó, Budapest

It was found that there were considerable genetic variations of these behavioral test measurements /8/, and further analysis with recombinant inbred lines showed an invariably polygenic genetic determination in the background /10/.

Attempts have already been made towards the synthesis of ethological approach to animal behavior and the study of its variation, primarily in mice /1, 15/. An ethological analysis of the behavior of paradise fish in various new environments have been published earlier /4/. A similar comparison of inbred paradise fish strains in a small, novel place has also been described /8/. The behavioral response was characterizd by recording the sequence of behavioral units, which had been defined within the ethogram of this animal /5, 6/. A considerable amount of genetic variation among the strains was found in several behavioral units.

In the present article we take further our investigations in analysing the genetic variation on two inbred strains and nine recombinant inbred (RI) lines derived from their cross.

Materials and Methods

Subjects

Female paradise fish of two inbred strains denoted by the capital letters U and S, and 11 recombinant (RI) lines derived from their cross, as defined by Bailey (1971), were used. The RI strains were maintained under strict inbreeding with gynogenesis and sib-mating (see /9, 12/), and individuals used in the present experiment were obtained from the eighth generation. Estimated inbreeding coefficient based on known recombination frequencies was 0.95.

Setting

The fish were reared in communal aquaria and were housed in groups of thirty of mixed sexes in 80 litre filtered tanks. Temperature was controlled at 27 ± 1 ^{0}C and fluorescent lights were on from 7 AM to 9 PM (see more details in /8/). At 130–160 days of age subjects were taken to the laboratory for two-day habituation before observations. They were housed then in 6 litre individual tanks, which were not visually isolated.

Behavioral measurements

The individual fish was placed for five minutes in an unfamiliar, transparent glass aquarium (20x20x20 cm) enclosed in an isolation chamber. The observation was made from behind a plastic screen. The sequence of 11 exactly defined behavior units was recorded by the same trained observer

with the help of a small personal computer (ATARI 400). This had been programmed to accept keyboard inputs as codes for behavior units, and recorded sequence and duration of each subsequent inputs. The software developed in this laboratory has been described in detail elsewhere /13/. At the end of five minutes sessions the computer supplied statistics on the frequency and cumulated relative duration of each behavior unit.

The behavior units (or as also mentioned, behavior elements) were defined to cover the entire length of observation session, describing types of movements, postures, and location of the fish. Here we only give a short list of these units, since they have been described in greater detail in earlier publications /5/. A functional interpretation of these units and their relations with environmental changes have also been discussed /4—6/.

The behavior units were defined as follows:

Move	(MOV)	= slow, even locomotion without using caudal fin
Swim	(SWI)	= fast locomotion
Escape	(ESC)	= rapid movement, forceful swimming perpendicular to the glass side
Creeping	(CRE)	= very slow swimming, the fish is propelled by pectoral fin fanning only, other fins are closed
Staccato	(STA)	= a series of quick starts and sudden stops
Air gulping	(A-G)	= being an Anabantoid, paradise fish regularly swim to the surface and gulp air to breath
Floating	(FLO)	= the immobile fish floats under surface, not deeper than 1—2 cm with body axis parallel with the surface. Fanning pectoral fins keep the body in position
Hanging	(HIM)	= as FLO, but in the middle range of the test aquarium
Resting	(RES)	= as FLO and HIM, but staying on the ground. The anal or caudal fin touches the bottom
Oblique	(OBQ)	= The body axis is inclined approx. 20—30 degree from the horizontal plane. The fish stays near the wall, often in a corner. Fins are closed except fanning pectoral ones.
Freezing	(FRZ)	= the fish is motionless, gills and occasionally the eyes move.

Statistical evaluation of data

BMDP Statistical Software- 1981 version was used. P1D, P2D procedures were used for data description and test of normality. P7D was used to find suitable transformations and for 1-way ANOVA.

In the biometrical analysis of the distribution of strain means, the results of Jinks and Pooni /11/, and Pooni and Jinks /14/ were applied.

Results

A total of 307 animals were measured, 18—20 individuals per day. Care was taken to randomize subjects belonging to different groups to avoid significant block effect. Comparison of blocks by nonparametric Kruskal-Wallis test did not show significant differences either across the blocks or the time of the day.

Table 1 contains the means of raw scores obtained in the progenitor and UXS RI lines. Correlation of group means and variances indicated some genotype -- microenvironmental interactions in all variable except ESC and A-G. Scale transformations were applied accordingly to eliminate this interaction. One-way ANOVA comparison of group means showed significant strain effects in all measures, except RES (see also Table 1).

A further attempt to find subgroups among the strain means by Duncan test indicated 2--5 partially overlapping non-significant ranges. Therefore no simple genetic model could have been constructed for the explanation of observed variation between the progenitor strains.

The distribution of transformed line means showed a marked asymmetry, a shift compared to the progenitor strains in several units (Fig. 1), such as SWI, A-G, HIM and FRZ, suggesting non-allelic genic interaction.

Although we have used only 9 RI lines in the present experiment, the characteristics of their distribution, i.e. the mean, variance and the number of strains scoring higher and lower than the higher and lower scoring parents, are used to estimate components of the parental means and the minimum number of effective factors (Table 2).

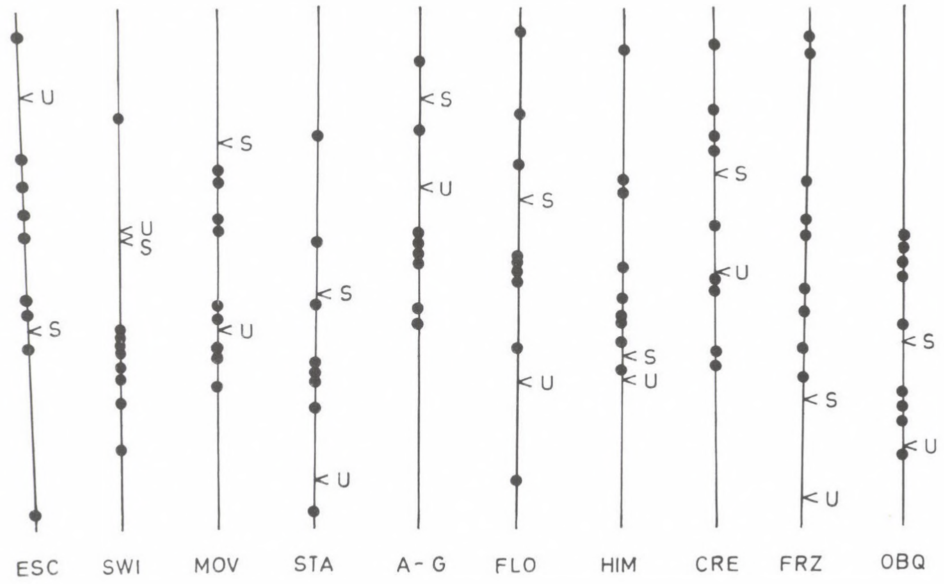

Fig. 1. Distribution of transformed means of recombinant inbred lines (●) and the progenitor strains

Table 1a

Behavior unit measurements (mean ± SE) of the parental and UXS RI strains, and results of comparisons by one-way analysis of variance

Strain	n	Behavior Units					
		ESC t%	SWI t%	MOV t%	STA t%	CRE t%	A-G n
U	40	59.0 ± 2.9	25.2 ± 2.5	1.5 ± 0.5	0.3 ± 0.2	2.0 ± 0.5	13.2 ± 0.6
S	40	40.0 ± 3.5	25.0 ± 2.2	5.0 ± 0.9	3.5 ± 1.2	4.8 ± 0.8	16.6 ± 1.0
UXS-2	40	42.1 ± 3.5	19.0 ± 2.4	4.0 ± 0.8	1.7 ± 0.5	3.3 ± 0.6	10.7 ± 0.6
UXS-4	40	49.3 ± 3.1	11.4 ± 1.5	0.9 ± 0.3	1.4 ± 0.5	7.5 ± 1.0	10.7 ± 0.7
UXS-5	40	53.7 ± 3.3	18.6 ± 2.3	1.4 ± 0.4	3.7 ± 1.0	5.8 ± 0.9	9.3 ± 0.7
UXS-8	10	51.6 ± 6.3	16.0 ± 2.7	1.3 ± 0.6	1.4 ± 0.8	0.6 ± 0.4	18.2 ± 3.2
UXS-15	10	24.4 ± 6.6	33.3 ± 3.5	1.9 ± 0.7	1.6 ± 0.9	1.8 ± 1.2	7.6 ± 1.1
UXS-24	27	38.7 ± 4.6	14.7 ± 1.9	0.6 ± 0.3	10.7 ± 1.9	5.3 ± 0.7	10.1 ± 0.8
UXS-26	10	47.9 ± 6.7	13.4 ± 3.3	0.8 ± 0.4	4.4 ± 1.9	1.8 ± 1.1	11.2 ± 1.5
UXS-28	10	64.0 ± 4.7	15.8 ± 4.4	2.5 ± 0.6	0 ± 0	0.5 ± 0.4	15.3 ± 2.4
UXS-41	40	44.2 ± 3.8	13.0 ± 2.0	2.1 ± 0.5	8.8 ± 2.1	8.6 ± 0.9	8.2 ± 0.6
Statistical analysis							
Transformation		-	$\sqrt{x} + 0.01$	ln(x+1)	ln(x+1)	$\sqrt{x} + 0.01$	-
F (10,296)		4.99^{xxx}	4.48^{xxx}	4.91^{xxx}	9.57^{xxx}	5.99^{xxx}	17.73^{xxx}

$^{xxx}P < 0.0001$

Table 1b

Behavior unit measurements (mean ± SE) of the parental and UXS strains, and results of comparisons by one-way analysis of variance

Strain	n	Behavior Units (t%)				
		FLO	HIM	RES	OBQ	FRZ
U	40	3.8 ± 0.7	2.3 ± 0.8	1.4 ± 0.4	0 ± 0	0.1 ± 0.1
S	40	6.0 ± 0.8	2.8 ± 0.7	1.1 ± 0.4	1.8 ± 0.7	4.8 ± 2.2
UXS-2	40	11.0 ± 1.6	4.8 ± 0.6	1.8 ± 0.5	4.5 ± 1.5	3.1 ± 1.8
UXS-4	40	6.4 ± 1.3	2.3 ± 0.5	1.6 ± 0.4	4.0 ± 1.4	11.0 ± 3.6
UXS-5	40	2.4 ± 0.5	3.5 ± 0.6	2.0 ± 0.4	0.5 ± 0.3	5.1 ± 1.6
UXS-8	10	5.3 ± 1.7	15.8 ± 4.4	1.0 ± 0.4	0 ± 0	1.2 ± 0.4
UXS-15	10	7.2 ± 1.2	8.7 ± 2.1	2.9 ± 1.0	0.5 ± 0.4	12.7 ± 5.1
UXS-24	27	4.2 ± 0.9	3.2 ± 0.9	1.7 ± 0.5	3.6 ± 1.4	13.7 ± 3.6
UXS-26	10	8.0 ± 2.5	7.8 ± 1.8	2.2 ± 1.6	2.6 ± 2.3	3.6 ± 1.4
UXS-28	10	4.3 ± 1.1	3.0 ± 0.9	1.2 ± 0.2	0.3 ± 0.3	3.9 ± 1.3
UXS-41	40	4.6 ± 0.7	3.3 ± 0.7	1.1 ± 0.4	3.4 ± 1.4	7.7 ± 3.6
Statistical analysis						
Transformation		$\sqrt{x + 0.01}$	$\ln(x + 1)$	$\ln(x + 1)$	$\ln(x + 1)$	$\ln(x + 1)$
F (10,296)		5.75^{xxx}	7.32^{xxx}	1.12^{ns}	4.90^{xxx}	7.78^{xxx}

$^{xxx}P < 0.0001$;

^{ns}non-significant

Table 2

Observed characteristics of the distribution of UXS line means and estimated h^2, [i], [d], and k values^{2}

Behavior Unit	Mid-Parent Value m+ [i]	UXS Lines Mean m	SE	$>\bar{P}_1$[d]	$<\bar{P}_2$	[i]	r_d [d]	[d]	h^2	k
ESC	49.5	46.25	11.01	0 (0)	1 (0)	3.25^{ns}	9.50^{xxx}	19.8^{xxx}	0.84	4
SWI	4.77[b]	3.80	0.70	1 (1)	6 (8)	0.97^{ns}	0.01^{ns}		0.84	4
MOV	0.89	0.65	0.30	0 (1)	1 (1)	0.24^{ns}	0.38^{xxx}	0.36^{xxx}	0.72	2
STA	0.50	0.84	0.61	1 (0)	0 (0)	-0.34^{ns}	0.38^{xxx}	1.00^{xxx}	0.88	3
CRE	1.28	1.42	0.88	2 (1)	0 (1)	-0.14^{ns}	0.40^{xx}	1.20^{xxx}	0.91	2
A-G	13.8	9.46	1.59	0 (0)	8 (9)	4.34^{xx}	1.10^{x}		0.74	3
FLO	1.89	2.06	0.52	1 (0)	0 (0)	-0.17^{ns}	0.37^{xx}	0.91^{xxx}	0.77	4
HIM	1.16[b]	1.99	0.82	4 (6)	0 (1)	-0.83^{ns}	0.09^{ns}		0.90	3
OBQ	0.21	0.46	0.33	2 (2)	0 (1)	-0.25^{ns}	0.21^{xx}	0.43^{xxx}	0.74	2
FRZ	0.25	1.16	0.49	5 (8)	0 (0)	-0.91^{x}	0.21^{x}		0.77	3

[a] Notation conforms with Mather and Jinks (1971)

[b] Parental lines do not differ significantly

[c] Rounded to the next higher integer

[d] Estimated numbers of RI lines falling outside of parental range are in parenthesis

$^{x}P < 0.05$; $^{xx}P < 0.01$; $^{xxx}P < 0.001$; ^{ns}non-significant

From the mean of all RI lines ($\underline{m}$) and the corresponding mid-parent value ($\underline{n}$+ $[\underline{i}]$) the interaction component can be estimated. The additive component was estimated from the proportion of lines outside of the parental range, taking significant interaction into account, where needed. The minimal number of effective factors was estimated from the largest and lowest RI means and the variance of all RI lines, as $\underline{k} = (\bar{L}_1 - \bar{L}_2)^2/4D$ (see /7/). h^2 — the narrow heritability in absence of significant interaction was estimated from the variance components: $h^2 = \sigma_b^2/(\sigma_b^2 + \sigma_w^2)$.

Discussion

In the present experiment an attempt was made to analyze the genetic background of an observed strain difference in reaction of the paradise fish placed in a relatively small new environment.

Significant genotype-dependent differences were found in measurements of all but one behavior units — in some cases, as of SWI and HIM, despite of the progenitor strains being similar to each other.

The distribution of strain means did not allow fitting of a simple genetic model in any behavior units, since phenotypic classes could only be partially distinguished, because of overlapping subgroup ranges.

Therefore, assuming polygenic genetic determination of parental variation the observed characteristics of RI strain distribution were used in estimating additive, and non-allelic interaction components of the parental means. A minimal number of effective factors was also calculated, though it should be emphasized that even these values of $\underline{k}$ were probably underestimated if either of the two extreme recombinant lines was not represented in the sample (which was quite possible in a sample of only 11 strains) or the additive effects of the genes were far from being equal.

Despite of these inherent uncertainties we can compare the present results with a previous similar analysis of more traditional psychological test measurements /10/ where these estimated $\underline{k}$ values were somewhat higher, indicating perhaps a more complex genetic background.

We found that the variation of the behavioral units could be often described by the simple model of additive-dominant effects, i.e. in the majority of the variables there were no significant interaction effect. This means that by using behavioral elements as phenotypes we obtained a more complete description of the genetic architecture compared to that obtained

by using test variables /10/. Moreover, the estimated parameters ([d], r_d, h^2, k) are also more reliable. This is shown by the good agreement of the observed and predicted numbers of RI lines falling outside the parental range, of which the later is calculated from [d], [i], and D.

Our previous correlational studies /4, 10/ have also indicated functional relationships among certain behavioral units. Therefore it seems logical to suppose some common genetic factors influencing their variation. A multivariate analysis could be a next step to explore this possibility.

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DIALLEL GENETIC ANALYSIS OF THE ELEMENTS OF PARADISE FISH'S (MACROPODUS OPERCULARIS L.) BEHAVIOR IN FAMILIAR AND NOVEL SITUATIONS

R. GERLAI and V. CSÁNYI

Department of Behavior Genetics, Eötvös Loránd University, Göd, Hungary

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Elements of the paradise fish's ethogram were recorded in 1 familiar and 3 different unfamiliar situations and the inheritance of these behavioral elements was investigated employing a five times replicated diallel cross between 3 inbred strains. A generalized Hayman Analysis of Variance and a Variance Covariance Analysis were performed to estimate genetic effects and parameters, such as, additive genetic variance, different sorts of dominance variance, reciprocal effects, direction and degree of dominance, ratio between the frequency of dominant and of recessive alleles, minimum number of effective factors and heritabilities, etc. Knowing the genetic architecture, we make inferences about the possible evolutionary past of the behavioral elements and explain why selection might favor certain types of paradise fish's behavior in particular circumstances.

In several cases a possibility of "monogenic" inheritance emerged. We explain this finding and conclude that in a cross experiment where the inheritance of phenotypical units are investigated by employing only a few genetically different strains this result may be expected.

Keywords: Diallel cross — Prardise fish — Behavioral elements — Genetic parameters

Introduction

There have been several analytical methods and concepts developed in animal psychology (see e.g. /6/) that enabled animal behavior to be

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Send offprint requests to: Dr. R. Gerlai, Department of Behavior Genetics, Eötvös University, Jávorka S. u. 14, H-2131 Göd, Hungary

Akadémiai Kiadó, Budapest

reliably studied under laboratory conditions. When, however, the question was to understand natural behavior and its evolution, ethologists have stressed (e.g. /1, 4/) that elements of the ethogram, that is, the species-specific behavioral repertoire should be observed as relevant behavioral phenotypes instead of possibly irrelevant test scores.

Inter-specific variability in behavior can provide a good opportunity to hypothesize steps in the evolution of behavior, a so-called classical ethological approach /51/. However, intra-specific genetic analysis can yield more accurate and statistically sound results. Surprisingly, with a few exceptions (e.g. /2, 46, 57, 60/) intra-specific genetic analysis of elements of the ethogram has been neglected, even though a genetical approach could increase the understanding of behavior.

A diallel cross-analysis can render detailed information about the variable genetic background of behavioral traits and may allow the investigator to make inferences about their evolutionary past. A further advantage of this method is that it requires only one generation time to breed all the experimental populations needed for the genetic analysis.

A diallel cross consists of n inbred strains and their all possible F1 hybrids, including reciprocal crosses. When employing the diallel cross method, several alternative approaches, summarized by Wearden /62/, are available. The most frequently applied method is the analysis developed by Hayman and Jinks /35, 36, 39/ for replicated diallel cross. A worked example of a generalized method of this cross design has also been available /12/.

Modifications of this design have already developed: reciprocal crosses are sometimes omitted if maternal (or paternal) influences are assumed to be absent; half diallel /40, 12/, or only a part of all possible crosses is raised (partial diallel; /11, 42, 55/). There have been methods available when the parental strains are not completely homozygous at all loci /23, 25, 43, 50/, and when only one replication is bred (e.g. /37, 38/). In short, these alternative methods require less sophisticated breeding and experimental design but rely upon more assumptions and give less exact and detailed information about the inheritance.

We used the most informative replicated diallel cross analysis developed by Hayman /35, 36/, whose theory was summarized by Mather and Jinks /49/ and whose genetic analysis was generalized by Crusio et al. /12/.

In the beginning, the diallel cross system was applied in plant genetic studies (e.g. /65/) and the methods and theory of the genetic analysis were developed for these subjects. However, these methods can be

applied to any quantitative traits of any species, for example to the behavioral phenotype as well. Thus, it has been widely employed in the genetic analysis of behavior, mostly in rats and mice, e.g., open-field behavior in rats /7/, avoidance learning in rats /30, 63, 64/, emotional defecation in mice /9/, reversal learning in mice /10/, alcohol preference in mice /31/, locomotor activity and jumping behavior in mice /37, 38/, exploratory and learning behavior in mice /44, 45/, maternal behavior in mice /61/, exploratory behavior in mice /13/ and exploratory behavior and structural variation of the Hippocampus in mice /14, 15/. The genetic background of mating speed in Drosophila /29/ has also been investigated by the diallel analysis.

The paradise fish is not a widely used species in behavior genetics. However, its ethology is not completely unfamiliar /27/, and it has been used in a selection experiment /28/ and also in a classical cross analysis /19/.

A coding system for recording the behavioral elements of the paradise fish's ethogram has been defined in our laboratory, and various functional relationships of these elements have been studied /19, 22, 53/. Using this ethological code, predator avoidance /16, 17, 18/, effects of drugs /24/ and open-field behavior of the paradise fish /32/ have been investigated.

In novel environments animals display a rich and varied behavioral repertoire /13/, and one can assume that the behavioral responses given by animals to the stimuli of unfamiliar environments are highly significant in terms of survival, that is, from an evolutionary point of view. It can also be assumed that the adaptivity of a certain behavior is highly dependent on the environmental circumstances.

The genetic architecture of a behavioral phenotype has been shaped by natural selection during a species' evolutionary history. Thus, it has been argued /8, 48, 56/ that a quantitative genetic analysis would allow us to make inferences about both the past action of selection and the adaptive significance of the behavioral trait in question the most important information relevant to it being the presence or absence of additive genetic variance and the type and direction of dominance. If we find additive genetic variance and/or ambidirectional dominance, the most plausible conclusion we may draw is to suppose stabilizing selection as an evolutionary past of the given trait. If, however, significant directional dominance is found, directional selection may be referred as an evolutionary past.

According to the results of some studies done on other speices (e.g.

on mice /13/) intermediate level of exploratory activity could be inferred as adaptive in open-field situation. On the other hand, in the case of a different situation, such as a small unfamiliar restricted place, we found /5/ that paradise fish tended to show defensive behavior instead of exploration. In this case extreme behavioral responses, either active or passive, can be supposed to be adaptive. To test these ideas we designed 4 different test situations and investigated the genetic architecture of the behavioral elements measured in them.

Materials and Methods

Animals and Housing

The paradise fish has from our point of view many advantages over other commonly used animals, such as rodents. It is a diurnal animal with a well developed visual communication system and certain changes in its inner state are clearly visible to the naked eye. Being a simpler vertebrate, its actions are relatively less sophisticated and can be described precisely in terms of simple behavior elements. An aquarium can be maintained and controlled easily, and if it is isolated from the observer it offers a very constant and undisturbed environment. Paradise fish are prolific and regularly produce 1000 young at each spawning.

The cross system used in this study is called diallel cross /35, 36/. For this cross, we chose three inbred strains which showed marked behavioral differences: the "U", the "P" and the "C", which is a color variation of this species, proved to be highly different /19, 34/, and bred their reciprocal F1 generations (UP = U-female x P-male; UC = U-female x C-male; PU = P-female x U-male; PC = P-female x C-male; CU = C-female x U-male and CP = C-female x P-male). At the time of recording, the parental strains had reached the 22nd generation of sib-mating. One pair of parents was used to produce each cross, that is the crosses were single families.

The fish originating from different crosses and strains were raised simultaneously in groups of thirty in 80 liter glass aquaria (60x40x35 cm). The water was filtered, the temperature was held constant at 28 ^{o}C, and a 14/10 light/dark cycle was maintained. Each aquarium contained water plants, Hygrophila polysperma, which occures in the natural habitat of the paradise fish. The animals were fed daily on laboratory made fish food, consisting of beef liver, hake, eggs, wheat bran and vitamins. Paradise fish reach maturity at 79--90 days and can live as long as 6 years under laboratory conditions. In these experiments, 120--220-day-old, fully mature fish were tested. The animals were moved to the recording room, where they were housed individually in 6 liter glass aquaria (15x15x30) for three days and then they were tested in a randomized order. To avoid possible subjectivity, before testing each individual fish was given a number and their exact genotypes were checked only when all recordings had already been done.

We measured the fish in five blocks (replications) and each block contained 4—5 female and 3--4 male individuals per cross, however, only the females' behavioral data are presented and analyzed here since the sample sizes of the males from UC and PC crosses were insufficiently low (the exact sample sizes of females are presented on Table 1). Each fish was

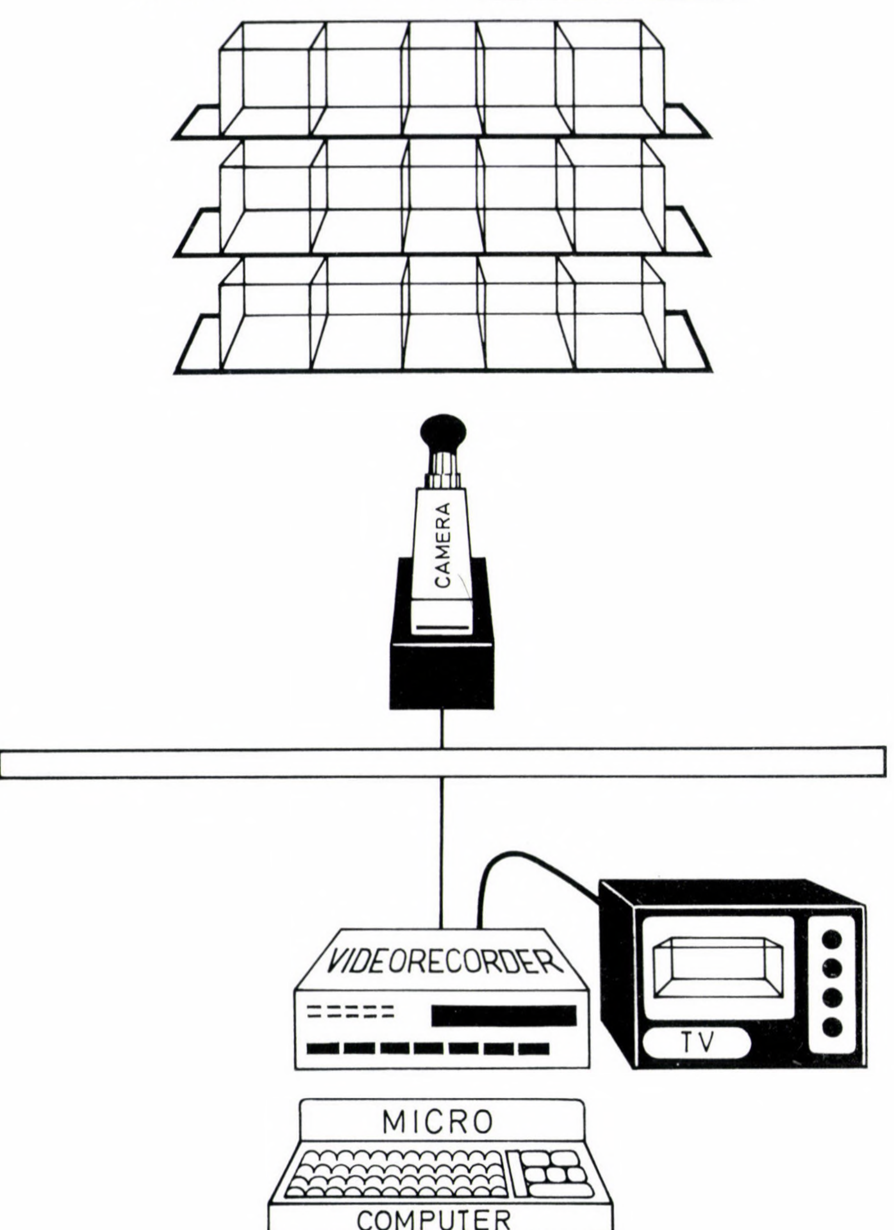

Fig. 1.

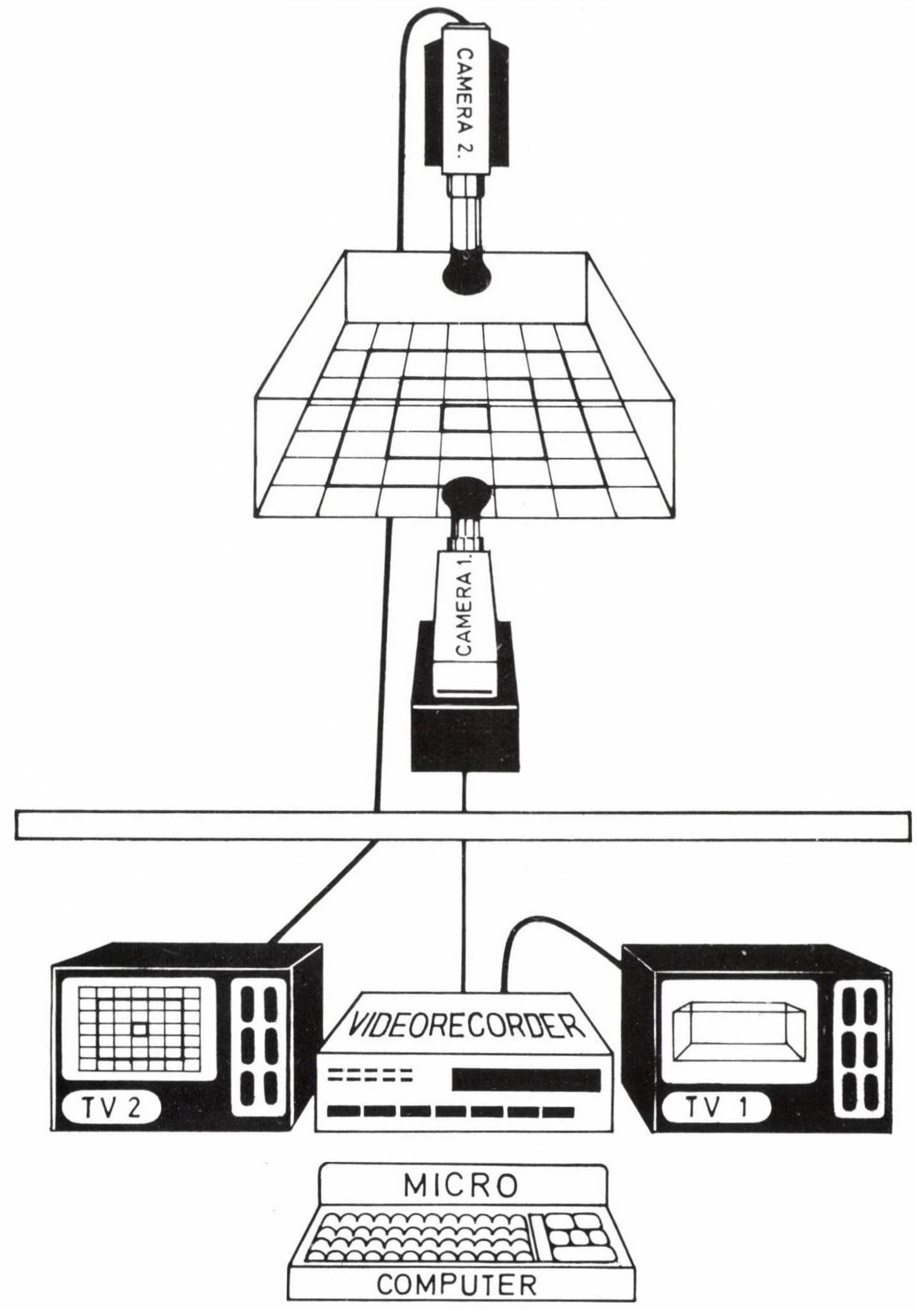

Fig. 2.

tested individually for 5 min in four different situations, which followed each other in the following order:

First situation: Familiar 6 liter holding aquaria in the recording room, where the animals had been kept for 3 days before testing (Fig. 1).

Second situation: An open-field, which was a 70x20x70 cm aquarium painted white on all but one side. A network of squares (10x10 cm) was painted on the bottom (Fig. 2). The fish were individually netted into a plastic pot and were placed into the center of the open-field.

Third situation: small (20x20x20 cm) unfamiliar aquarium with transparent glass walls (Fig. 4).

Fourth situation: After the 5 min testing in the 3rd situation we stopped recording and from the 10th min we restarted recording with the presence of a rotating disc having placed above the aquarium (Fig. 4).

In all test situations the aquaria were illuminated from above by white phototubes and all the conditions were similar to those of during rearing. For the open-field test a television camera was aimed at the transparent side of the tank and a second one was placed above it, in all other test situations the fish were monitored from the side. During the recording session the experimenter left the recording room and observed the fish's behavior on monitors.

In the case of the open-field, latency time to emerge (LAT) from the pot was recorded, and the animal's movement was monitored for 5 min thereafter. The total number of squares entered (locomotion scores) in various parts of the open-field were recorded. Designations are as follows (Fig. 3): OIT is the activity score in the inner part of the open-field. OOT is the activity score in the outer part of the open-field, which was further divided and assigned as OUT and OST, where OUT is the activity score in the "U-segment" of the outer part of the open-field, and OST is the activity score in the segment of the outer part located next to the transparent side of the open-field. This classification of the activity scores is based on previous studies /32, 34/.

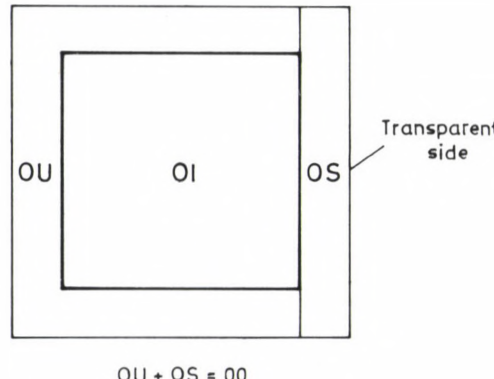

Fig. 3.

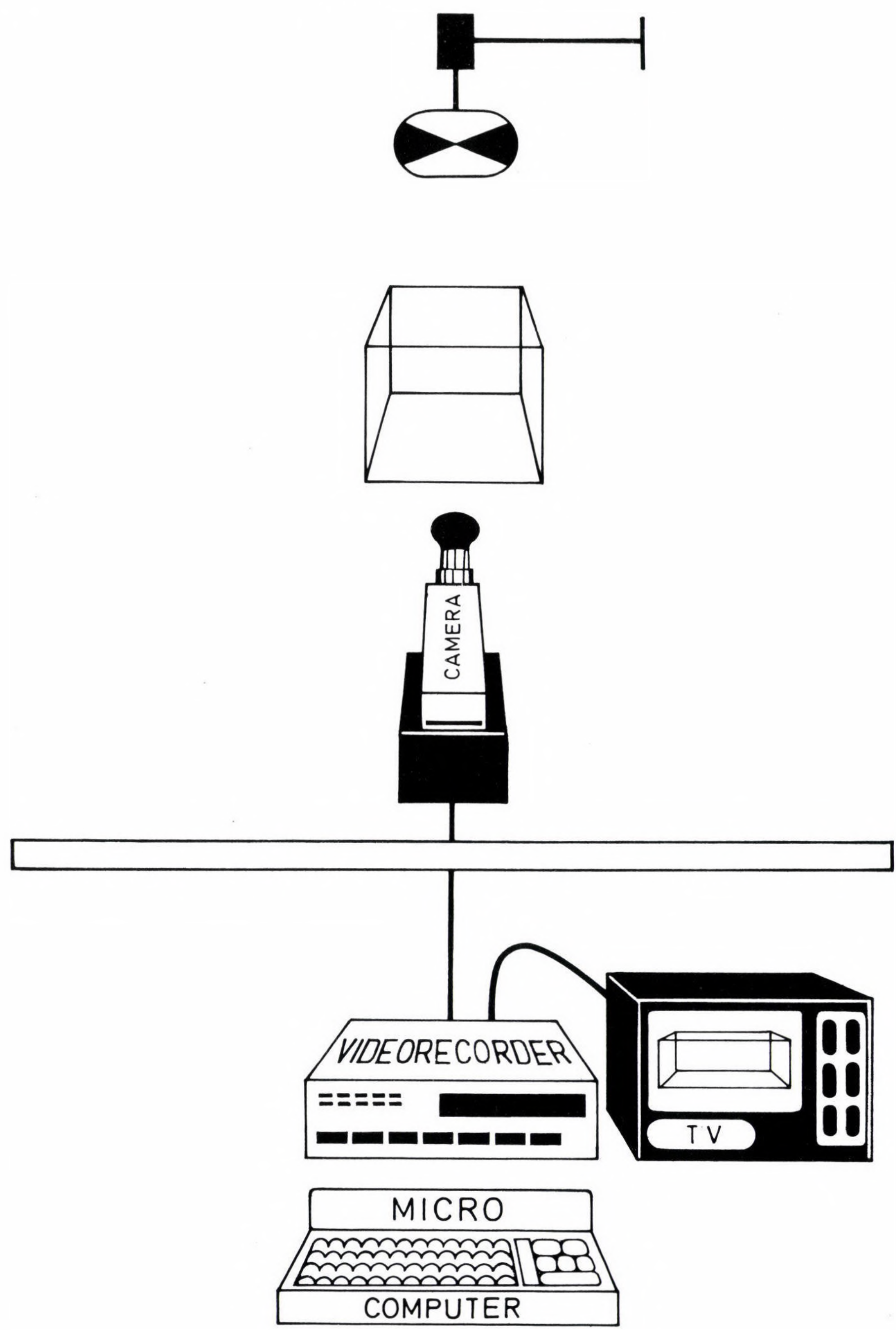

Fig. 4.

The behavioral elements measured in the four situations were defined as follows with relative duration (a), or frequency (b) indicated /21/: Escape (a) ESC: rapid to and fro movement, with forceful swimming perpendicular to the glass wall. Swim (a) SWI: fast locomotion mainly with the use of the caudal fin. Move (a) MOV: slow, even locomotiom without using caudal fin. Staccato (a) STA: a series of quick starts and sudden stops during locomotion. Creeping (a) CRE: the fish is propelled forward only by pectoral fin fanning, all other fins are closed, pectoral fins beat very quickly. Erratic movement (a) ERA: an intense, extremely quick, zig-zag-like locomotion. Air gulping (b) A-G: being an anabantoid fish, Macropodus from time to time swims to the surface and gulps air. Picking (b) PIC: oriented movement, the jaw is streched out to grasp small pieces of food or visible spots in the environment. Floating-under-surface (a) FLO: the immobile animal floates just beneath the surface, it holds position by beating its pectoral fins. Hanging-in-midwater (a) HIM: as FLO but at medium depth. Resting-on-bottom (a) RES: as FLO and HIM, but staying on the bottom of the tank. The anal or caudal fin touches the bottom. Oblique-plan-position (a) OBQ: the body axis of the immobile animal is inclined by 20--40 degrees from the horizontal plane. The dorsal, caudal and anal fins are closed, the pectoral fins are quickly fanning as in CRE. Freezing (a) FRZ: the fish is motionless, only the opercula and occasionally the eyes move. The ERA element was measured only in the 4th situation instead of PIC element since the former was completely absent in the other 3 situations while the latter was absent in the 4th one. The 3rd characters of designations of the behavior elements were changed according to which environment were the elements measured in.

Methods of genetic analysis

Prerequisites

A number of criteria have to be met before proceeding with the estimation of the components of phenotypic variance and genetic parameters and these are as follows /35/: 1) diploid segregation; 2) independent action of nonallelic loci, that is, no epistasis; 3) no multiple allelism; 4) fully inbred parental strains, that is, homozygozity at all loci in parents; 5) independent distribution of alleles among parental strains, that is, no gene dispersion or association; 6) additive action of genotype and environment, that is, no genotype-environment interaction; 7) Since we used parametric statistical tests the distribution of data should be normal and the variances should be homogeneous.

Criteria 1 and 4 are fulfilled if highly inbred strains of a diploid species are used. Our paradise fish strains had been sib-mated for 22 generations at the time of the experiment, theoretically leading to a high inbreeding coefficient (F) /26/. If, however, selection against homozygozity occurs, the real value of inbreeding could be lower than predicted. However, the biochemical /33, 52/ and behavioral /19, 34/ homogeneity of the strains seems to support the idea of the parental strains being highly inbred.

Criteria 6 and 7 can be tested partly in the same way since in the case of no genotype-environment interaction the phenotypic variances of non-segregating populations, that is, the variance of inbreds and F1 hybrids should be homogeneous /49/. This requirement can be tested with Bartlett's test, and according to Kirk /47/, with Pearson product-moment correlations between means and standard deviations, means and variances, and squared means and variances. The criterion of normality was tested by calculating skewness and kurtosis and by visual inspection of distributions. Criteria

6 and 7 may be fulfilled by applying adequate scale transformations. Transformations were chosen according to the recommendations of the BMDP '81 (Biomedical Data Processing) software and their adequacy tested according to Mather and Jinks /49/.

Prerequisites 2, 3 and 5 can be tested by a variance-covariance analysis proposed by Hayman /36/.

Analysis

The analysis of variance and the variance-covariance analysis of the diallel cross followed the methods of Hayman /35, 36/ described in detail by Crusio et al. /12/.

The diallel matrices on which the analysis was applied were arranged as follows:

		fathers		
		U	P	C
mothers	U	UU	UP	UC
	P	PU	PP	PC
	C	CU	CP	CC

According to our design we had 5 such matrices (replications or blocks). Each cell of each matrix contained a mean score of 4 or 5 individuals.

First, we applied Hayman's ANOVA of diallel crosses, in which the (a) item tests primarily for additive variation, the (b) item tests for dominance effects, which can further be divided into 3 variance components: (b1) tests for directional dominance, (b2) for additional dominance effects owing to unequal strain-distributions of dominant alleles, and finally, (b3) for residual dominance effects. The latter cannot be tested in a 3x3 diallel cross. Item (c) tests for general maternal (reciprocal) effects and (d) tests for special maternal effects, that is the maternal effects that occure only in certain crosses. Further, between-cell variance (Between-cell) and between blocks variance (Blocks) can be estimated. The interaction between the main effects and the blocks were then calculated /12/, which let us estimate the significance of each item. The interaction of the between-cell variance with the blocks estimates the (E) environmental variance.

Second, a variance-covariance analysis was conducted. In this part we leave aside possible reciprocal differences, therefore, we average over reciprocals. In short, we calculated the variance (Vr) of the entries on each row (array) for the blocks separately and calculated the covariance (Wr) for each array entry with the corresponding non-recurrent parent so that one pair of Wr,Vr values is obtained for each array. If assumptions 2, 3 and 5 underlying a diallel-cross analysis are fulfilled, that is no epistasis, no multiple allelism and independent distribution of alleles

among parents, the linear regression of Wr on Vr should have a slope of unity when there is present some dominance /49/.

The variance-covariance analysis allow us to estimate several genetic parameters (e.g. /12/), such as, D: additive genetic variance; H1 and H2: dominance variance; F, which indicates whether dominant or recessive alleles are in excess, its value is positive if there are more dominant allels than recessives present in the inbred lines, and negative if the opposite is true; $(\mathrm{Sum}\ (h))^2$: the square of the mean deviation of the F1's from their midparent values. Its value is high when all the h's have the same sign, that is, in the case of directional dominance; 1/4(D-H1): The intercept of Wr,Vr regression line on the y axis, if $D > H1$ it is above the origo (partial dominance), if $D < H1$ it is below the origo (over dominance). The significance of the genetical parameters listed above were estimated according to Hayman's /36/ theoretical SE's. To estimate directional dominance Spearman rank correlation between Wr+Vr value and Pr (phenotypical value) of strain r was calculated (r(W+V,P)). Significant correlation indicates the presence of directional dominance. The sign of such a correlation is opposite to the direction of the dominance. b(W,V): the slope of the Wr,Vr regression line. It should not differ significantly from 1 when the assumptions are met. $(H/D)^{0.5}$: degree of dominance; C(h/d): the mesure of consistency of the ratio (u-v)h/d over all loci (where u is the frequency of dominant alleles and v is the frequency of recessive alleles). If this ratio is consistent the value of C(h/d) is 1, however, if the ratios vary independently over loci its value is 0; u/v: the ratio of dominant alleles to recessives; k: the minimum number of effective factors (segregation units) that show dominance. k seriously underestimates the real number of effective factors when dominance is ambidirectional, and even in the case of directional dominance k still should be considered as a minimum estimate /49/. h^2n: narrow heritability, the ratio of additive genetic variance and total phenotypic variance; h^2b: broad heritability or degree of genetic determination, the ratio of genetic variance and total phenotypic variance. Dominance order: the r inbred lines can be arranged according to the order of their Wr+Vr values, small Wr+Vr value indicates high dominance compared to the other lines and vica-versa.

Results

The behavioral data of the 9 populations are summarized in Tables 1, 2, 3, and 4. The scale transformations and investigation of normality and homogeneity of variances are summarized in tables 5—8 and in 5(a)—8(a). The details of results of the genetic analysis can be studied by perusing Tables 9—12 (variance analysis); 13—16 and 13(a)—16(a) (variance-covariance analysis).

Only the more important findings are described and discussed in the text. Generally, appropriate scale transformations were found for most of the variables.

The presence of genetic variance can be demonstrated readily by the significancy of the "Between-cell" item. The non-significant "Blocks" items

Table 1

Raw scores (means and standard deviations) for the behavior of the 9 populations of the diallel cross measured in the familiar environment (Mean above, SD below)

	UU	UP	UC	PU	PP	PC	CU	CP	CC
ES1	14.2 14.6	11.4 19.5	20.2 26.3	13.2 27.1	3.6 10.9	3.2 14.4	11.2 22.1	4.7 14.2	13.9 19.2
SW1	6.3 5.1	4.7 6.2	4.5 7.5	5.1 5.9	4.3 6.3	1.8 3.0	3.0 3.5	4.1 5.9	3.1 3.5
MO1	47.7 15.1	44.9 19.7	23.8 19.2	35.9 20.9	40.7 22.7	7.8 10.0	30.5 21.8	22.2 21.9	32.9 25.1
ST1	0.10 0.31	2.38 8.53	1.83 4.05	0.33 0.97	1.05 1.96	1.03 2.37	2.36 3.03	0.79 2.50	0.0 0.0
CR1	0.20 0.89	0.05 0.22	1.97 6.13	0.29 0.90	1.75 6.15	1.79 4.70	1.36 4.53	0.92 3.13	1.24 2.91
PI1	1.3 1.7	2.1 3.0	0.8 1.5	0.5 0.8	1.6 2.4	0.1 0.2	1.7 2.6	1.0 2.3	0.6 1.3
AG1	5.6 2.5	3.7 2.8	2.8 2.8	3.9 2.3	3.1 2.0	0.8 1.5	4.3 5.2	1.5 1.5	3.1 3.4
FL1	5.2 8.0	2.4 4.0	2.7 4.5	3.5 5.0	2.6 5.3	1.2 3.5	2.3 5.1	1.4 2.5	1.7 3.5
HI1	8.8 6.7	13.8 11.9	9.1 12.7	12.7 18.1	14.9 10.3	8.5 16.3	14.3 16.5	13.5 16.7	10.2 16.0
RE1	7.1 7.1	7.7 7.3	8.2 9.2	6.2 8.0	15.1 22.3	17.2 18.8	7.7 7.3	12.6 20.6	12.1 23.9
OB1	0.20 0.62	0.52 2.40	0.76 2.53	0.0 0.0	0.0 0.0	0.30 1.43	3.46 10.48	0.38 1.84	1.24 2.82
FR1	9.1 14.5	11.2 18.3	26.2 30.2	22.1 31.3	15.8 24.6	56.7 33.4	22.8 27.7	38.5 41.1	23.2 35.6
Sample sizes	20	21	29	21	20	33	22	24	17

Table 2

Raw scores (means and standard deviations) for the behavior of the 9 populations of the diallel cross measured in the open-field (Mean above, SD below)

	UU	UP	UC	PU	PP	PC	CU	CP	CC
ES2	38.0	32.3	31.7	18.0	13.2	13.0	26.0	12.1	6.6
	19.1	17.2	19.9	13.1	14.8	13.7	14.7	12.8	9.8
SW2	25.2	27.0	12.5	17.0	20.0	6.8	13.9	14.7	8.1
	11.7	14.9	8.9	8.5	10.7	6.2	7.2	12.7	8.4
MO2	28.1	24.7	23.0	33.0	34.9	11.1	31.1	15.8	24.9
	13.6	12.5	10.6	10.5	17.6	14.2	12.9	13.1	17.6
ST2	1.6	3.8	9.0	10.6	7.6	27.1	8.7	25.4	5.9
	3.1	4.3	8.8	12.0	12.3	16.1	7.9	15.2	8.3
CR2	2.3	8.0	19.4	16.0	19.3	30.5	15.3	22.0	38.0
	4.4	10.3	18.3	11.6	21.0	16.7	11.8	16.8	20.7
PI2	0.7	0.0	0.0	0.6	0.5	0.1	0.0	0.0	0.4
	1.6	0.0	0.0	2.2	1.5	0.3	0.2	0.0	1.5
AG2	12.2	8.5	6.5	7.5	6.5	3.9	10.6	4.8	5.8
	3.2	2.4	2.8	1.9	2.6	2.0	3.5	2.3	2.0
FL2	2.3	2.2	1.0	2.3	1.8	1.4	1.4	1.8	9.6
	1.7	1.6	1.5	3.5	3.3	2.5	2.0	3.2	16.2
HI2	0.70	0.29	0.03	0.19	0.50	0.21	0.41	0.21	0.24
	1.13	0.56	0.19	0.51	1.05	0.78	0.73	0.59	0.75
RE2	0.30	0.10	0.48	0.43	0.30	0.18	0.36	1.50	0.18
	1.13	0.30	0.99	1.17	0.80	0.64	0.73	6.31	0.73
OB2	0.3	0.3	1.4	1.4	1.2	6.7	1.0	2.8	4.6
	0.6	0.9	2.3	2.0	1.5	9.2	1.5	3.7	7.5
FR2	0.0	0.0	0.52	0.0	0.40	2.21	0.59	2.92	0.82
	0.0	0.0	2.79	0.0	1.19	9.31	1.65	6.21	2.43
OIT	54.0	36.5	24.9	37.2	40.3	26.7	29.6	30.4	35.2
	85.0	17.1	12.3	17.0	15.6	9.0	10.1	12.6	16.7
OUT	48.2	77.2	35.4	60.2	63.3	25.5	36.4	38.1	20.8
	28.3	33.8	18.8	23.4	25.6	18.1	19.2	21.2	15.0
OST	138.3	117.6	82.6	81.5	70.3	48.8	66.9	58.4	32.8
	51.3	53.8	48.0	47.5	47.6	38.3	21.3	36.6	17.3
LAT	4.0	10.1	23.4	13.4	17.4	38.3	20.5	38.5	70.6
	3.7	8.9	10.9	6.3	8.6	34.6	10.0	17.9	54.6

Sample sizes as in Table 1

Table 3

Raw scores (means and standard deviations) for the behavior of the 9 populations of the diallel cross measured in the small unfamiliar aquarium (Mean above, SD below)

	UU	UP	UC	PU	PP	PC	CU	CP	CC
ES3	38.7	64.0	38.2	41.6	32.2	26.8	26.8	17.0	4.6
	17.3	17.8	23.2	19.2	22.8	21.4	19.6	15.0	6.2
SW3	12.8	10.1	4.6	8.8	14.4	4.5	5.0	4.9	1.3
	8.2	7.2	4.9	7.2	10.4	5.1	5.4	7.3	3.6
MO3	42.8	17.6	35.3	26.8	34.9	32.7	47.7	30.9	31.2
	17.7	14.5	16.6	13.1	16.3	17.9	20.3	15.9	23.4
ST3	0.5	1.9	9.3	8.9	9.4	15.9	9.5	19.8	0.8
	1.4	4.2	11.4	13.7	17.7	17.9	13.3	18.8	1.2
CR3	0.4	2.7	8.2	10.0	5.7	12.6	6.2	16.5	38.3
	1.0	5.2	9.9	10.1	5.5	10.2	4.3	7.3	22.6
PI3	0.8	0.0	0.2	0.1	0.0	0.1	0.0	0.0	0.1
	3.6	0.0	0.6	0.4	0.0	0.4	0.0	0.0	0.5
AG3	12.1	10.7	6.2	9.2	8.4	4.2	9.1	5.3	5.3
	4.4	2.3	2.2	2.4	1.8	2.1	3.7	2.9	4.3
FL3	1.25	1.33	0.17	0.62	0.85	0.15	0.64	0.25	0.88
	1.52	1.56	0.38	1.24	1.14	0.44	0.79	0.74	2.26
HI3	1.00	0.33	0.28	0.24	0.30	0.21	0.46	0.21	1.06
	1.38	0.58	0.53	0.44	0.66	0.65	0.74	0.72	1.56
RE3	0.55	0.67	1.28	1.10	0.95	2.97	1.23	1.96	2.18
	0.89	1.02	1.91	1.22	1.61	2.56	1.48	2.58	4.08
OB3	0.25	0.24	1.28	1.00	0.60	0.91	0.59	1.71	1.06
	0.64	0.77	1.79	1.90	1.00	1.33	1.05	3.50	1.98
FR3	0.0	0.05	0.62	0.33	0.15	4.52	0.77	6.42	18.24
	0.0	0.22	1.76	1.07	0.67	12.02	2.20	17.04	27.13

Sample sizes as in Table 1

Table 4

Raw scores (means and standard deviations) for the behavior of the 9 populations of the diallel cross measured in the small unfamiliar aquarium with the presence of a rotating disc above the aquarium (Mean above, SD below)

	UU	UP	UC	PU	PP	PC	CU	CP	CC
ES4	65.1	52.1	50.1	33.0	18.9	35.9	33.5	27.5	8.6
	17.3	20.8	29.1	28.6	21.9	28.0	22.8	24.0	14.0
SW4	4.8	4.9	2.0	2.1	3.3	3.8	3.1	2.1	0.4
	4.4	6.5	3.1	3.7	4.6	5.2	4.3	3.5	1.3
MO4	18.9	22.0	13.5	17.9	25.7	16.5	17.2	14.3	18.3
	9.7	11.0	10.0	18.7	18.8	14.5	11.7	12.6	18.5
ST4	0.7	3.2	9.9	3.2	12.0	15.6	13.0	17.5	1.8
	1.3	5.5	13.5	3.9	16.4	18.1	11.2	17.9	3.5
ER4	0.3	0.4	0.4	0.6	0.9	0.5	0.8	0.6	0.2
	0.7	1.2	0.7	1.3	1.3	1.1	1.3	1.0	0.4
CR4	2.7	4.0	3.1	9.2	9.7	7.3	5.1	8.5	18.3
	5.7	6.1	6.6	15.8	7.2	10.7	4.0	12.5	19.1
AG4	10.2	8.0	5.5	5.0	5.8	4.6	7.7	4.1	3.6
	5.0	3.7	3.4	3.8	3.5	3.1	4.6	3.8	3.7
FL4	0.80	2.28	0.66	0.81	1.50	0.64	0.36	0.29	1.06
	0.95	2.88	0.86	1.44	2.09	0.96	0.95	1.23	2.36
HI4	0.15	0.38	0.03	0.05	0.0	0.18	0.05	0.08	0.35
	0.49	0.97	0.19	0.22	0.0	0.47	0.21	0.28	1.06
RE4	2.9	2.7	2.3	3.1	5.8	3.2	1.8	2.7	1.2
	3.4	2.1	2.8	3.1	9.0	3.0	1.9	3.0	2.1
OB4	1.3	2.8	0.8	1.9	2.7	2.6	3.3	2.0	6.0
	1.4	5.7	1.7	2.2	2.8	3.7	5.2	3.1	5.9
FR4	1.5	4.0	12.8	27.0	18.5	11.0	17.7	23.6	42.9
	3.6	5.6	19.5	36.6	26.6	17.3	22.0	32.0	40.0

Sample sizes as in Table 1

Table 5

The data distribution for the variables measured in the familiar environment

	Transformation	Distribution	
		(Skewness)	(Kurtosis)
ES1	LN(1+X)	0.43 t(43)=1.182	2.96 t(43)=0.058
SW1	no	0.80 t(43)=2.200 x	3.52 t(43)=0.710
MO1	$(0.01+X)^{0.5}$	0.11 t(43)=0.317	2.77 t(43)=0.315
ST1	LN(1+X)	0.76 t(43)=2.090 x	4.27 t(43)=1.732
CR1	LN(1+X)	0.85 t(43)=2.339 x	2.45 t(43)=0.754
PI1	LN(1+X)	1.00 t(43)=2.726 xx	3.64 t(43)=0.879
AG1	$(0.01+X)^{0.5}$	0.71 t(43)=1.948	5.05 t(43)=2.807 xx
FL1	LN(1+X)	0.59 t(43)=1.628	3.33 t(43)=0.453
HI1	no	0.48 t(43)=1.315	3.25 t(43)=0.347
RE1	LN(1+X)	-0.19 t(43)=0.508	3.07 t(43)=0.090
OB1	LN(1+X)	0.92 t(43)=2.531 x	4.22 t(43)=1.689
FR1	$(0.01+X)^{0.5}$	0.16 t(43)=0.441	2.58 t(43)=0.574

$^{x}P < 0.05$; $^{xx}P < 0.01$; $^{xxx}P < 0.001$

Table 5(a)

Homogeneity of variances for the variables measured in the familiar environment

	Bartlett-test	$r(M,s^2)$	$r(M,s)$	$r(M^2,s)$
ES1	6.593	0.24	0.33	0.23
SW1	11.440	0.50	0.62	0.51
MO1	5.955	0.01	-0.00	-0.15
ST1	-	0.32	0.39	0.11
CR1	12.840	0.96^{xxx}	0.94^{xxx}	0.86^{xxx}
PI1	24.301^{xx}	0.73^{x}	0.75^{x}	0.70^{x}
AG1	11.764	0.17	0.18	0.13
FL1	14.418	0.13	0.02	-0.08
HI1	12.366	-0.19	-0.27	-0.30
RE1	8.074	0.24	0.19	0.25
OB1	-	0.96^{xxx}	0.95^{xxx}	0.89^{xxx}
FR1	7.212	-0.42	-0.49	-0.49

$^{x}P < 0.05$; $^{xx}P < 0.01$; $^{xxx}P < 0.001$

Table 6

The data distribution for the variables measured in the open-field

	Transformation	Distribution (Skewness)		Distribution (Kurtosis)	
ES2	$(0.01+X)^{0.5}$	-0.20	t(43)=0.546	3.00	t(43)=0.006
SW2	$(0.01+X)^{0.5}$	0.36	t(43)=0.998	2.72	t(43)=0.385
MO2	no	0.17	t(43)=0.457	3.24	t(43)=0.325
ST2	$(0.01+X)^{0.5}$	0.21	t(43)=0.582	2.63	t(43)=0.511
CR2	$(0.01+X)^{0.5}$	-0.10	t(43)=0.281	3.69	t(43)=0.947
PI2	LN(1+X)	1.35	t(43)=3.716***	4.89	t(43)=2.583*
AG2	no	0.79	t(43)=2.159*	4.56	t(43)=2.140*
FL2	LN(1+X)	0.15	t(43)=0.421	2.09	t(43)=1.248
HI2	LN(1+X)	0.70	t(43)=1.903	2.98	t(43)=0.027
RE2	LN(1+X)	0.86	t(43)=2.364*	3.46	t(43)=0.634
OB2	LN(1+X)	0.51	t(43)=1.397	3.30	t(43)=0.413
FR2	LN(1+X)	0.26	t(43)=0.717	4.05	t(43)=1.434
OIT	1/X	2.88	t(43)=7.892***	16.67	t(43)=18.715***
OUT	$(0.01+X)^{0.5}$	0.02	t(43)=0.053	2.12	t(43)=1.203
OST	LN(1+X)	-0.41	t(43)=1.127	2.68	t(43)=0.436
LAT	LN(1+X)	0.60	t(43)=1.654	3.53	t(43)=0.726

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Table 6(a)

Homogeneity of variances for the variables measured in the open-field

	Bartlett-test	$r(M,s^2)$	$r(M,s)$	$r(M^2,s)$
ES2	8.509	-0.13	-0.14	-0.15
SW2	9.178	-0.50	-0.48	-0.41
MO2	9.280	0.33	0.26	0.26
ST2	7.632	-0.16	-0.20	-0.36
CR2	10.207	-0.12	-0.21	-0.25
PI2	-	0.93***	0.96***	0.91***
AG2	13.422	0.54	0.55	0.55
FL2	1.989	-0.03	-0.03	-0.06
HI2	13.942	0.87**	0.84**	0.78*
RE2	7.579	0.67*	0.71*	0.74*
OB2	22.855**	0.69*	0.70*	0.70*
FR2	-	0.96***	0.97***	0.87***
OIT	53.856***	0.74*	0.86**	0.89**
OUT	17.988*	-0.19	-0.18	-0.19
OST	11.740	0.02	0.13	0.09
LAT	19.431*	-0.39	-0.47	-0.39

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Table 7

The data distribution for the variables measured in the small unfamiliar aquarium

	Transformation	Distribution			
		(Skewness)		(Kurtosis)	
ES3	$(0.01+X)^{0.5}$	0.05	$t(43)=0.141$	2.58	$t(43)=0.577$
SW3	$(0.01+X)^{0.5}$	0.15	$t(43)=0.400$	1.70	$t(43)=1.784$
MO3	no	-0.16	$t(43)=0.446$	3.13	$t(43)=0.181$
ST3	LN(1+X)	-0.28	$t(43)=0.759$	3.48	$t(43)=0.664$
CR3	$(0.01+X)^{0.5}$	0.05	$t(43)=0.157$	3.94	$t(43)=1.284$
PI3	LN(1+X)	2.43	$t(43)=6.651^{***}$	9.55	$t(43)=8.974^{***}$
AG3	$(0.01+X)^{0.5}$	-0.87	$t(43)=2.384^{*}$	5.87	$t(43)=3.925^{***}$
FL3	LN(1+X)	0.08	$t(43)=0.228$	1.98	$t(43)=1.402$
HI3	LN(1+X)	0.67	$t(43)=1.846$	4.02	$t(43)=1.399$
RE3	LN(1+X)	-0.02	$t(43)=0.067$	2.58	$t(43)=0.572$
OB3	LN(1+X)	0.83	$t(43)=2.273^{*}$	2.87	$t(43)=0.182$
FR3	LN(1+X)	0.65	$t(43)=1.787$	7.07	$t(43)=5.573^{***}$

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

Table 7(a)

Homogeneity of variances for the variables measured in the small unfamiliar aquarium

	Bartlett-test	$r(M,s^2)$	$r(M,s)$	$r(M^2,s)$
ES3	6.464	0.10	0.09	0.01
SW3	3.157	0.26	0.26	0.23
MO3	8.115	0.30	0.38	0.35
ST3	17.351^{*}	0.51	0.57	0.50
CR3	18.951^{*}	0.62	0.51	0.61
PI3	-	0.97^{***}	0.99^{***}	0.95^{***}
AG3	15.547^{*}	-0.50	-0.49	-0.47
FL3	11.682	0.69^{*}	0.73^{*}	0.60
HI3	13.419	0.49	0.46	0.40
RE3	9.533	-0.05	-0.07	-0.04
OB3	4.351	0.53	0.61	0.52
FR3	-	0.95^{***}	0.96^{***}	0.90^{***}

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

Table 8

The data distribution for the variables measured in the small unfamiliar aquarium with the presence of a rotating disc above the aquarium

	Transformation	Distribution			
		(Skewness)		(Kurtosis)	
ES4	$(0.01+X)^{0.5}$	-0.29	t(43)=0.788	2.64	t(43)=0.483
SW4	LN(1+X)	0.80	t(43)=2.183	3.46	t(43)=0.634
MO4	$(0.01+X)^{0.5}$	0.10	t(43)=0.269	2.82	t(43)=0.249
ST4	LN(1+X)	-0.10	t(43)=0.274	2.77	t(43)=0.318
ER4	LN(1+X)	0.41	t(43)=1.136	2.56	t(43)=0.596
CR4	LN(1+X)	-0.07	t(43)=0.202	2.52	t(43)=0.654
AG4	no	0.57	t(43)=1.550	2.76	t(43)=0.332
FL4	$(0.01+X)^{0.5}$	0.62	t(43)=1.687	4.16	t(43)=1.590
HI4	LN(1+X)	1.29	t(43)=3.538***	4.90	t(43)=2.601*
RE4	LN(1+X)	-0.11	t(43)=0.307	2.06	t(43)=1.266
OB4	LN(1+X)	0.16	t(43)=0.429	2.33	t(43)=0.914
FR4	LN(1+X)	-0.05	t(43)=0.128	2.66	t(43)=0.463

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

Table 8(a)

Homogeneity of variances for the variables measured in the small unfamiliar aquarium with the presence of a rotating disc placed above the aquarium

	Bartlett-test	$r(M,s^2)$	$r(M,s)$	$r(M^2,s)$
ES4	13.825	-0.03	-0.11	-0.24
SW4	19.466*	0.63	0.65	0.60
MO4	9.343	-0.55	-0.56	-0.55
ST4	7.598	0.53	0.59	0.48
ER4	3.860	0.00	0.01	-0.02
CR4	8.859	0.46	0.45	0.54
AG4	7.854	0.42	0.44	0.44
FL4	9.186	0.18	0.24	0.25
HI4	-	0.89**	0.91***	0.87**
RE4	6.298	0.56	0.52	0.60
OB4	14.424	0.87**	0.84**	0.83**
FR4	10.449	0.35	0.41	0.32

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

found for many variables indicate that in most cases the behavior did not change over blocks, that is accross recording sessions. Interestingly, however, this was not the case fo SW1, SW2, SW3 and SW4.

Among the variables measured in the first environment (familiar small aquarium) additive genetic variance was found for ES1, SW1, MO1, AG1, FL1, OB1 and FR1. Directional dominance (b1) was detected for MO1 and ST1 by the variance analysis. The variance-covariance analysis supported this finding only for MO1 ($(\mathrm{Sum}(h))^2$ was significant and r(W+V,P) almost reached significance). Additional dominance variance (b2) was detected for MO1, ST1, AG1 and FR1. All dominance effects seem to be due to overdominance. Average maternal effect (c) was found significant for FR1 and special maternal effect for ST1 only. Genetic variance was not detected for CR1, PI1, HI1 and RE1 at all. The sign of F proved to be negative for almost all variables, which means that there are more recessive alleles in the parental strains than dominant ones. However, the value of F proved to be significantly less than zero only for SW1, FL1 and OB1. Violation of the assumptions were indicated for ST1 only. The estimated numbers of effective factors (k) were below 1. In MO1 the estimated number of effective factors can be considered as a real minimum estimate, since here we found directional dominance. In ST1, k probably underestimates the real number of genes, since there may be ambidirectional dominance present and it can be biased because criteria 2, 3 and 5 were not fulfilled. Having been ambidirectional dominance found for AG1 too, k must underestimate the number of effective factors. Further, for AG1 we found significant additive genetic variance, thus the number of loci that do not show dominance should be added to the k value to get the total number of effective factors. This may not apply to MO1 where the (a) item might not be free from dominance effects (b2), so we may accept the lack of additive genetic effects revealed by non-significant D of the variance-covariance analysis.

The genetic analysis for the variables measured in the second situation (open-field) did not reveal any genetic variance for PI2, HI2, RE2 and OIT. Among the other variables all but one (FL2) proved to show significant additive genetic variance. Directional dominance was revealed by the variance analysis for ST2, AG2 and FL2. The variance-covariance analysis supported this finding obviously for FL2 only. Additional dominance was found for MO2, ST2 and OUT. The level of dominance among these variables seems to be intermediate for OUT and AG2, and for the other variables overdominance was found. Significant average reciprocal effect (c) was obtained

Table 9

F-values obtained in the Hayman analysis of variance.
Familiar environment

	a	b	b1	b2	c	d	Between Cell	Blocks
ES1	4.81^{*}	2.28	1.62	2.63	0.48	1.34	3.18^{**}	0.52
SW1	6.15^{*}	0.88	2.14	0.11	0.17	5.02	1.62	5.61^{***}
MO1	9.86^{**}	16.22^{***}	40.08^{**}	9.45^{**}	3.21	0.30	9.14^{***}	0.692
ST1	0.04	12.95^{***}	10.04^{*}	14.26^{**}	0.16	16.1^{*}	3.58^{*}	1.66
CR1	0.84	1.48	0.68	1.50	1.50	0.01	0.59	1.69
PT1	2.34	1.66	3.08	1.40	1.73	0.40	1.67	1.02
AG1	16.44^{***}	5.74^{*}	$7.34^{(*)}$	4.74^{*}	1.96	0.07	7.21^{***}	0.35
FL1	5.05^{*}	0.38	2.10	0.07	0.05	1.51	1.57	2.90^{*}
HI1	0.43	0.88	0.03	1.62	3.18	0.02	1.10	0.89
RE1	1.28	0.91	0.01	1.17	0.13	2.17	1.0	0.86
OB1	9.90^{**}	0.38	0.06	0.60	0.64	1.28	$2.06^{(*)}$	2.05
FR1	8.97^{***}	7.32^{**}	$6.65^{(*)}$	8.31^{*}	9.27^{**}	3.42	7.88^{***}	2.81^{*}

$^{(*)}P < 0.1$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$
The degrees of freedom for the items are the following: a(2,8); b(3,12); b1(1,4); b2(2,8); c(2,8); d(1,4); Betw(8,32); Blo(4,32)

Table 10

F-values obtained in the Hayman analysis of variance.

Open-field aquarium

	a	b	b1	b2	c	d	Between Cell	Blocks
ES2	16.48**	2.95$^{(*)}$	7.54$^{(*)}$	1.15	2.55	1.19	7.66***	1.55
SW2	37.16***	0.65	0.54	0.76	5.13	0.01	9.72***	5.42**
MO2	5.59*	8.01**	2.64	24.15***	10.94**	0.27	6.69***	0.40
ST2	10.85**	30.34***	62.06**	17.17**	1.51	7.74*	12.20***	2.92*
CR2	56.41***	0.33	0.11	0.48	3.43$^{(*)}$	0.46	12.83***	0.93
PI2	0.56	1.46	1.42	1.82	2.31	0.92	1.32	3.31
AG2	39.62***	3.01$^{(*)}$	10.23*	0.99	16.62**	23.33**	19.55***	3.66*
FL2	2.41	5.65*	14.31*	3.88$^{(*)}$	0.85	1.22	4.25**	1.91
HI2	1.62	0.85	2.76	0.32	0.98	2.22	1.17	1.48
RE2	0.00	1.56	1.62	1.53	0.50	1.04	0.76	1.88
OB2	7.56*	2.20	0.39	2.45	4.54*	0.01	5.11***	1.08
FR2	5.85*	1.77	0.70	2.41	1.66	0.23	2.26*	0.57
OIT	1.53	0.06	0.08	0.04	0.56	0.25	0.79	2.30$^{(*)}$
OUT	155.18***	6.23**	1.48	7.04*	3.68$^{(*)}$	0.57	25.45***	1.32
OST	32.46***	0.33	0.72	0.24	3.46$^{(*)}$	0.15	10.39***	3.57*
LAT	118.03 ***	1.85	2.15	0.93	1.84	5.09$^{(*)}$	22.00***	3.9 *

Significance levels and degrees of freedoms as in Table 9

Table 11

F-values obtained in the Hayman analysis of variance. Small unfamiliar aquarium

	a	b	b1	b2	c	d	Between Cell	Blocks
ES3	85.25***	3.73*	15.51*	0.52	6.29*	3.62	17.26***	0.14
SW3	45.93***	2.96(*)	18.95*	0.74	0.38	0.07	15.44***	6.68***
MO3	1.39	4.83*	0.85	5.99*	4.88*	0.02	3.03*	1.10
ST3	20.75***	10.99***	92.54***	4.90*	0.96	16.19*	7.67***	1.12
CR3	75.95***	4.09*	2.20	4.49*	6.57*	51.58***	28.74***	1.02
PI3	2.21	0.07	0.09	0.03	1.84	1.05	0.85	1.79
AG3	21.01***	1.37	0.36	2.28	6.47*	12.71*	9.83***	0.58
FL3	4.50*	4.45*	5.85(*)	3.02	2.13	21.90**	4.87***	2.19(*)
HI3	3.20(*)	1.94	3.66	0.27	0.00	0.52	1.69	0.20
RE3	3.14(*)	3.29 (*)	6.85(*)	2.43	3.13(*)	3.53	3.22(*)	1.25
OB3	2.16	0.71	0.89	0.67	0.41	2.35	1.33	1.13
FR3	25.90***	2.38	1.58	2.99	0.25*	0.09	7.91***	1.20

Significance levels and degrees of freedoms as in Table 9

Table 12

F-values obtained in the Hayman analysis of variance.
Small unfamiliar aquarium with rotating disc placed above

	a	b	b1	b2	c	d	Between Cell	Blocks
ES4	33.18***	5.50*	10.84*	3.39(*)	13.93**	1.67	13.94***	0.68
SW4	8.81**	2.69(*)	0.46	2.79	0.66	6.77(*)	4.49***	9.25***
MO4	5.75*	0.20	0.55	0.05	0.46	2.17	1.41	4.54**
ST4	9.33**	14.22***	12.29*	15.69**	3.72(*)	0.30	7.92***	0.30
CR4	8.23*	2.20	2.87	1.14	13.62***	0.00	5.65***	0.71
ER4	2.38	0.99	0.10	1.21	0.74	0.18	1.05	0.08
AG4	15.52**	0.70	0.68	0.71	0.85	26.00**	5.18***	4.23**
FL4	4.55*	1.34	1.30	1.35	3.87(*)	6.27(*)	2.76*	1.94
HI4	0.22	1.36	0.11	1.90	0.93	3.02	1.07	0.51
RE4	5.97*	0.43	0.19	0.52	0.03	0.01	1.48	1.83
OB4	3.70(*)	1.82	1.51	2.66	2.30	6.11(*)	2.61*	0.29
FR4	24.57***	1.86	0.07	2.23	6.05**	5.14(*)	7.49***	1.73

Significance levels and degrees of freedoms as in Table 9

for MO2, AG2 and OB2, and special reciprocal effect (d) for ST2 and AG2. The criteria were significantly violated in CR2 and OST only. The number of effective factors were estimated low again. In ES2 the estimated number must be considered as inappropriate since there was no significant dominance variance found. Being the dominance ambidirectional in MO2, k must be an underestimation. In ST2 we could not decide whether the dominance was directional or not, thus the estimated number of effective factors may be considered as a real minimum estimate. The same could apply to AG2, however, here the criteria of the distribution being normal was not fulfilled, thus the genetic estimations could be biased. In the case of FL2 the picture is quite clear: it seems that directional dominance explains the whole genetic variance found, that is, there is no other genetic effects. Being the dominance directional and being no other loci involved than those of showing dominance, k must be considered as an appropriate minimum estimate of the number of effective factors. The estimated number of effective factors obtained for LAT cannot be regarded as appropriate since there was no dominance found. Heritabilities, in general, were estimated greater than those of obtained for the variables of the first situation.

The genetic analysis of the variables measured in the 3rd situation (small unfamiliar aquarium) did not reveal genetic variance for PI3, HI3 and OB3. Excluding these 3, significant additive genetic variance was obtained for all but one (MO3) variable. The variance analysis revealed directional dominance for ES3, SW3, ST3 and possibly for FL3 and RE3. However, among these variables the correlation between Wr+Vr and P reached significance only in FL3. Additional dominance variance (b2) was estimated significant for MO3, ST3 and CR3. Overdominance was revealed for ST3 and FL3, and the dominance effects found for the other variables proved to be intermediate. Average reciprocal effect (c) was found for ES3, MO3, CR3, AG3 and FR3, and special (d) for ST3, CR3, AG3 and FL3. The prerequisites were significantly violated in CR3 only. The estimated numbers of effective factors were low again. In the case of ES3 the prerequisite of directional dominance revealed by the variance analysis may not hold, thus the value obtained as the number of effective factors should be regarded as an underestimate. The same applies to SW3 and ST3. However, the number of effective factors estimated for FL3 should be considered as an appropriate minimum estimate. There was no dominance found for FR3, so the k value must be ir-

Table 13

The variance-covariance analysis. Familiar environment

	E	D	H1	H2	F	$(\Sigma h)^2$	1/4(D-H1)	r(W+V,P)	bW,V
ES1	0.52***	0.31***	0.18	0.17	-0.25	0.03	0.03	0.457	0.969
SW1	7.26***	1.93***	0.0	0.0	-1.47***	2.06**	1.02***	0.418	1.064
MO1	1.30***	0.48	5.82*	5.31	-1.57	3.97**	-1.33	0.493(*)	0.835
ST1	0.09**	0.03	0.30(*)	0.26(*)	0.09	0.08	-0.07	-0.262	-0.445***
CR1	....	....	....	....	....	....	....	.....	.....
PT1	....	....	....	....	....	....	....	.....	.....
AG1	0.18***	0.16**	0.39*	0.36*	-0.23	0.25*	-0.06	-0.146	0.901
FL1	0.22***	0.08***	0.0	0.0	-0.05***	0.01**	0.03***	0.335	0.831
HI1	....	....	....	....	....	....	....	.....	.....
RE1	....	....	....	....	....	....	....	.....	.....
OB1	0.08***	0.04***	0.0	0.0	-0.02***	0.0	-0.02*	0.660**	0.715
FR1	1.59**	0.0	7.44*	6.78*	-1.18	5.04*	-1.87	0.013	0.194

(*)P > 0.1; *P > 0.05; **P > 0.01; ***P > 0.001; (....)not calculated

Table 14

The variance-covariance analysis. Open-field aquarium

	E	D	H1	H2	F	$(\sum h)^2$	1/4(D-H1)	r(W+V,P)	bW,V
ES2	1.41***	4.19***	0.27	0.37	-0.39	0.58**	0.98**	-0.432	1.088
SW2	0.51***	1.67***	0.0	0.0	-0.48***	0.0	-0.42***	-0.196	1.372
MO2	45.75*	21.34	211.14*	179.42$^{(*)}$	26.80	56.49	-47.45	0.422	0.644
ST2	0.81*	0.22	6.48*	5.95*	-0.92	4.84**	-1.57	-0.213	0.685
CR2	0.82***	5.65***	0.0	0.0	0.60***	0.0	1.48***	-0.111	1.436*
PI2	....	....	....	....	....	....	....	.....	.....
AG2	1.79***	12.17***	2.01***	2.04***	-2.33***	2.33***	2.54***	0.403	1.139
FL2	0.15***	0.22***	0.59**	0.52**	0.26	0.30*	-0.09	0.571*	0.815
HI2	....	.....	....	....	....	....	....	.....	.....
RE2	....	....	....	....	....	....	....	.....	.....
OB2	0.15***	0.15***	0.04	0.03	-0.12**	0.0	0.03	0.424	1.446
FR2	0.08***	0.0	0.02	0.02	-0.03	0.0	-0.01	0.254	0.389
OIT	....	....	....	....	....	....	....	.....	.....
OUT	0.43***	3.30***	1.38*	1.12*	-1.26*	0.0	0.48	0.568*	0.754$^{(*)}$
OST	0.11***	0.55***	0.0	0.0	0.02***	0.0	0.15***	-0.186	1.424*
LAT	0.14***	1.55***	0.13***	0.14***	0.09***	0.19***	0.36***	-0.396	0.967

Significance levels as in Table 13

Table 15

The variance-covariance analysis. Small unfamiliar aquarium

	E	D	H1	H2	F	$(\Sigma h)^2$	1/4(D-H1)	r(W+V,P)	bW,V
ES3	1.01***	5.96***	1.76***	1.81***	-1.21***	2.40***	1.05***	-0.401	0.916
SW3	0.33***	2.82***	0.20**	0.22***	0.33***	0.29***	0.66***	0.422	1.215$^{(*)}$
MO3	133.56***	0.0	257.52*	210.45$^{(*)}$	21.80	0.0	-66.96	-0.028	1.066
ST3	0.42***	0.42***	1.99***	1.83***	0.12	1.47***	-0.39*	-0.128	0.776
CR3	0.49***	8.36***	1.08**	0.89*	2.23***	0.05	1.82***	0.535*	1.317*
PI3	....	....	....	....	....	....	....	.....	.....
AG3	0.14***	0.43***	0.06	0.06	-0.17**	0.0	0.09	-0.035	1.251$^{(*)}$
FL3	0.05***	0.01*	0.06**	0.06**	-0.03$^{(*)}$	·0.06***	-0.01	0.612*	0.919
HI3	....	....	....	....	....	....	....	.....	.....
RE3	0.09***	0.0	0.10$^{(*)}$	0.09$^{(*)}$	-0.03	0.05$^{(*)}$	-0.03	-0.453$^{(*)}$	0.839
OB3	....	....	....	....	....	....	....	.....	.....
FR3	0.24***	1.06***	0.30$^{(*)}$	0.26$^{(*)}$	0.31$^{(*)}$	0.09	0.19	0.778***	1.089

Significance levels as in Table 13

Table 16

The variance-covariance analysis. Small unfamiliar aquarium with a rotating disc placed above

	E	D	H1	H2	F	$(\Sigma h)^2$	1/4(D-H1)	r(W+V,P)	bW,V
ES4	1.32***	9.80***	3.81**	3.52*	3.93**	2.73**	1.50	-0.532*	1.006
SW4	0.18***	0.50***	0.06	0.05	0.22***	0.0	0.11*	0.032	1.663***
MO4	0.88***	0.34***	0.0	0.0	-0.09***	0.0	0.17***	0.204	1.151$^{(*)}$
ST4	0.35***	0.52***	1.81***	1.58***	0.36	0.79***	-0.32	-0.106	0.970
CR4	0.30***	0.72***	0.16*	0.17**	0.15**	0.25***	0.14*	0.473$^{(*)}$	1.410$^{(*)}$
ER4	....	....	....	....	....	....	....	.....	.....
AG4	3.78***	9.40***	0.0	0.0	1.42*	0.0	2.40**	0.028	1.167
FL4	0.13***	0.01	0.06	0.05	-0.03	0.0	-0.01	0.607	1.158
HI4	....	....	....	....	....	....	....	.....	.....
RE4	0.22***	0.16***	0.0	0.0	0.04$^{(*)}$	0.0	0.05$^{(*)}$	-0.014	1.459
OB4	....	....	....	....	....	....	....	.....	.....
FR4	0.46***	1.91***	0.38$^{(*)}$	0.31	0.86***	0.0	0.38	-0.261	1.120

Significance levels as in Table 13

Table 13(a)

The variance-covariance analysis. Familiar environment

	$(H/D)^{0.5}$	C(H/D)	u/v	k	h_n^2	h_b^2	Dominance order
ES1	0.75	0.0	0.31	0.15	0.40	0.46	U,P,F
SW1	....	....	....	....	0.15	0.15	.....
MO1	3.48	0.0	0.36	0.75	0.40	0.81	F.U.P
ST1	2.97	1.20	2.72	0.30	0.0	0.49	P.U.F
CR1	....	....	....	....	0.11	0.11	.....
PI1	....	....	....	....	0.08	0.10	.....
AG1	1.54	0.0	0.37	0.69	0.56	0.80	F,U,P
FL1	....	....	....	....	1.23	1.23	.....
HI1	....	....	....	....	0.0	0.02	.....
RE1	....	....	....	....	0.0	0.02	.....
OB1	....	....	....	....	0.28	0.28	.....
FR1	....	....	....	0.74	0.24	0.68	U,F,P

(....)not calculated

Table 14(a)

The variance-covariance analysis. Open-field aquarium

	$(H/D)^{0.5}$	C(H/D)	u/v	k	h_n^2	h_b^2	Dominance order
ES2	0.26	0.0	0.70	1.58	0.70	0.73	U,P,F
SW2	....	....	....	....	0.70	0.70	.....
MO2	3.15	0.52	1.50	0.32	0.15	0.64	U,F,P
ST2	5.49	0.0	0.44	0.82	0.32	0.90	U,P,F
CR2	....	.....	....	....	0.98	0.98	.....
PI2	....	.....	....	....	0.02	0.10	.....
AG2	0.41	0.0	0.62	1.14	0.88	0.94	P,F,U
FL2	1.65	1.10	2.16	0.58	0.04	0.55	U,P,F
HI2	....	....	....	....	0.02	0.04	.....
RE2	....	....	....	....	0.01	0.01	.....
OB2	0.49	0.0	0.08	....	0.54	0.57	U,F,P
FR2	....	....	....	....	0.19	0.25	.....
OIT	....	....	....	....	0.14	0.14	.....
OUT	0.65	0.0	0.54	....	1.02	1.14	F,U,P
OST	....	....	....	....	0.85	0.85	.....
LAT	0.29	0.0	1.23	1.40	1.05	1.10	F,P,U

Table 15(a)

The variance-covariance analysis. Small unfamiliar aquarium

	$(H/D)^{0.5}$	C(H/D)	u/v	k	h^2_n	h^2_b	Dominance order
ES3	0.54	0.0	0.69	1.33	0.91	1.02	U,P,F
SW3	0.27	0.0	1.57	1.34	0.90	0.94	F,U,P
MO3	....	....	....	....	0.07	0.36	P,F,U
ST3	2.18	0.23	1.14	0.80	0.25	0.74	P,U,F
CR3	0.36	0.89	2.18	0.05	1.07	1.14	U,P,F
PI3	....	....	....	....	0.07	0.07	.....
AG3	0.38	0.0	0.32	....	0.85	0.89	U,F,P
FL3	2.22	0.0	0.29	0.99	0.24	0.41	F,U,P
HI3	....	....	....	....	0.13	0.25	.....
RE3	....	....	....	0.55	0.16	0.33	U,F,P
OB3	....	....	....	....	0.12	0.12	.....
FR3	0.53	0.81	1.77	0.34	0.72	0.85	U,P,F

(....) not calculated

Table 16(a)

The variance-covariance analysis.

Small unfamiliar aquarium with a rotating disc placed above

	$(H/D)^{0.5}$	C(H/D)	u/v	k	h^2_n	h^2_b	Dominance order
ES4	0.62	1.16	1.95	0.78	0.70	0.90	P,U,F
SW4	0.34	1.72	4.79	....	0.33	0.36	.....
MO4	....	....	....	....	0.17	0.17	.....
ST4	1.86	0.52	1.45	0.50	0.26	0.77	U,P,F
CR4	0.47	0.0	1.57	1.41	0.50	0.58	U,P,F
ER4	....	....	....	....	0.0	0.06	.....
AG4	....	....	....	....	0.51	0.51	.....
FL4	3.21	0.0	0.18	....	0.11	0.19	F,U,P
HI4	....	....	....	....	0.0	0.06	.....
RE4	....	....	....	....	0.23	0.23	.....
OB4	0.97	0.0	1.87	0.98	0.11	0.24	.....
FR4	0.44	1.21	3.07	....	0.55	0.62	P,F,U

relevant. The heritabilities seem to be even higher than those estimated for the variables measured in the 2nd situation.

The genetic analysis for the variables measured in the 4th situation revealed no genetic variance for ER4 and HI4. Among the other variables all but one (OB4) proved to show additive genetic variance. Directional dominance was indicated for ES4 and ST4 by the variance analysis. However, the covariance-variance analysis supported this finding only for ES4 and found another variable (FL4) where the presence of directional dominance is probable. Significant general reciprocal effect (c) was obtained for ES4, CR4 and FR4 and special reciprocal effect (d) for AG4 only. The F values proved to be greater than zero in several variables, which means that there are more dominant genes present in the inbred lines than recessive ones. The criteria were seriously violated in the case of SW4 and may be slightly violated in MO4 and CR4. The number of effective factors was estimated lower than 1 for ES4. Being the dominance found to be directional, this value can be regarded as an appropriate minimum estimate. However, there was some additive genetic variance found, thus the total number of effective factors explaining the genetic part of the phenotypic variance must be greater than the number of loci showing dominance only. The k values obtained for ST4 can underestimate the number of effective factors showing dominance since the dominance found for this variable may be ambidirectional. In the cases of CR4 and OB4 no or very little dominance was indicated by the analysis, thus the values obtained for k must be regarded as irrelevant. The heritabilities, in general, seem to be slightly smaller than those obtained for the variables measured in the 2nd and 3rd situations.

Discussion

Studies in which the genetics of both the behavioral and the neural phenotype is investigated are rare (e.g. /14. 15/). One would argue that the behavior is so complex that we should expect polygenic inheritance for behavioral traits and for the underlying neural mechanisms.

However, the obvious complexity of behavior, cannot exclude the finding of "monogenic" or "oligogenic" inheritance since only the variable part of the genetic effects can be detected and consequently, if few strains are used the number of variable loci can be low.

The possibility of major gene effect, that is only a few variable

loci being involved in the inheritance, emerges for several behavioral elements measured in the 4 different environments. A similar result has been obtained in a classical cross analysis of paradise fish /19/.

The behavioral elements can be regarded as organized co-ordinated interactions of different muscles. These elements have proved to be units /19, 22/ in the sense that the correlations among them are changeable and these correlations depend on the situation in which the behavior is observed. In another sense they are also units, because they have identity and they are discrete, that is, there are no continuous transitions among them. The assumption of functional genetic units responsible for particular behavioral elements seems to be reasonable. Segregation can break down the genetic arrangements, the so-called recombination load /59/. The closer genes are linked, the lower is the probability of recombination, and the lower the load. Functionally related genes are frequently found to be located close to each other on the chromosome (/58/ pp. 585—587), and these functional units, as we suppose, may be equivalent to effective factors revealed by a genetic analysis. This would explain why behavior genetic studies (e.g. /2, 3, 19, 41, 54/) frequently find apparently monogenic inheritance of the elements of ethogram. However, the estimated monogenic inheritance, even if it is really monogenic, does not mean monogenic control, it only means that the genetic part of phenotypic variability is due to different alleles of one locus.

In general, it seems that behavioral elements measured in different situations do not represent the same phenes and their genetic background may be different too. We have previously found /19, 22/ that in different environments paradise fish show different behavioral strategies. We define behavioral strategy as a sort of composite variable based on correlations of the behavioral units and it can be regarded as a higher order character, which organizes the behavioral units /19/. In different environments animals may use different behavioral strategies, that is, the organization of the behavioral units may be different. If the genetic background of the behavioral strategies in paradise fish is variable, as we have previously found /19/, it can affect the genetic analysis of the behavioral units. Even if a behavioral element appears to be the same in different environments it can be a part of different behavioral strategies, that is, it can be under control of different genetic backgrounds. This explains why behavioral elements have different genetic backgrounds in different environments.

Information about the genetic background of a trait allows the investigator to make inferences about its evolutionary past /8/.

The additive genetic variance obtained for ES1, SW1, FL1 and OB1, and the ambidirectional dominance detected for FR1, AG1, ST1 indicate that for these behavioral elements an evolutionary past connected with stabilizing selection, that is, with selection against extremes can be inferred. The directional dominance found for MO1 indicates a possible role of directional selection against high phenotypic values in the past.

It seems that in a familiar environment it is not adaptive for the paradise fish to perform MOV element and to be either too active or too passive. If, however, we compare the population means measured in the four situations, we can see that intermediate level of activity at home is much lower than in the novel situations and intermediate level of passivity means more pronounced passive behavior. A possible explanation for selection against extremes can be that high activity may draw predators' attention up and, on the other hand, too passive behavior would not let the fish look for mates or food.

In the open-field situation the additive genetic variance obtained for ES2, SW2, CR2, OB2, FR2, OST and LAT, and the ambidirectional dominance found for MO2 indicate stabilizing selection as an evolutionary past for these traits. Probably, this applies to OUT, AG2 and ST2 as well, however, in the latter two cases directional selection cannot be excluded. Directional selection against high phenotypic values of FL2 is inferred for this variable. Concluding from the results of a classical cross analysis, we have previously found /19/ selection against high expressions of activity and passivity measures in the open-field situation.

Thus, in an open-field, intermediate levels of active and passive behaviors seem to be adaptive again. However, in general, it means more active behavior comparing to the behavior in home range. The adaptive value of intermediate activity, presumably, can be interpreted as a result of responses given to two opposing selection pressures: First, the high activity, fast moving, etc. may be disadvantageous because the fish that behave like this may be more vulnerable to predators. Second, a too passive behavior would not allow the fish to collect sufficient amount of information about its new surroundings. Getting acquainted with a new environment seems to be essential for paradise fish, since it has been proved that they can avoide predators more successfully in familiar environments /5/. However, floating under the surface (FL2) is, obviously, a maladaptive behavior,

presumably because of the danger of fishing birds, and directional selection against it was inferred.

Among the variables measured in the small unfamiliar aquarium (3rd situation) additive genetic variance was found for AG3, and ambidirectional dominance for MO3. Thus, in the cases of these two behavioral elements stabilizing selection is inferred. In the cases of ES3, SW3, ST3 and RE3 we could not decide with confidence whether the dominance was directional or not. However, it seems reasonable to accept directional selection for high phenotypic values of ES3 and RE3 and low values of SW3. The high phenotypic values of ES3 seem to be very high, which means extremely strong tendency to leave. The highest value of RE3, however, is still low, that is, escaping seems to play more important role in this situation. In the case of FL3, similar to FL2, directional selection against high phenotypic values can be inferred.

Thus, it seems that in a small restricted unfamiliar environment it is adaptive for the paradise fish to flee mostly or be occasionally passive and not to explore the small area. The probability of a successful defense against a predator in a restricted environment is thought to be smaller /5/ than in an open-field, thus it seems reasonable to accept that in this situation extreme values of defense are adaptive. Floating under the surface is still maladaptive probably because of increased vulnerability to birds.

In the case of the 4th environment (rotating disc above the small unfamiliar aquarium) stabilizing selection is inferred as a possible evolutionary past for SW4, MO4, CR4, AG4, RE4 and FR4 since there was only additive genetic variance found for these variables. In the case of ST4 there may be stabilizing selection too. However, directional selection for high values of ES4 and low values of FL4 is the most probable evolutionary past of these elements. An other remarkable finding in that swimming element (SW1) occurs much less in the two small novel tanks then is the open-field, which may mean that exploration plays less important role in the former two.

Thus, in this environment intermediate levels of most of the behavioral elements seem to be adaptive. Corresponding with our prediction, escaping from this environment is an adaptive and floating under the surface a maladaptive behavior.

However, the possible difficulties of drawing conclusions from results of a diallel analysis have been stressed (e.g. /43/). First, we

should assume a "base" population from which our inbred lines orginated. The individuals should have been chosen randomly for inbreeding from this population so that the lines having been inbred represent the genetic structure of that particular base population /26/. Obviously, the more lines have been inbred, the better. Unfortunately, the definition of base population does not seem to be clear. Because allele frequencies in populations can change over time, we should have had to take our sample in a definite short period, or we should assume that the base population was in Hardy-Weinberg equilibrium over the time when the samples were taken. Otherwise, our inbred lines would not represent this population.

Secondly, another difficulty arises from the process of inbreading. The inbred lines would represent a population that existed in the past if random genetic drift only had acted on the lines during inbreeding, that is, there should not have been, for example, mutation or selection. Since one usually cannot assess the genetical change of the lines being studied caused either by domestication, a sort of selection process, or by mutation, the only thing we can do is to study the effect of the "mixture" of natural and artificial selection. According to the estimations of genetic parameters our inbred lines contain more recessive alleles then dominant ones in most of the behavioral elements. This may mean that under laboratory conditions the selection has been relaxed. Many studies are done on lines of unknwon origin, which is also the case here. However, even if the origin is known one cannot say that the conclusions from the results of a genetical analysis are about that particular base population.

Despite rejecting the relevance of the possible inferences made about the evoultionary past, we should consider one more point. Even though the behavioral phenotype is flexible, the genotype behind it must be as rigid as genotypes of any other kind of traits, that is, we can neither say that the genetic structure, which has been built up by evolution for thousands of years, has been absoultely changed by domestication or laboratory breeding, nor suppose that the results of a genetic analysis are biologically irrelevant artifacts only.

Similarities with the results of other studies done on other species (e.g. /12, 13/) reveal a sort of biological generality of the animals' adaptive behavioral strategies in open-fields. However, the adaptive responses depend on the environmental stimuli, thus they can change according to the circumstances of a given situation.

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NEURONAL PATHWAYS INVOLVED IN THE OPTOKINETIC HEAD NYSTAGMUS OF THE FROG

GY. LÁZÁR, S. BENNANI, P. TÓTH

Department of Anatomy, University Medical School, Pécs, Hungary

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The morphology of projection neurons in the basal optic nucleus and the pretectal area and the interconnections of these brain regions were studied with the aid of the cobalt-filling technique. It was found that the nucleus-sends long descending axons to the medullary reticular formation. The two basal optic nuclei are reciprocally interconnected and do not give a direct descending pathway. The pretectal nuclei and the basal optic nucleus are also reciprocally coupled. It is supposed that the described pathways mediate commands for horizontal and vertical nystagmic head movements.

Keywords: Basal optic nucleus — Pretectum — Projection neurons — Descending pathways — Frog — Cobalt filling

Introduction

It is generally accepted that neurons in the pretectal area are preferentially sensitive to horizontal temporo-nasal movement of the patterned whole visual environment /1, 2, 4/ and are involved in the mediation of horizontal optokinetic nystagmus /5, 8, 13/. Vertical whole field movements are detected in the basal optic nucleus (BON) /1, 3, 4/. After bilateral transection of the basal optic root (BOR), which carries retinal fibres to the BON, vertical optokinetic head nystagmus cannot be evoked

Send offprint requests to: Dr. Gy. Lázár, Department of Anatomy, University Medical School, Szigeti u. 12, H–7643 Pécs, Hungary

Akadémiai Kiadó, Budapest

/6/. When the BOR was transected unilaterally, optokinetic stimulation in the vertical plane evoked also horizontal nystagmic head movements in addition to movements in the vertical plane. Removal of the eye, ipsilateral to the BOR lesion, increased the occurrence of horizontal head nystagmus in this experimental situation /7/. This phenomenon suggests that the normal optokinetic nystagmus is the result of a finely balanced activity of the paired pretecta and BONs and the vestibular system, and asymmetric visual input can disturb the equilibrium of their activity.

To maintain interactions among these structures probably complicated anatomical connections are necessary. It has already been shown by Montgomery et al. /12/ that the nucleus lentiformis mesencephali (NLM), a pretectal nucleus, is interconnected with the ipsilateral BON, and from another pretectal nucleus (posterior thalamic nucleus, PTN) descending fibres originate, which can be followed to the lower part of the brain stem. Cochran et al. /1/ also found descending pretectal fibres, which terminated in the abducens nucleus. These pathways are probably involved in the mediation of horizontal optokinetic nystagmus.

In these studies horseradish peroxidase was used to label neurons and their axons. While this technique gave good results in showing axon terminals, retrograde filling of dendrites was rather poor and the mophology of neurons could not be studied in detail.

We attempted to use the cobalt-filling technique to investigate the morphology of projection neurons in the pretectum and the BON. We expected to obtain more information about the connectivity of these structures. The final goal of these studies was to try to disclose the anatomical background of the unusual head movements in the above mentioned experimental situation.

Materials and Methods

Fifty eight frogs (Rana esculenta) were used. The animals were anaesthetized with Urethane (0.2 g/100 g body weight) and the brain was exposed by removing the bone with a dental drill above the pretectal region or the BON. Cobaltic lysine complex (CLC) in the concentration of 0.4 M (pH 7.2) was iontophoretically injected (1 to 2 μA anodal current for 30 to 40 min) into the selected brain area. The animals survived for 24 to 36 h and then they were decapitated and the brains were processed to show cobalt-labelled structures according to Lázár et al. /9/. Based on the location of the cobalt foci in the brains 5 experimental groups could be formed as follows:

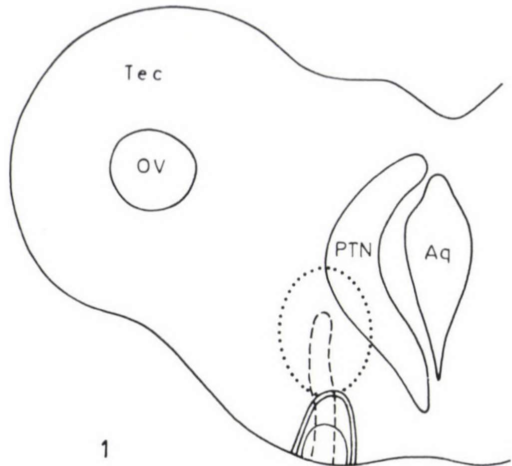


Fig. 1. The location of 5 cobalt injections within (continuous lines, dashed line) or close (dotted line) to the basal optic nucleus. Line drawing of a transverse section from the frog's mesencephalon. Aq -- cerebral aqueduct; OV -- optic ventricle; PTN -- posterior thalamic nucleus; Tec -- optic tectum. Abbreviations apply also to Fig. 2

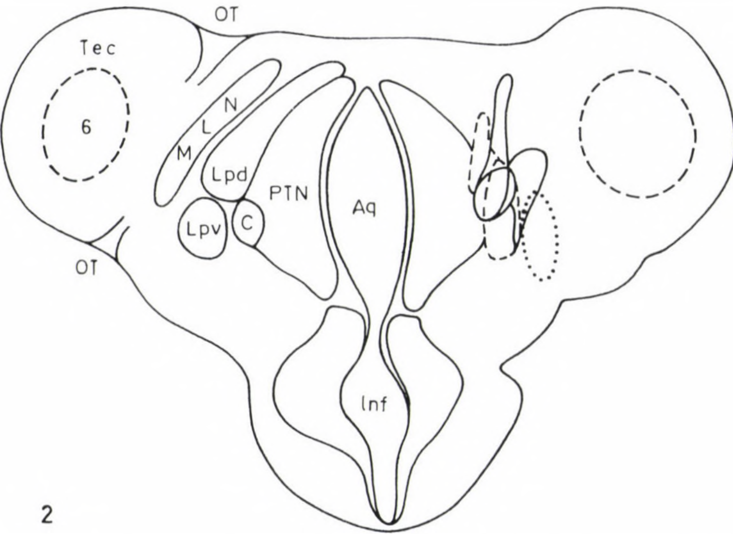
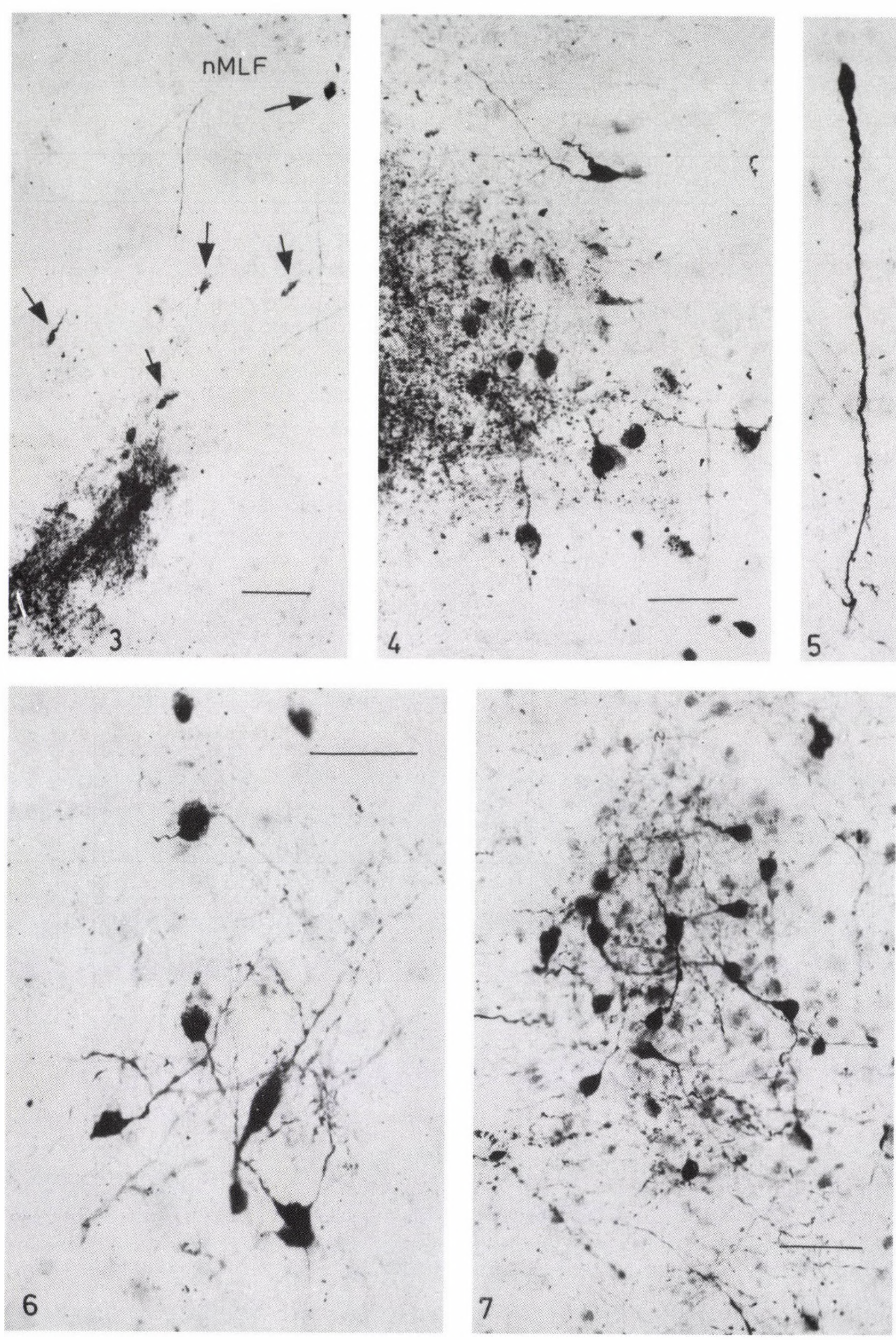


Fig. 2. The location of 6 cobalt injections in the pretectal region (right side of the drawing). Transverse section through the rostral pole of the optic tectum. Pretectal nuclei are indicated on the left side. C -- central thalamic nucleus; Inf -- infundibulum, Lpd, Lpv -- lateral thalamic nucleus, posterodorsal, posteroventral division; NLM -- nucleus lentiformis mesencephali; OT -- optic tract; 6 -- sixth layer of the tectum in tangentional transverse section


nMLF
3
4
5
6
7

1) The cobalt focus is restricted to the BON (4 animals).
2) The cobalt focus is in the midline between the two BONs (2 animals).
3) The cobalt foci are in the tegmentum with partial involvement of the BON (5 animals).
4) Pretectal cobalt injections involving the NLM and part of the lateral thalamic nucleus (LTN, 8 animals), the PTN (4 animals) and the pretectal neuropil (PN, 3 animals).
5) Injections into the ventral funiculus of the medulla (4 animals).

Only those preparations were evaluated which showed intense cobalt labelling in axons or neurons (30 brains). Figure 1 shows the location of cobalt foci of group 3 animals. Pretectal CLC injections are shown in Fig. 2. The nomenclature introduced by Neary and Northcutt /14/ will be used in the description of the results.

Results

Cobalt injections into the BON (Group 1)

In all animals in this group the amount of injected cobalt was very small and the BON was only faintly labelled. A few perikarya were incompletely stained within the BON, dendrites, however, were not filled (Figs 3, 4). Labelled neurons could be detected also in the tegmentum surrounding the BON, and in the nucleus of the medial longitudinal fasciculus (nMLF) (Figs 3, 5). These cells had piriform perikarya with long apical dendrites which terminated in the neuropil of the BON. The axons of BON cells were not stained.

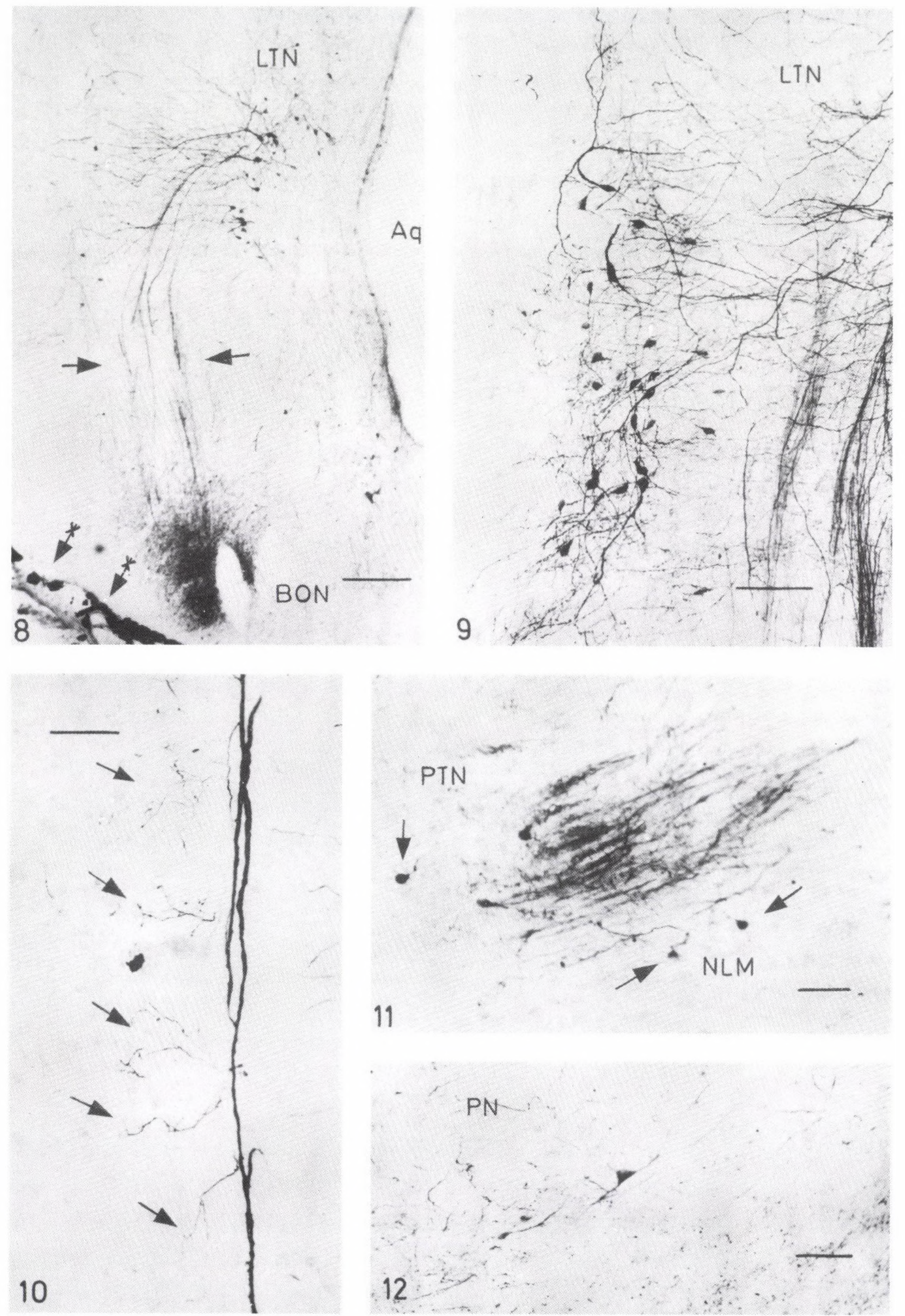
Fig. 3. Injection site of cobalt lysine complex (CLC) in the basal optic nucleus (BON) (black area in the left lower corner). In addition to cobalt-filled cells here, labelled neurons (arrows) are visible dorsal to the BON and in the nucleus of the medial longitudinal fasciculus (nMLF). Transverse section. Medial is to the right. Scaler bar = 100 µm and applies also to Fig. 5

Fig. 4. Labelled neurons in the BON after CLC injection into the ventral part of the nucleus. Transverse section. Ventral is to the left. Scale bar = 5 µm

Fig. 5. Cobalt-filled cell in the nMLF after CLC injection into the BON. The cobalt entered the cell through a ventrally projecting dendrite

Fig. 6. Cobalt-filled neurons in the BON in transverse section. The CLC was iontophoretically injected into the rostral part of the ansulate commissure. Scale bar = 50 µm

Fig. 7. Cobalt-filled cells in the ventral part of the BON in horizontal section. The cobalt focus was located dorsolaterally to the BON and involved the dorsalmost part of the nucleus. Scale bar = 50 µm


LTN
Aq
BON
8
LTN
9
10
PTN
NLM
11
PN
12

Projection neurons of the BON

One population of these neurons could be filled by cobalt injections placed between the two BONs (Group 2). Several small round perikarya (diameter 10--12 µm) were filled in these animals in both BONs (Fig. 6). Short dendritic segments were also filled. Much less in number, neurons with fusiform perikarya could also be stained (Fig. 6). The results obtained by these injections show that the two BONs are reciprocally interconnected throguh the rostral part of the ansulate commissure.

Another population of projection neurons were filled bilaterally after large cobalt injections placed dorsal or dorsolateral to the BON. These neurons were mainly located dorsally at the periphery of the nucleus. The dendrites pointed towards the central part of the nucelus and only a few side branches were filled. Some neurons had one or two short dendrites also in the horizontal plane (Fig. 7). Axons could be followed from the BON to the posterior commissure. Cobalt-filled axons were seen, however, also in the ansulate commissure.

Occasionally one or two cells were filled in the BON on both sides after pretectal injections (group 4). These cells were similar to those found after tegmental injections.

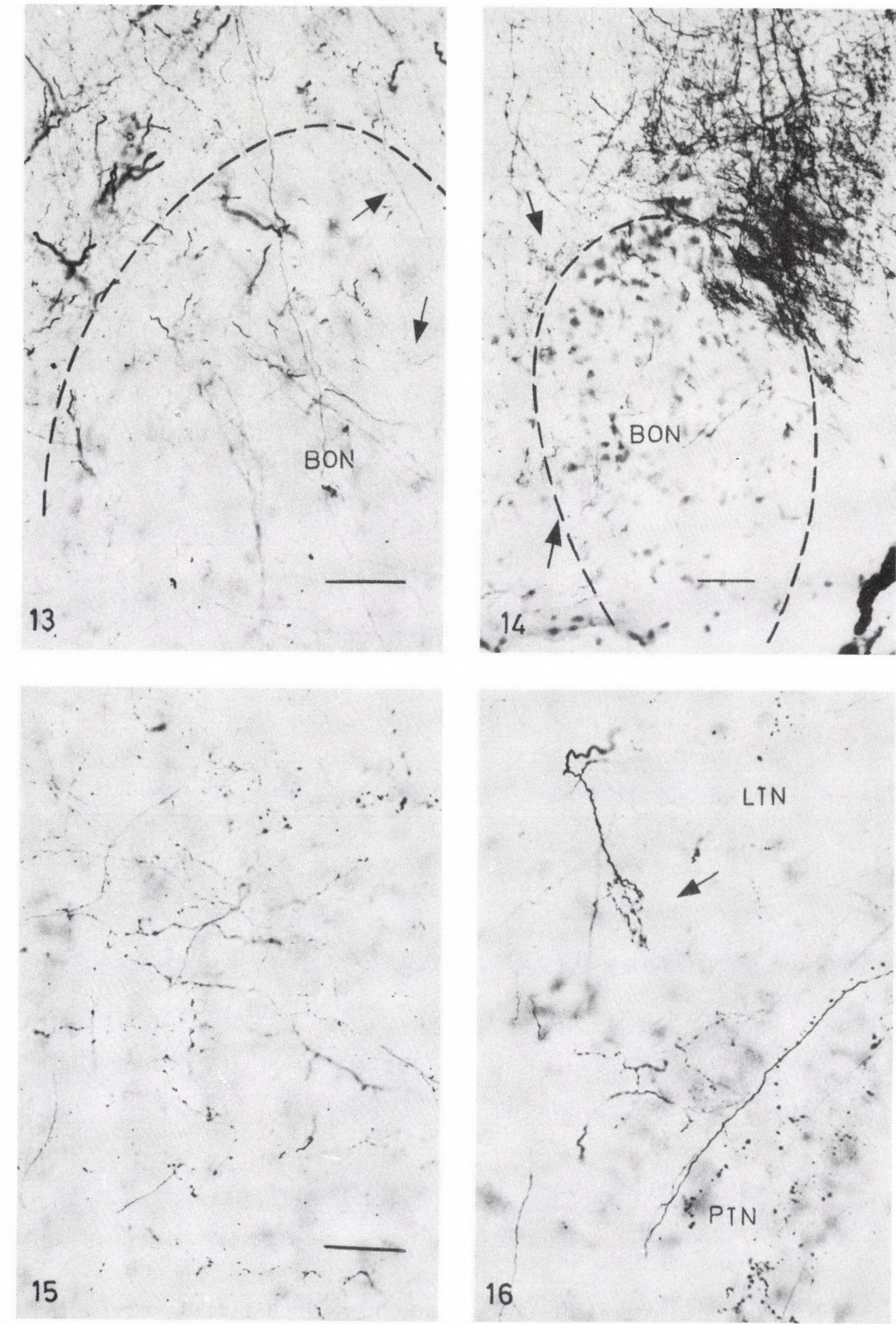
Fig. 8. Cobalt focus (black area in the middle lower portion of the figure) in the tegmentum mesencephali lateral to the basal optic nucleus (BON). The micropipette interrupted the descending axons (arrows) of projection neurons located in the posterior thalamic nucleus and these neurons were back-filled with cobalt. Arrows with asterisk point at pigment granules on the ventral surface of the brain. Transverse section. Aq -- aqueductus cerebri; LTN -- lateral thalamic nucleus. Scale bar = 250 µm

Fig. 9. Cobalt-filled cells in the nucleus lentiformis mesencephali in the same preparation as shown in Fig. 8. Two fascicles of descending axons are visible on the right. Transverse section. Dorsal is up. Scale bar = 100 µm

Fig. 10. Cobalt-filled axon in the ventral funiculus close to the midline with collaterals (arrows) terminating in the medullary reticular formation. The cobalt focus involved descending pretectal axons and part of the nucleus of the medial longitudinal fasciculus. Horizontal section. Scale bar = 100 µm

Fig. 11. Cobalt-filled axons (middle of the figure) and labelled neurons (arrows) in the pretectal area contralateral to a pretectal cobalt injection. Horizontal section. Scale bar = 100 µm

Fig. 12. Cobalt-filled multipolar neuron in the nucleus lentiformis mesencepahli. CLC was injected into the contralateral posterior thalamic nucleus. Horizontal section. PN -- pretectal neuropil. Scale bar = 100 µm


BON
13
BON
14
15
LTN
PTN
16

Morphology of pretectal projection neurons

In one animal the cobalt focus was located just lateral to the BON and the axons which traversed the injected area were intensely filled with cobalt. A relatively loose, but well defined bundle of axons could be followed to the pretectal area (Fig. 8), where large number of neurons were labelled in the NLM and the PTN. Most of the caudally projecting neurons in the NLM have fusiform perikarya. The two main dendrites are oriented dorsoventrally and their side branches point rostrally and medially. A few cells have piriform perikarya and their dendrites point medially (Fig. 9).

Most cobalt-filled neurons in the PTN have piriform perikarya. Long apical dendrites project laterally and terminate in the pretectal optic neuropil and overlap rostrally projecting dendrites of the NLM (Fig. 8). Many cells have shorter apical dendrites, which split to a dorsal and ventral branch at the lateral border of the PTN. A few cells with triangular perikarya also occur.

The cobalt-filled pretectal axons descend close to the midline in the ventral funiculus of the medualla and can be followed to the uppermost part of the spinal cord. They give collaterals to the medullary reticular formation (Fig. 10). Pretectal cobalt injections involving the NLM labelled piriform cells in the contralateral PTN and NLM. When the PTN was injected, one or two cells were filled with cobalt in the contralateral NLM (Fig. 12),

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Fig. 13. Cobalt-filled axon terminals in the basal optic nucleus (BON) after CLC injection into the ipsilateral nucleus lentiformis mesencephali. Note the paucity of terminating fibres. Transverse section. Scale bar = 50 μm

Fig. 14. Cobalt-filled axon terminals (arrows) at the periphery of the BON. Above the nucleus descending pretectal fibres and axons of the posterior commissure can be seen. Transverse section. Scale bar = 100 μm

Fig. 15. Terminal network of fine beaded axons in the posterior thalamic nucleus. The cobalt was injected into the posterodorsal division of the lateral thalamic nucleus and the nucleus lentiformis mesencephali on the contralateral side. Transverse section. Black dots arranged in lines (right upper corner) are degenerated terminals. The scale bar is 50 μm and applies also to Fig. 16

Fig. 16. The same preparation as shown in Fig. 15. In the lateral thalamic nucleus (LTN) the terminal arborization of a thick fibre can be seen (arrow). The posterior thalamic nucleus (PTN) contains beaded axon terminals; many of them are degenerated (black dots)

or in the contralateral PTN. All these projection neurons were similar to those giving the descending pathway, but the cells were incompletely filled and details of their morphology could not be disclosed.

Afferent connections of the BON

When the cobalt focus was located between the two BON's (group 2), fine beaded terminals could be detected in both nuclei among labelled perikarya. The number of terminal branches was low and the terminal twigs were short.

Tegmental cobalt injections also labelled terminals in the BON. They entered the nucleus dorsally and branched similarly as in the case of group 2 animals.

With the exception of cobalt injections into the PN, in all cases after pretectal cobalt injections a few labelled terminals could be detected in the BON ipsilaterally (Figs 13, 14), contralaterally or on both sides.

Afferent connections of the pretectum

Cobalt injections in the tegmentum (group 3) labelled several fibres, which terminated bilaterally in the NPT and NLM. Contralaterally terminating fibres were very low in number and their terminal branching was poor. In the ipsilateral NPT fine, beaded terminals were running parallel with the dorsoventrally oriented rows of perikarya, while in the NLM loose plexuses of labelled fibres could be found.

Cobalt injected into the NLM labelled terminal fibres in the PTN bilaterally, and PTN cobalt injections resulted in labelling on both sides in the NLM. After cobalt injections into the PTN, also the contralateral PTN contained fine, beaded terminal networks of fibres (Fig. 15), and in the LTN some thick fibres formed small broom-like terminals (Fig. 16).

Both the tegmental and pretectal injections labelled fibres and their terminals in several other tegmental and diencephalic structures. The description of these connections, however, will not be included in this paper.

Medullary cobalt injections

The purpose of these injections was to try to identify descending axons from the BON. The injection site was always in the rostral part of

the medulla. The rostral extent of the cobalt focus was about 1 mm from the caudal border of the BON. In these cases no cobalt-filled neurons were found in the BONs. A large CLC injection infiltrated the whole ventral and lateral parts of the medulla. Even in this case the BON standed out clearly against the cobalt stained surrounding area, and only traversing cobalt-filled axons could be identified in the nucleus. In this case, some of the pretectal projection neurons were nicely stained.

Discussion

Cobalt injection in the group 1 of animals labelled a few cells outside the BON. Cells in the nMLF were filled through their dendrites, which means that the terminals of retinal fibres overlap these dendrites giving the possibility of direct retinal input to these cells. It was shown by Matesz and Székely /10/ that dendrites from the oculomotor nucleus also reach the BON. It is not known, however, whether monosynaptic coupling of retinal axons with these cells exists or not.

The problem of descending pathway for vertical head movements

In these experiments we did not find descending axons which could have originated from the BON. In our earlier studies we injected large amount of cobalt into different pathways of the medulla and also did not find labelled cells in the BON. Similar result was obtained with the horseradish peroxidase technique /11/. These negative findings suggest that descending impulses which control vertical head movements reach their target cells through indirect pathways. These may be the medial longitudinal fasciculus or the reticulospinal tract.

Descending pathways for horizontal head movements

Cobalt labelling proved to be an excellent technique to show the morphological features of pretectal projection neurons. Many of these cells send dendrites into the optic neuropils and may have direct retinal input. The dendrites of other back-filled cells arborize outside of the optic neuropils and thus may be triggered through interneurons.

We suppose that neurons labelled in the NLM and the PTN and having their descending axons in the vicinity of the BON, may convey the commands

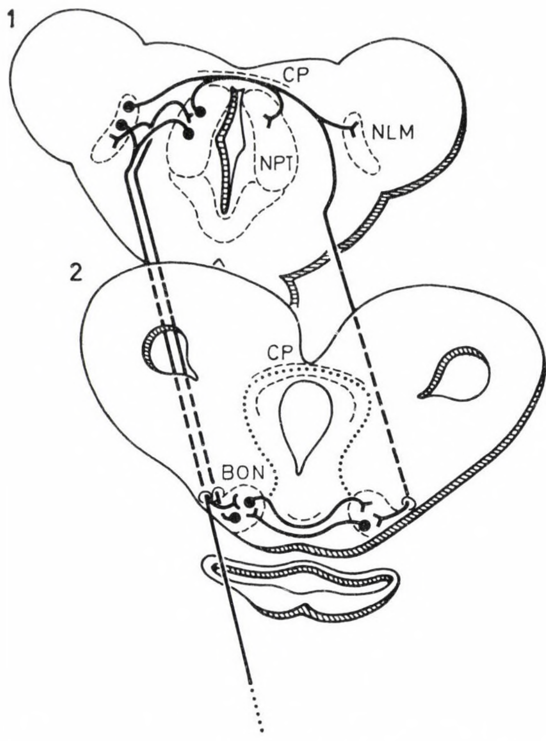

Fig. 17. Line drawing to show interconnections of some pretectal nuclei and the basal optic nucleus (BON). The two planes of the drawings are the same as shown in Figs 2 and 1, respectively. In the 1st plane of the figure the interconnections of the pretectal nuclei, the nucleus lentiformis mesencephali (NLM) and the posterior thalamic nucleus (NPT) are shown. Descending fibres terminate in the 2nd plane in the BON and can be followed ipsilaterally towards the medulla. Tectal layers are not indicated. Commissural fibres between the two BONs are shown in the 2nd plane.
CP - posterior commissure

for horizontal optokinetic head movements. It is probable that most of these descending axons form synapses in the medullary part of the reticular formation. A hypothesis, how these pathways may regulate head movements has been put forward recently /7/.

Interconnections of the pretectum and the BON

Cobalt injections into the ansulate commissure showed that the two BONs are reciprocally coupled. It is also possible that they are interconnected through the posterior commissure, because tegmental cobalt injections located above the BON also labelled cells on both sides in the BON.

In this case, however, the possibility that the contralaterally labelled neurons project only to the tegmentum cannot be excluded.

Reciprocal connections were also found between the ipsilateral NLM-PTN complex and the BON. Montgomery et al. /12/ showed that the BON projects to the core of the NLM where contralateral retinal fibres terminate. We found similar distribution of BON afferents in the pretectum after large injections of CLC into the BON and the surrounding tegmentum.

The two PTNs are also reciprocally coupled and may project to the contralateral BON through the posterior commissure. Contralateral fibres might originate also from the NLM. The existence of contralaterally originating afferents to the BON, however, cannot be proved in the present material, because tegmental injections gave similar result.

The findings of the present investigations are summarized in Fig. 17. The multiple connections between the pretectal nuclei and the BONs, and the connections with the nMLF and the reticular formation suggest that this neuronal system should be very versatile and form the anatomical basis for the unusual head movements found in other experiments /7/.

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LUMINOL DEPENDENT CHEMILUMINESCENCE OF QUARTZ AND ZYMOSAN STIMULATED NEUTROPHILS

F. LISZT and K. JOBST

Department of Clinical Chemistry, University Medical School of Pécs, Hungary

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The effect of the opsonization by zymosan and quartz particles on the chemiluminescence was investigated on human neutrophil granulocyts. Opsonization of zymosan enhanced the chemiluminescence response, while opsonized quartz inhibited the chemiluminescence reaction. Calcium ionophore A 23187 treatment did not influence the chemiluminescence of quartz but the light signal in the presence of quartz decreased rapidly. In parallel experiments the protein pattern of zymosan treated neutrophils was investigated by high resolution two-dimensional polyacrylamide gel electrophoresis.

Keywords: Neutrophil granulocyte — Quartz — Zymosan — Ionophore A 23187

Introduction

The chemiluminescence (CL) of luminol has been widely used to detect the generation of reactive oxygen metabolites by various phagocytes since 1972 /1/. It is accepted as a method for evaluating the intensity of the respiratory burst. The luminol or lucigenin dependent CL of polymorphonuclear leukocytes (PMNL) and macrophages have been shown to be triggered by various inorganic dusts (quartz, carbon, asbestos etc.) or organic components e.g. zymosan /3, 4, 8, 9/.

The present study was aimed at to investigate the effect of opsonization of zymosan and quartz particles on the CL in human neutrophils. In

Send offprint requests to: Dr. K. Jobst, Department of Clinical Chemistry, University, Medical School of Pécs, H–7643 Pécs, Ifjúság u. 13, Hungary

Akadémiai Kiadó, Budapest

some cases the protein pattern of zymosan stimulated PMNL was investigated by high resolution two dimensional electrophoresis. This way we could decide how this stimulus might cause specific changes in the pattern of synthetized protein during phagocytic process to reveal the short term effects.

Materials and Methods

Heparin anticoagulated (10 U/ml) venous blood from healthy donors was obtained. The cells were isolated by a modified procedure according to Hjort and Johnson /5/ on a two step discontinuous (55--75%) Percoll gradient. After centrifugation the distribution of the cells was 75--85% PMNL and 25--15% lymphocytes, the viability was 95--98%. The PMNL were resuspended in Hanks solution and the count was adjusted to 6×10^6/ml.

We used DQ--12 quartz (FRG locality of Dörentrup) with diameter of 2--5 μm. Zymosan was purchased from Serva (Heidelberg, FRG) with diameter of 4--7 μm. Calcium ionophore (Boehringer Mannheim, FRG) was applied in a final concentration of 1 μmol/l.

Serum treated quartz and zymosan were prepared by incubating quartz and zymosan particles at a concentration of 50 mg/ml with 50% fresh pooled serum in Hanks solution at 37 °C for 30 min. The mixtures were centrifuged for 10 min and then washed twice with Hanks solution before being resuspended to the same concentration in Hanks solution.

For the luminol dependent chemiluminescence study 100 ul of the PMNL suspension, 500 μl of 0.2 mmol/l luminol and 1.5 ml of Hanks solution were preincubated for 10 min at 37 °C. The reaction was initiated by adding 20 ul particle suspension. A control without particle stimulation was carried out to exclude the inducing effect of luminol. CL was measured by an XP 2020-Q photomultiplier tube (Applied Photophysics, England) coupled to an ICA 70 (KFKI, Budapest, Hungary) multichannel analyzer and Biolumat 9505 analyzer (Laboratorium Prof. Dr. Berthold, Wildbad, FRG -- generous gift of the Alexander von Humboldt Foundation). In parallel with the CL measurements, the protein pattern of zymosan stimulated PMNL was investigated by high resolution two dimensional polyacrylamide gel electrophoresis. Isolated PMNL were incubated in methionine deficient MEM Dulbecco medium supplemented with fetal bovine serum (5 ml/l) and 40 μCi of (^{35}S)-methionine. Approximately 5×10^6 cells with 20 μl zymosan suspension were cultured per well in flat bottom multiwell tissue culture plates. Cultures with and without zymosan were incubated for 18 h at 37 °C in humidified atmosphere containing 5% CO_2. At the end of the labeling period cells were harvested by brief centrifugation in a Beckman Microfuge and the pellets were lysed in O'Farrel lysis buffer /7/. After centrifugation at 100 000 g for 30 min the lysed samples were analyzed by high resolution two-dimensional polyacrylamide gel electrophoresis /7/. The protein spots were visualized by fluorography using KODAK RP Royal X-Omat film /2/.

Results and Discussion

Comparing the CL of opsonized and non-opsonized quartz and zymosan, the highest peak was measured after non-opsonized quartz treatment (Fig. 1). Our results support that both opsonized and non-opsonized forms of zymosan induce CL in human neutrophils, but the repsonse is much higher in opsonized form.

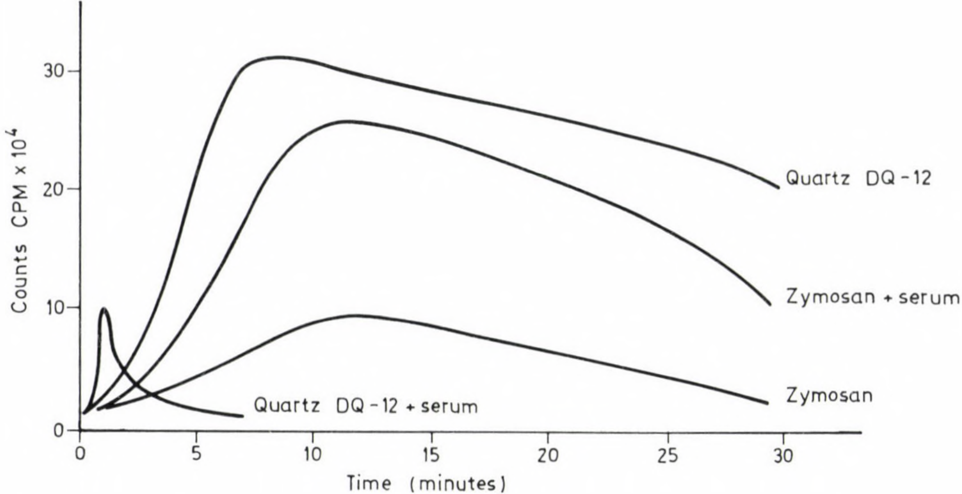

Fig. 1. Effect of quartz and zymosan opsonized with serum on the chemiluminescence of human neutrophils. Experiments were carried out in five parallels. CL of stimulated neutrophils corrected with control (unstimulated neutrophil) was represented on the graph

Before the onset of light emission there is brief lag period (approximately 30 s). The maximum of CL is usually within the 10th and 15th min of the stimulation in the non-opsonized quartz and both types of zymosan addition. The CL response of opsonized quartz is quite different from all of the three other curves. Opsonized quartz produces a very rapid decrease of the light emission and almost total inhibition of CL after 5 min The inhibitory effect of serum can best be explained by an inactivation of the particle surface by non-specific protein adsorption. The in vivo cytotoxic effect of these dusts suggests that this inhibition is due to a scavenging of oxygen radicals by proteins, there is a secondary interference in CL measurement /6/. It is also possible, that opsonized quartz is so avidly phagocytosed by neutrophils that it quickly causes cell lysis

Fig. 2. Two-dimensional polyacrylamide gel electrophoresis protein pattern of neutrophil granulocytes. In Figs 2 and 3 the gels are oriented with the acidic end to the left and higher molecular masses at the top. First dimesnion is 4% polyacrylamide gel, pH 3.5—10, second dimension is 12% polyacrylamide gel, 0.1% SDS

and death and thus CL inhibition. Comparing the protein patterns of control and opsonized zymosan phagocytosed samples (Figs 2 and 3) we could not detect any significant differences. This stimulus did not cause any specific changes in the protein pattern.

The CL responses of quartz and opsonized zymosan to the addition of A 23187 show (Fig. 4) that the reactions of the dusts are quite different. Higher ionized calcium level in cytosol due to A 23187 does not influence the CL of quartz. On the contrary, the CL of opsonized zymosan decreased very fast compared to control opsonized zymosan curve. The reduction, but not complete inhibition, suggests that the CL response depends on the ionized calcium concentration in the cytosol. However, we cannot rule out that two types of CL response, a calcium dependent and a calcium independent, may exist.

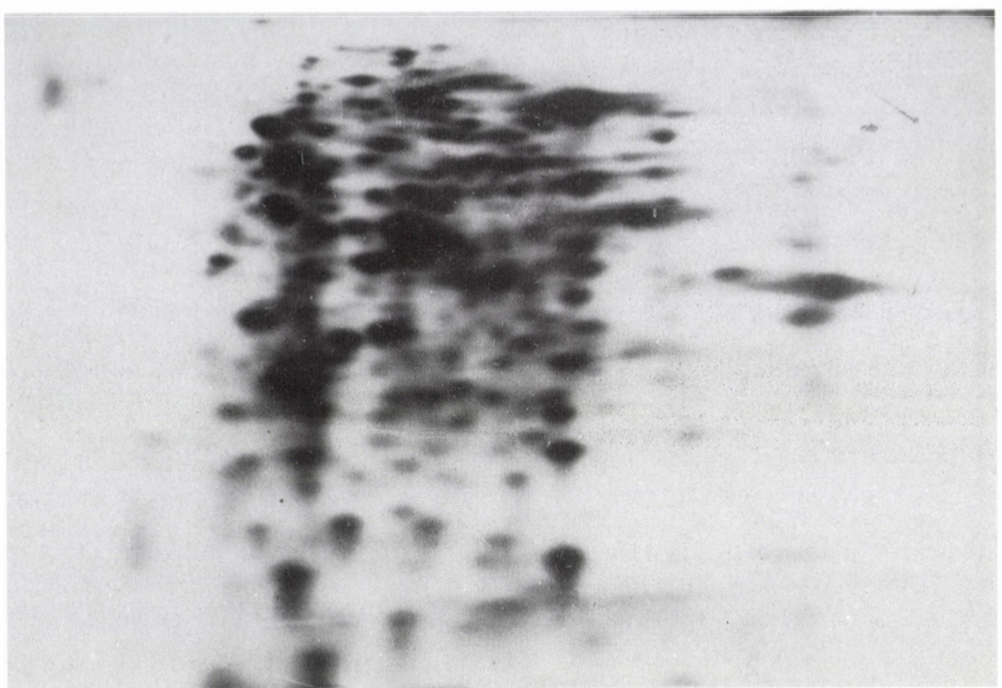

Fig. 3. Two-dimensional polyacrylamide gel electrophoresis protein pattern of neutrophil granulocytes incubated with zymosan

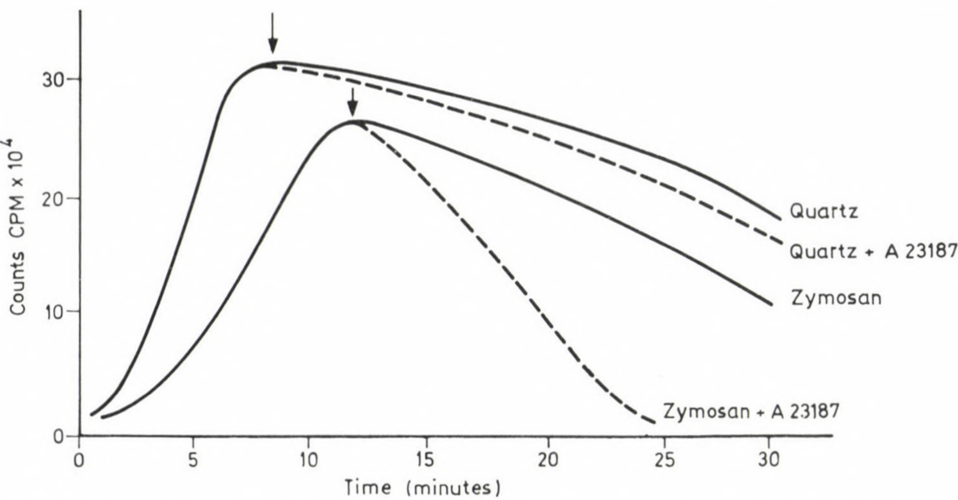

Fig. 4. Effect of calcium ionophore A 23187 on the chemiluminescence of quartz and opsonized zymosan. The arrows indicate the addition of A 23187 to the system

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EFFECT OF DIFFERENT CRYOPROTECTANTS AND TRANSFER TEMPERATURES ON THE SURVIVAL RATE OF HEMP (CANNABIS SATIVA L.) CELL SUSPENSION IN DEEP FREEZING

ZS. JEKKEL, L.E. HESZKY and A.H. ALI

Department of Genetics and Plant Breeding, University of Agricultural Sciences, Gödöllő, Hungary

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Adequate cell dehydration is the precipitating element in the successful cryopreservation of plant cells and organs. This could be achieved by using different cooling rates, transfer temperatures and cryoprotectants.

Experiments were performed to determine these critical points in the freeze preservation procedure of Cannabis sativa (L.) suspension cultures. The explants were frozen at a cooling rate of 2 °C/min, while the transfer temperatures were -10 °C, -20 °C, -30 °C, -40 °C and -50 °C. The applied cryoprotectants were the DMSO, glycerol, proline and PEG in different concentration. The highest viability (58%) was obtained by using 10% DMSO and at -10 °C transfer temperature. The optimum transfer temperature varied remarkably by different cryoprotectant concentrations indicating the importance of their interactions.

Keywords: Hemp — Cannabis sativa — Cell suspension culture — Freeze preservation — Cryoprotectant — Transfer temperature

Introduction

Maintenance and preservation of germplasm collections representing a wide source of genetic diversity of cultivated plants and their wild relatives are considered to be essential for plant breeding programs. However, the demand for increased food production tends to result in the production of high-yielding varieties of crop plants with a narrow genetic base.

Send offprint requests to: Dr. L.E. Heszky, Department of Genetics and Plant Breeding, University of Agricultural Sciences, H-2103 Gödöllő, P.O.Box 303, Hungary

Akadémiai Kiadó, Budapest

The storage of seeds is a conventional method generally used in the gene banks. There are some species involving the recalcitrant seeds and the vegetatively propagated crops, the seeds of which cannot be stored. When the cell-plant system operates these germplasms can be maintained in somatic tissue cultures. Although this approach seems to be attractive — under unchangeable metabolic activity conditions — there are numerous difficulties which might have serious repercussions. Risks of loss through microbial contamination, or equipment failure and the sheer expense of maintaining stock cultures are all contributory factors. The main limit however is the genetic instability.

Prolonged preservation of plant material should guarantee genetic stability that is it should prevent progressive changes in the genom. Storage at an ultralow temperature — when all metabolic activity of living cells are approaching a standstill such as that of liquid nitrogen (-196 ^{0}C) is one of the most promising approaches being purposed to achieve this goal.

Adequate cell dehydration is the precipitating element in the successful cryopreservation of plant cells. This could be achieved by using different cooling rates, transfer temperatures and cryoprotectants.

There have been a number of reports of successfully cryopreserved species as suspension culture (Acer pseudoplatanus /7/, Atropa belladonna /7/, Coleus blumei /8/, Daucus carota /2/, Digitalis lanata /1/, Medicago sativa /4/, Nicotiana plumbaginifolia /5/, Nicotiana silvestris /5/, Nicotiana tabacum /2, 5/, Oryza sativa /3, 9/, Phoenix dactylifera /10/, Saccharum sp. /3/, Zea mays /11/).

Most of the published methods depended on slow cooling (0.5—2 ^{0}C/min) of cells to transfer temperature (-30 to 100 ^{0}C) in the presence of cryoprotectants followed by transfer to liquid nitrogen. Generally applied chemicals were dimethyl sulfoxide (DMSO), glycerol, a variety of glycols, sugars and proline.

However, there is no information about the effect of cryoprotectants at various transfer temperatures.

This report is an account of experiments performed to determine temperature points critical from this respect and describe a simple protocol, which we use to preserve cultured cells and aggregates of Cannabis sativa (L.) at liquid nitrogen temperature.

Materials and Methods

Plant material and culture conditions

Hemp (Cannabis sativa L.) cv. 'Kompolti kétlaki' suspension culture was established from the callus produced by anther culture. The anthers were gently husked and sterilized with $HgCl_2$ (0.2%) for 1o min after having been rinsed in ethanol (96%) for 1 min. Before putting on the agar medium sterilized anthers were washed three times with distilled sterile water.

The medium for callus induction was solid MS /6/ containing kinetin (1.5 mg/l) and naphthaleneacetic acid (NAA 1.0 mg/l). After three weeks when the visible callus appeared the cultures were transferred to the same basic medium supplemented with kinetin (10 mg/l). Cultures were incubated in photoperiod of 16 h light, 8 h dark, at 26--27 ^{o}C, with light intensity of 3000 Lux. In order to reduce the size of calli after some passages the explants were cut into small pieces with sterile razor blade and maintained in liquid MS medium supplemented with kinetin (10 mg/l). Cultures were held in 100 ml Erlenmeyer flasks containing 50 ml of the liquid medium, inoculated with 10 ml of culture and transferred weekly. Incubation was at 26--27 ^{o}C on a rotary shaker (120 r/min).

Pretreatment

Actively growing cell aggregates were usually harvested for freezing experiments on the 3th or 4th days after inoculation. Cryoprotectants dimethyle-sulfoxide (DMSO), glycerol, proline and polyethylene glycol (6000 Mw PEG), were used in concentration range of 2.5--5.0--7.5--10.0--12.5--15.0 %. The cryoprotectant solutions and cell suspension were chilled on ice and the cryoprotectants gradually added to an equal volume of cell suspension in four aliquots over a period of one hour. After keeping the flasks in refrigerator at 5 ^{o}C for 16 h, the explants were dispensed into sterile polypropylene ampoules (50 pieces into each 2 ml ampoule) and transferred to a controlled freezing apparatus.

Freezing and thawing

In order to determine the adequate freezing rate, the cooling regime had to be optimalized. In the first step -- samples were frozen without any cryoprotectants, studying the clear effects of the freezing rate. Presumably the cooling rate at which survival can be found is the optimal one.

Five different cooling rates were compared such as 0.5, 0.75, 1.0, 2.0 ^{o}C/min and 1000--2000 ^{o}C/min. The freezing was carried out in a DIGIT-COOl-5000 programmed freezer (ISV, France). Since only by using a 2 ^{o}C/min cooling rate were surviving explants found cells in cryoprotective solution were frozen at this cooling level.

The freezing program began at +4 ^{o}C and held till -50 ^{o}C. From -10 ^{o}C in every 5 min (at -10, -20, -30, -40 and -50 ^{o}C transfer temperatures) 2 samples were taken out of the freezing chamber. One of these 2 samples was immersed to the liquid nitrogen and after 10 min storage placed to a water bath. The other ampoule was thawed directly from the transfer temperature, without liquid nitrogen storage. Thawing was carried out by using +40 ^{o}C water bath. The ampoules were swirled in the bath until ice in the tubes had just disappeared (50--60 s).

Assessment of viability

Cell viability was determined either after the addition of triphenyl tetrazolium chloride (TTC) to the frozen cells or by the transfer of cells to the culture medium containing agar for assaying their growth capability.

Half of the thawed cell clumps (25 pieces) were transferred onto a plate of standard semisolid medium and the green, growing aggregates were counted. The other cell aggregates (25 pieces) were treated with an equal volume of 1% of TTC (W/v) in 0.05 M phosphate buffer (pH 7.5). The mixtures were incubated for 16 h at 20 °C in dark. Dehydrogenases in recovering cells reduce the TTC to formazan with red color. Proportion of the red coloured calli indicates the survival rate and growing capacity.

Results and Discussion

Our experimental method allows studying different cryoprotectants, transfer temperatures and their combined effects on the viability of cultured hemp cells during deep freezing. After the freezing-thawing procedure, most of the explants turned brown and did not show any sign of growth for the first 3--4 weeks therefore, the cell viability was assessed generally at the 6th week after freezing.

The results are shown in Tables 1—4. In each treatment 3 different types of control were used: (i) frozen non-treated (Control 1); (ii) non-frozen treated (Control 2); (iii) without prefreezing putting samples directly into liquid nitrogen (Control 3).

The applied concentrations of cryoprotectants (DMSO, PEG, proline, glycerol) did not show any toxic effect. Viability of non-frozen treated controls (Cont. 2) was 100% in every case.

Down to -30 °C all these chemicals had considerable cryoprotective effects as compared to the frozen non-treated control (Cont. 1).

The maximum cryoprotective effect was obtained by using DMSO (Table 1). Dimethyle sulfoxide 10% and transfer temperature -10 °C gave a 58% viability after 10 min storage in liquid nitrogen. At DMSO level 12.5% and 15% increased survival (26%, 20%). With the exeption of 15% DMSO level no survival rate cells were found at -20 °C transfer temperature (TT). At -30 °C TT the concentrations of 10% and 12.5%, at -40 °C TT the 12.5% concentration and at -50 °C TT also the 10% and 12.5% DMSO resulted in remarkable survival rates.

Somewhat reduced cryoprotective effect was observed, when polyethylene glycol (PEG) was applied (Table 2). Surviving cells were found at

Table 1

The effect of DMSO on the viability (%) of hemp suspension cultures at different transfer temperatures

DMSO %	Transfer temperature (°C)											
	Survival rate without storage in LN						Survival rate after 10 min storage in LN					
	Cont. 2.	-10	-20	-30	-40	-50	Cont. 3.	-10+LN	-20+LN	-30+LN	-40+LN	-50+LN
2.5	100±0.0[x]	100±0.0	100±0.0	92±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
5.0	100±0.0	100±0.0	100±0.0	100±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
7.5	100±0.0	100±0.0	100±0.0	100±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
10.0	100±0.0	100±0.0	100±0.0	100±0.0	0±0.0	0±0.0	0±0.0	58±0.0	0±0.4	23±0.0	0±0.0	17±0.4
12.5	100±0.0	100±0.0	100±0.0	100±0.0	10±1.2	6±1.2	0±0.0	26±0.0	0±0.0	28±0.0	12±0.0	16±0.0
15.0	100±0.0	100±0.0	100±0.0	100±0.0	28±0.8	12±0.0	0±0.0	20±1.22	4±0.0	2±0.0	2±0.0	0±0.0
Cont 1.	-	100±0.0	100±0.0	7±0.0	0±0.0	0±0.0	0±0.0	7±0.0	0±0.0	0±0.0	0±0.0	0±0.0

LN = liquid nitrogen storage
Cont. 1. = Control 1 = frozen non-treated control
Cont. 2. = Control 2 = non-frozen treated control
Cont. 3. = Control 3 = direct immersion to liquid nitrogen
x = Mean±SE
LSD at 1% = 1.54% (viability)

Table 2

The effect of PEG on the viability (%) of hemp suspension cultures at different transfer temperatures

PEG %	Transfer temperature (°C) — Survival rate without storage in LN						Survival rate after 10 min storage in LN					
	Cont. 2.	-10	-20	-30	-40	-50	Cont. 3.	-10+LN	-20+LN	-30+LN	-40+LN	-50+LN
2.5	100±0.0[x]	100±0.0	100±0.4	78±1.6	69±3.6	28±1.2	0±0.0	35±0.8	32±1.6	26±0.4	18±0.4	4+0.0
5.0	100±0.0	100±0.0	95±2.4	63±0.8	25±1.6	0±0.0	0±0.0	20±4.1	7±0.4	0±0.0	0±0.0	0±0.0
7.5	100±0.0	100±0.4	100±0.0	51±1.6	0±0.0	0±0.0	0±0.0	0±0.0	4±0.0	0±0.0	0±0.0	0±0-0
10.0	100±0.0	100±0.0	100±0.0	28±1.2	9±0.0	2±0.0	6±0.0	6±0.0	3±0.4	0±0.0	0±0.0	0±0.0
12.5	100±0.0	100±0.0	100±0.0	25±1.2	0±0.0	0±0.0	0±0.0	5±0.0	2±0.4	0±0.0	0±0.0	3±0.4
15.0	100±0.0	100±0.0	100±0.0	37±0.8	0±0.0	0±0.0	0±0.0	13±0.4	3±0.4	0±0.0	0±0.0	0±0.0
Cont. 1.	-	100±0.0	100±0.0	7±0.0	0±0.0	0±0.0	0±0.0	7±0.4	0±0.0	0±0.0	0±0.0	0±0.0

LN = liquid nitrogen storage
Cont. 1. = Control 1 = frozen non-treated control
Cont. 2. = Control 2 = non-frozen treated control
Cont. 3. = Control 3 = direct immersion to liquid nitrogen
x = Mean ± SE
LSD at 1% = 2.79% (viability)

Table 3

The effect of proline on the viability (%) of hemp suspension cultures at different transfer temperatures

PROLINE %	Transfer temperature (°C)											
	Survival rate without storage in LN						Survival rate after 10 min storage in LN					
	Cont. 2.	-10	-20	-30	-40	-50	Cont. 3.	-10+LN	-20+LN	-30+LN	-40+LN	-50+LN
2.5	100±0.0[x]	100±0.0	100±0.0	40±3.2	6±0.4	0±0.0	0±0.0	8±0.4	5±0.4	24±0.8	0±0.0	4±0.0
5.0	100±0.0	100±0.0	100±0.0	100±0.0	70±0.0	39±0.0	0±0.0	0±0.0	0±0.0	14±0.4	19±1.2	18±0.8
7.5	100±0.0	100±0.0	100±0.0	92±3.2	7±0.0	4±0.0	0±0.0	0±0.0	0±0.0	0±0.0	4±0.0	0±0.4
10.0	100±0.0	100±0.0	100±0.0	100±0.0	0±0.0	4±0.0	0±0.0	0±0.0	6±0.0	10±0.0	5±0.0	15±0.4
12.5	100±0.0	100±0.0	100±0.0	100±0.0	13±0.0	0±0.0	0±0.0	8±0.0	4±0.0	0±0.0	4±0.0	18±0.8
15.0	100±0.0	100±0.0	100±0.0	100±0.0	0±0.0	4±0.0	0±0.4	3±1.6	0±0.0	2±0.0	3±0.0	6±2.8
Cont. 1.	-	100±0.0	100±0.0	7±0.4	0±0.0	0±0.0	0±0.0	7±0.4	0±0.4	0±0.0	0±0.4	0±0.0

LN = liquid nitrogen storage
Cont. 1. = Control 1 = frozen non-treated control
Cont. 2. = Control 2 = non-frozen treated control
Cont. 3. = Control 3 = direct immersion to liquid nitrogen
x = Mean ± SE
LSD at 1% = 2.84% (viability)

Table 4

The effect of GLYCEROL on the viability (%) of hemp suspension cultures at different transfer temperatures

GLYCEROL %	Transfer temperature (°C)											
	Survival rate without storage in LN						Survival rate after 10 min storage in LN					
	Cont. 2.	-10	-20	-30	-40	-50	Cont.3.	-10+LN	-20+LN	-30+LN	-40+LN	-50+LN
2.5	100±0.0[x]	100±0.0	100±0.0	100±0.0	7±0.0	16±0.8	0±0.0	16±0.0	4±0.4	6±0.4	0±0.0	17±0.8
5.0	100±0.0	100±0.4	100±0.0	100±0.0	0±0.0	0±0.0	0±0.4	0±0.0	0±0.0	0±0.4	2±0.0	3±0.4
7.5	100±0.0	100±0.0	100±0.0	100±0.0	0±0.4	0±0.0	0±0.0	0±0.0	9±0.0	0±0.0	0±0.0	11±1.2
10.0	100±0.0	100±0.0	100±0.0	100±0-0	0±0.0	0±0.0	0±0.4	10±0.0	10±0.0	0±0.0	13±0.0	14±0.0
12.5	100±0.0	100±0.0	100±0.4	100±0.0	9±0.0	0±0.0	0±0.4	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
15.0	100±0.0	100±0.0	100±0.0	97±2.0	18±0.0	4±0.0	0±0.0	0±0.0	0±0.0	0±0.0	4±0.0	0±0.0
Cont. 1.	-	100±0.0	100±0.0	7±0.0	0±0.0	0±0.0	0±0.0	7±0.4	0±0.0	0±0.0	0±0.0	0±0.0

LN = Liquid nitrogen storage
Cont. 1. = Control 1 = frozen non-treated control
Cont. 2. = Control 2 = non-frozen treated control
Cont. 3. = Control 3 = direct immersion to liquid nitrogen
x = Mean ± SE
LSD at 1% = 1.19% (viability)

the lower PEG level. The viability was 35% and 20% in the case of PEG concentration 2.5% and 5% at -10 ^{0}C transfer temperature, respectively.

Similar to PEG proline (Table 3) and glycerol (Table 4) also showed some cryoprotection at the lower concentrations only. The highest viability (24%) was observed at 2.5% of proline, while the transfer temperature was -30 ^{0}C.

Glycerol did not bring about an acceptable survival rate after storage in liquid nitrogen (Table 4).

The intact hemp plant is generally very sensitive to frost or frost injury. The results presented here indicate that cultured cells retained this feature.

However, it would be premature to conclude that frost resistant species are the only ones available for freeze preservation. There are examples (e.g. date palm /10/) when tropical plant species were cryopreserved and survived after having been kept in liquid nitrogen. It can only be stated that recovery after the freeze-thaw trauma is easier achieved in the case of frost resistant species and that species sensitive to frost injury need much more attention in their freezing protocol.

Our results show that Cannabis sativa suspension cultures require the precise determination in every critical steps of freeze preservation.

Freezing cell aggregates at 5 different freezing rates resulted a zero viabillity response without any cryoprotectant treatment.

The survival rate varied remarkably when different types and concentrations of cryoprotectants were used. These also underline the importance of exact determination of the procedure. Since unfrozen samples of hemp treated with cryoprotectants grew equally well as untreated controls, it was concluded that the chemicals employed as protective agents had little or no lasting toxicity for the cells.

Surviving cells were obtained by using different concentrations of cryoprotectants, while the optimum transfer temperature varied remarkably. This observation suggest that these two factors should be studied from this respect. In other words no ideal transfer temperature could be determined without its cryoprotectant connection. By using different levels of cryoprotectants, the homogeneous nucleation temperature (T_h) -- and in this way the protective dehydration -- also takes place at different temperature, for the optimal transfer temperature is optimal only with a determined cryoprotectant level and vice-versa.

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BIOCHEMICAL INVESTIGATION OF THE RANA ESCULENTA COMPLEX IN THE KIS-BALATON NATURE RESERVE, HUNGARY

P. LŐW, M. SASS, T. CSÖRGŐ and J. TÖRÖK*

Department of General Zoology and *Department of Systematic Zoology and Ecology, Eötvös Loránd University, Budapest

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In this study it has been proved by PAGE of serum proteins that all the three members of the Rana esculenta complex occur in the Kis-Balaton Nature Reserve (Hungary). On the basis of the LDH isoenzyme pattern which is characteristic in green frogs we could distinguish all three variations of R. ridibunda and R. lessonae and one type of R. esculenta. The mobility of serum albumins on SDS-PAGE implies that the R. esculenta comes from hybridization of the two other species. The PAGE methods provide a reliable basis for the rapid taxonomic identifiaction of both adults and immature speciments of the three forms of frogs.

Keywords: Rana esculenta complex — PAGE — LDH — Albumin

Introduction

In central and eastern Europe three types of water frogs occur in broad sympatry: Rana esculenta Linnaeus (the edible frog of Europe), Rana ridibunda Pallas (the lake frog) and Rana lessonae Camerano (the pool frog). The adults are more or less easily distinguishable, the young, immature animals are, however, very similar to each other. Therefore, their identification on the basis of simple morphological criteria seems to be impossible. This causes a problem in ecological investigations of natural populations which consist mainly of immature animals. The systematic status of

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Send offprint requests to: P. Lőw, Department of General Zoology, Eötvös University, Budapest, Puskin u. 3, H–1088, Hungary

Akadémiai Kiadó, Budapest

the three taxons has long been disputed. Morphometric studies, crossing experiments /1, 2, 3, 4, 5, 6, 11, 13, 25, 28/, genetical data /12, 14, 15, 16, 17, 20, 23, 27/, experimental gynogenesis /10/ and the PAGE /9, 23, 24, 26, 30, 31/ and immunological comparison /28/ of several proteins prove that R. ridibunda and R. lessonae are two distinct species and that R. esculenta arose as a result of hybridization between the two /7/.

The current Hungarian identification guide /8/ mentions R. esculenta and R. ridibunda as two distinct species and R. lessonae as a variant of the former. Karyological investigations have documented the presence of all three forms of green frogs in eastern Hungary /20/. Moreover, they implied that these forms represent three distinct species.

The present study was undertaken to collect data on Hungarian green frogs by biochemical methods. We separated two serum proteins, lactate dehydrogenase (LDH) and albumin by electrophoresis and analyzed their isoenzyme patterns and mobility. Previous studies by others /26, 30, 31/ have already indicated that this method can be successfully applied for the taxonomic separation of the members of Rana esculenta complex. This study has also been suitable to establish the composition of the green frog population in the territory of the Kis-Balaton Nature Reserve.

Materials and Methods

The frogs were collected in the spring and autumn of 1987 on the Diás island, the only island of the Kis-Balaton Nature Reserve. The animals were transported to the laboratory and then about 300 μl of blood was collected from the brachial vein of each frog. The samples were stored for 60 min at room temperature and the serum fractions were separated by centrifugation on 5500 rpm for 15 min and stored at -20 °C. Our sample sizes were the following:

Rana lessonae 8
Rana esculenta 5
Rana ridibunda 13

For the separation of LDH isoenzymes native PAGE (7.5% at pH=8.9) system was used. After electrophoresis the gels were washed with cold 0.1 M Tris-HCl buffer of pH 8.3 and incubated for 30 min at 37 °C in a mixture of

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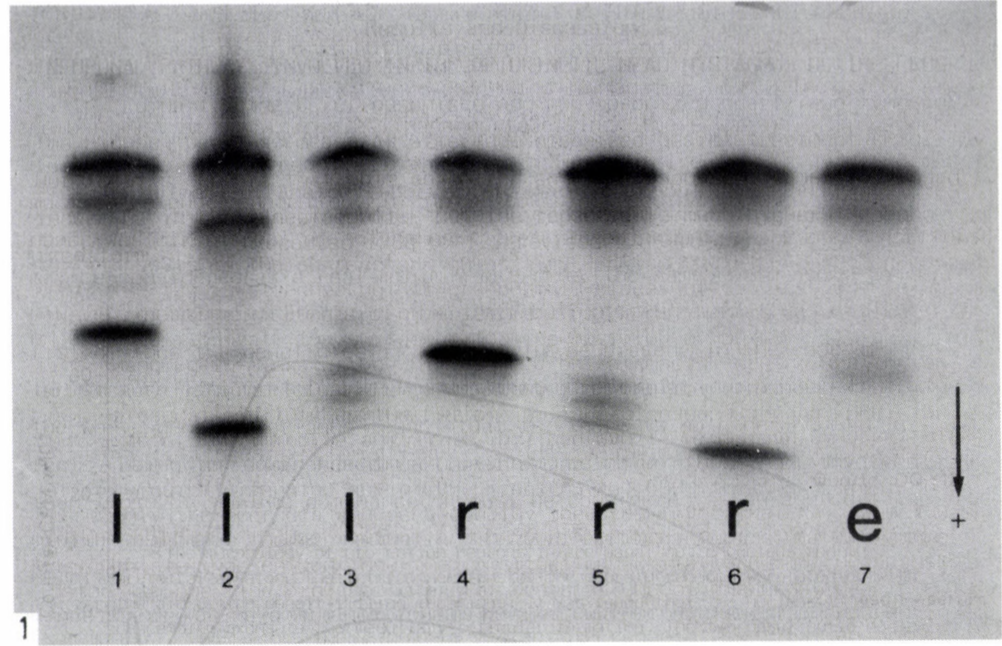
Fig. 1. The LDH isoenzyme patterns of Rana lessonae (l), Rana ridibunda (r) and Rana esculenta (e) (Numbers are explained in the text)

Fig. 2. The albumin bands (arrows) of Rana ridibunda (r), Rana esculenta (e) and Rana lessonae (l)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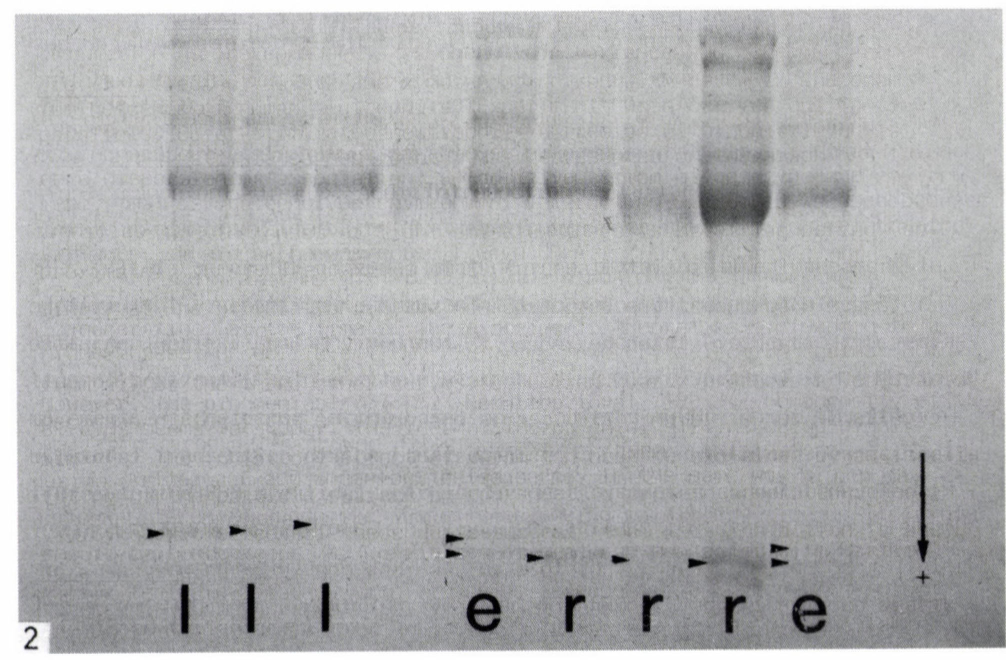
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0.036 M L-sodium lactate, 0.3 mg/ml NAD, 0.8 mg/ml nitrobulue tetrazolium and 0.14 mg/ml N-methyl-phenasonium-methosulphate in 0.5 M Tris-HCl buffer of pH 8.3 according to Goldberg /19/. For the detection of albumins we used SDS-PAGE (13%) system and Coomassie Brillant Blue R-250 staining.

Results

The LDH isoenzyme patterns found in this study are shown in Fig. 1. We identified three LDH isoenzyme patterns of R. ridibunda, three of R. lessonae and one of R. esculenta. 5 enzymatically active bands appear in two samples of either Rana lessonae (Fig. 1, No. 1, 2) or R. ridibunda (Fig. 1, No. 4, 6) of which the two outer are the most intensive. In the case of R. lessonae two of the inner bands are hardly stained, but they are observable on the original gels (Fig. 1, No. 4, 6). Further 5 enzyme containing bands appear on the other lanes (Fig. 1, No. 3, 5). This group of isoenzymes give only one wide band in R. esculenta samples (Fig. 1, No. 7).

The molecular weight of albumin found in R. lessonae is higher than that of R. ridibunda. In the blood of R. esculenta we detected both kinds of albumins (Fig. 2).

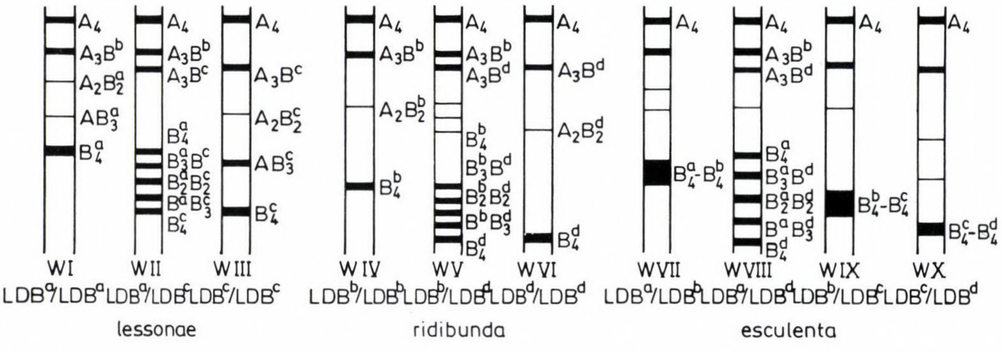

Fig. 3. The possible LDH isoenzyme bands of the Rana esculenta complex (31)

Discussion

R. lessonae and R. ridibunda are two morphologically less distinct species and they show very wide individual variations. Their hybrid form, R. esculenta has intermediate characteristics, which make this morphological sequence almost totally continuous as it is demonstrated in Table 1 taken from identification guides. Therefore, the outer morphological characteristics observable in the living individuals and representing the basis of determination of the Rana esculenta complex express only tendencial differences between the three forms. This is especially true for the immature animals, because their systematic identification on the basis of morphological signs is impossible. This problem may be overcome by PAGE of serum proteins as shown in this study.

Table 1

The morphological markers of the green frogs allowing their determination

	R. lessonae	R. esculenta	R. ridibunda
Body size	small app. 6 cm	medium app. 9 cm	large app. 12 cm
Colour	light-green without black spots	grass-green with black spots	oil-green
Skin	smooth	medium	rough
D.p./C.int. ratio	<2	2–3	2.5–4
Length of tibia	short	medium	long

The lactate dehydrogenase complex (LDH, EC 1.11.27.) of vertebrates consists of four subunits. These subunits are in most cases coded in two alleles. Product of type A (muscle) has a higher molecular weight, than that of type B (heart). When both gene products occur in one individual they may combine freely. In this way five isoenzymes with different molecular weights may be formed /18, 22/. In the case of the R. esculenta complex the situation is complicated by the fact that A genes are identical both in R. lessonae and R. ridibunda, but B genes are different and have two alleles in both species. These are B^a and B^c in R. lessonae and B^b and B^d in R. ridibunda. Five isoenzymes are formed when at the presence of A only one type

of B occurs. If both of the B types appear in one individual, they may combine with each other as well /15, 26, 30, 31/ (Fig. 3). In our samples of the Rana esculenta complex we could identify all three possible types of R. ridibunda and R. lessonae, and one of the types of R. esculenta (Figs 1, 3). From the 5 bands found in the samples of R. lessonae and R. ridibunda containing only one B product the two outer ones (A_4, B_4) are the most intensive. In the samples containing two B gene variants the inner combinations are hardly visible. According to this, either the combination of A and B is inhibited or these isoenzymes are less stabile or less active. The presence of the lighter five bands suggests that the B types combine easily among each other. In this case the two outer bands are less intensive because the homogeneous combinations of the subvariations arising in 1:1 rate are less probable. From the electrophoretic mobility the following sequence of molecular weights seems to be possible: $A \gg B^a > B^b \gg B^c > B^d$. In the parental species occur the two ones (B^a and B^c in R. lessonae and B^b and B^d in R. ridibunda) which have the most differing molecular weights. Therefore the isoenzyme containing bands appear as well separated strips. The single R. esculenta variant found in this study represents an intermediate between R. lessonae containing B^a and R. ridibunda containing B^b gene variation. In this case the isoenzymes with very similar molecular weight form a single but wide band (Fig. 1, No. 7). This observation strongly suggests that R. esculenta is a hybrid of R. lessonae and R. ridibunda. This conclusion is strengthened by our data on albumin mobility. Albumin is a major serum protein in all vertebrates with the fastest anodal mobility in electrophoresis. It is believed to have no quaternary structure and in electrophoretic patterns always appears as a single major band. Two such bands appear in heterozygotes. Although genetic variants are known variation is rare compared to some other polymorphic serum proteins like transferrin. Phylogenetically serum albumin is also conservative in its electrophoretic location /21/.

The pattern of serum albumins also suggests the hybridogenetic origin of R. esculenta. The molecular weight of serum albumin in R. lessonae is much higher than that of R. ridibunda while in the blood of R. esculenta we observed both types of serum albumin. By the PAGE methods we pointed out that R. lessonae and R. ridibunda are the parental species of the Rana esculenta complex in Hungary as well as in other countries /2, 16, 24, 25/ and that the Rana esculenta is the hybrid of them. All three forms of green frogs are spread over also the western part of this country. On the basis

of our experiences we recommend these relatively cheap, quick and expedient PAGE methods for the identification of green frogs.

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THE CORPUS LUTEUM OF THE PIG. SCANNING ELECTRON MICROSCOPIC STUDY OF SURFACE FEATURES AT DIFFERENT TIMES OF INCUBATION

EWA GREGORASZCZUK and EWA KRZYSZTOFOWICZ

Department of Animal Physiology, Laboratory of Animal Endocrinology and Tissue Culture, Institute of Zoology, Jagiellonian University, Krakow, Poland

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The surface ultrastructure of porcine early corpus luteum cells (days 1–3 of the luteal phase) was studied in SEM and correlated with progesterone secretion. Luteal cells were divided into 2 groups: small cells (10–20 μm) and large cells (20–30 μm) and their surface features were observed after 1, 3, and 5 h of incubation in the control medium and in a medium supplemented with prolactin (PRL). The surface morphology of control cells was characterized by numerous smooth blebs and the presence or absence of thin microvilli. Small and large cells showed a tendency to adhere to the glass during the experiment, but on the large cells the number of thin adhesive filopodia was greater. After the 1st and 3rd h of incubation with PRL the number of microvilli and numerous filopodia on the small cells increased substantially. Nodular blebs were scattered and appeared to protrude from the cell surface. Many small cells adhered to the glass by thick, layered and thin thread-like cytoplasmic processes. After the 5th h distinct smoothing of the surface of the small cells was seen. The number of microvilli seen on the PRL stimulated surface of the large cells was smaller and in some cases even entirely absent. After the 1st and 3rd h of the experiment the large cell surface was ruffled with minute folds. Numerous nodular blebs protruded from the cell surface. The number of adhesive filopodia attaching the cells to the glass decreased or vanished during the experiment. After the 5 h of incubation most of the cells had smooth surface with smooth blebs. Progesterone secretion was measured by radioimmunoassay. The cells in the medium without exogenous hormone (control) secreted relatively low levels of progesterone throughout 1–5 h of the incubation period. After addition of PRL to the medium the amount of secreted progesterone increased.

Keywords: Corpus luteum – Pig – Surface – In vitro incubation – Scanning electron microscopy

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Send offprint requests to: Dr. Ewa Gregoraszczuk, Institute of Zoology, Jagellonian University, Karasia 6, 30–060 Kraków, Poland

Akadémiai Kiadó, Budapest

Introduction

Ultrastructural observation of enzyme dissociated rat corpus luteum indicates the presence of 18—25 μm diameter cell population which possesses a ruffled plasma membrane as well as some blebs, ruffles and "cytoplasmic bodies" evaginating from the plasma membrane /18/. Tashjian and Hoyt /16/ established that the cells of the hormone-producing GH cell line, relatively rich in surface structure spread out and become smooth after thyrotropin releasing hormone administration. This finding demonstrates that the surface morphology of endocrine cells may change during hormone release. At the same time it suggests that scanning electron microscopy might prove an auxiliary tool in the study of hormone-producing in vitro systems. Luteal cells may also possess microvilli and intercellular canaliculi /2, 7/ as well as irregular patterns of folding of plasma membrane /9/. Centola /6/ correlated cell surface features with progesterone secretion in rat granulosa cell cultures. Studies of the surface morphology of the pig corpus luteum cells are not available so far. The purpose of this study, therefore, is to describe the fine surface structure of pig corpus luteum cells in vitro and to correlate these morphological features with progesterone secretion under the influence of prolactin (PRL).

Materials and Methods

Material

Porcine ovaries were obtained at a slaughterhouse. The approximate stage of the oestrus cycle of the ovaries was determined using criteria described by Akins and Morrissette /3/. Luteal cells were collected from newly forming corpora lutea of ovaries in an early luteal phase (days 1—3 of luteal phase).

Methods

Cells were incubated in medium 199 supplemented with 10% calf serum. Control cultures were incubated in this medium while other cultures were supplemented with 100 μg/ml prolactin. 1, 3 or 5 h later the medium was removed and frozen at -20 ^{0}C for further progesterone analysis. The cells were fixed for 12 min with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH=7.4), postfixed with 1% OsO in the same buffer. They were then rinsed and dehydrated using graded alcohols and amyl acetate and dried at the critical point in carbon dioxide. Finally, the coverslips with cells were coated with gold and examined in a JEOL SM-35 scanning electron microscope at 15—20 HV. Progesterone analysis: The media were assayed by the radio-

immunoassay for progesterone according to Abraham and co-workers /1/. In the progesterone assay a highly specific antibody was used raised in sheep against 11-alpha-hydroxyprogesterone hemisuccinate coupled to bovine serum albumin. Cross-reaction with pregnenolone was 2.9%. All related steroids that we tested was less than 1% /1, 2, 6, 7-3H/ progesterone (Amersham) (specific activity 80 Ci/mmol) was used as a tracer. Progesterone was detected directly in the culture medium without extraction. The sensitivity of the assay was 50 pg. Coefficients of variation within and between assays were less than 2.5% and 15% resepctively. The data were computed in nanograms of progesterone per 1 ml of medium during 1, 3 or 5 h of incubation. Standard error and Student test (st) were calculated to compare the secretion of PRL stimulated cultures with the control ones.

Results

Luteal cells of an early corpus luteum differ in size and therefore they were divided into 2 groups: small cells 10—20 μm and large cells 20—30 μm in diameter. In both groups of cells their surface features were observed after 1, 3 or 5 h of incubation. They were compared with the surface of control cells incubated in a similar system without prolactin.

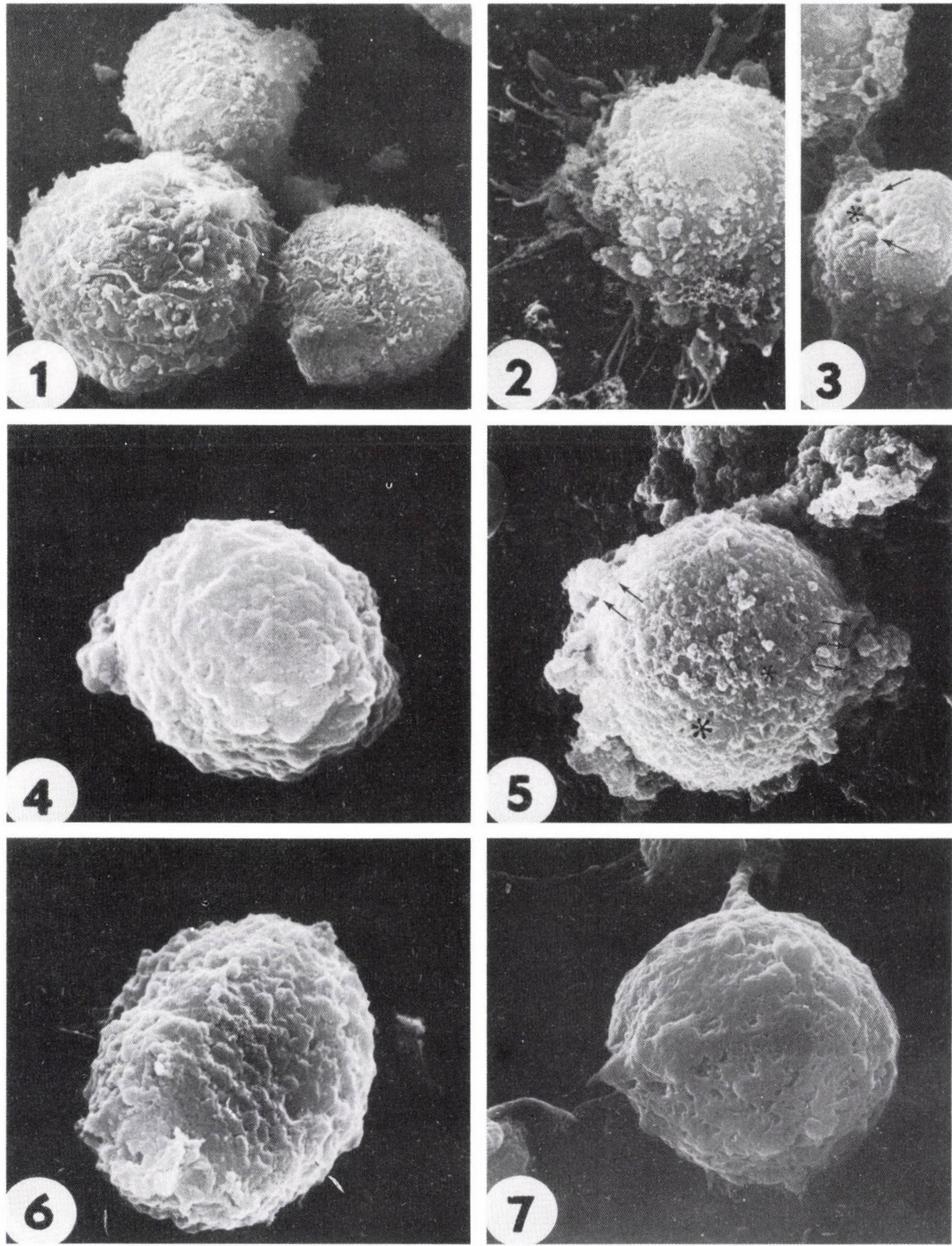
Small cells

After the 1st h of incubation in the control medium, the cells were rounded and on their surface thin and small microvilli were visible with numerous microblebs exposed on their ends. Similar microblebs and bigger blebs were visible also on the surface of slightly ruffled cells. The cells showed very little tendency to adhere to the glass without adhesive filopodia (Fig. 1).

Small cells treated with PRL possessed thicker villi ending in wide folds. On the surface of large blebs numerous new microvesicles were seen and the blebs became nodular. Many cells of this group adhered to the glass by thick, layered and thin thread-like adhesive filopodia (Figs 2, 3).

After the 3rd h of control incubation the small cells became oval and flattened with ruffled surface. Short microvilli were occasionally seen. The cells adhered to the glass without adhesive filopodia. Most of the cells had no blebs (Fig. 4).

In the experimental group, the surface of cells was still very rich. They showed numerous microblebs and short microvilli. The surface was ruffled strongly and also big nodular blebs were formed. Numerous micropits were observed between microblebs. The cells adhered to the glass (Fig. 5).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After the 5th h of control incubation, the cells were oval and flattened with more ruffled surface than after shorter control incubations. Short microvilli and little blebs were observed. The cells adhered to the glass by means of very thin, short filopodia (Fig. 6).

The cells treated with PRL showed smooth surface. The fusion of microvilli with microblebs and with nodular blebs was observed. The surface of most of cells was so smooth that they looked as if they were "pressed and polished" (Fig. 7).

Large cells

After the 1st h of control incubation, the cells were oval and the surface of the cells looked like a conglomerate of folds and furrows out of which large round lobular blebs with anodular surface protruded. Adherence to the glass by thin thread-like cytoplasmatic processes was observed. The surface was rich in numerous microvilli and additional microblebs (Fig. 8).

In the experimental group the number of microvilli decreased or vanished and from the surface there appeared numerous solid microblebs forming nodular tarsi on the surface of the cells. The cells were oval or

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Fig. 1. The small luteal cells after the 1st h of control incubation. Note the small and thin microvilli, microblebs and blebs on slightly ruffled cells'surface. X 3000

Fig. 2. The small luteal cell after the 1st h of experimental incubation with PRL (100 ng/ml). A lot of microblebs are seen on the surface. The cell adhere to the glass by thick, layered and thin thread-like adhesive filopodia. X 4500

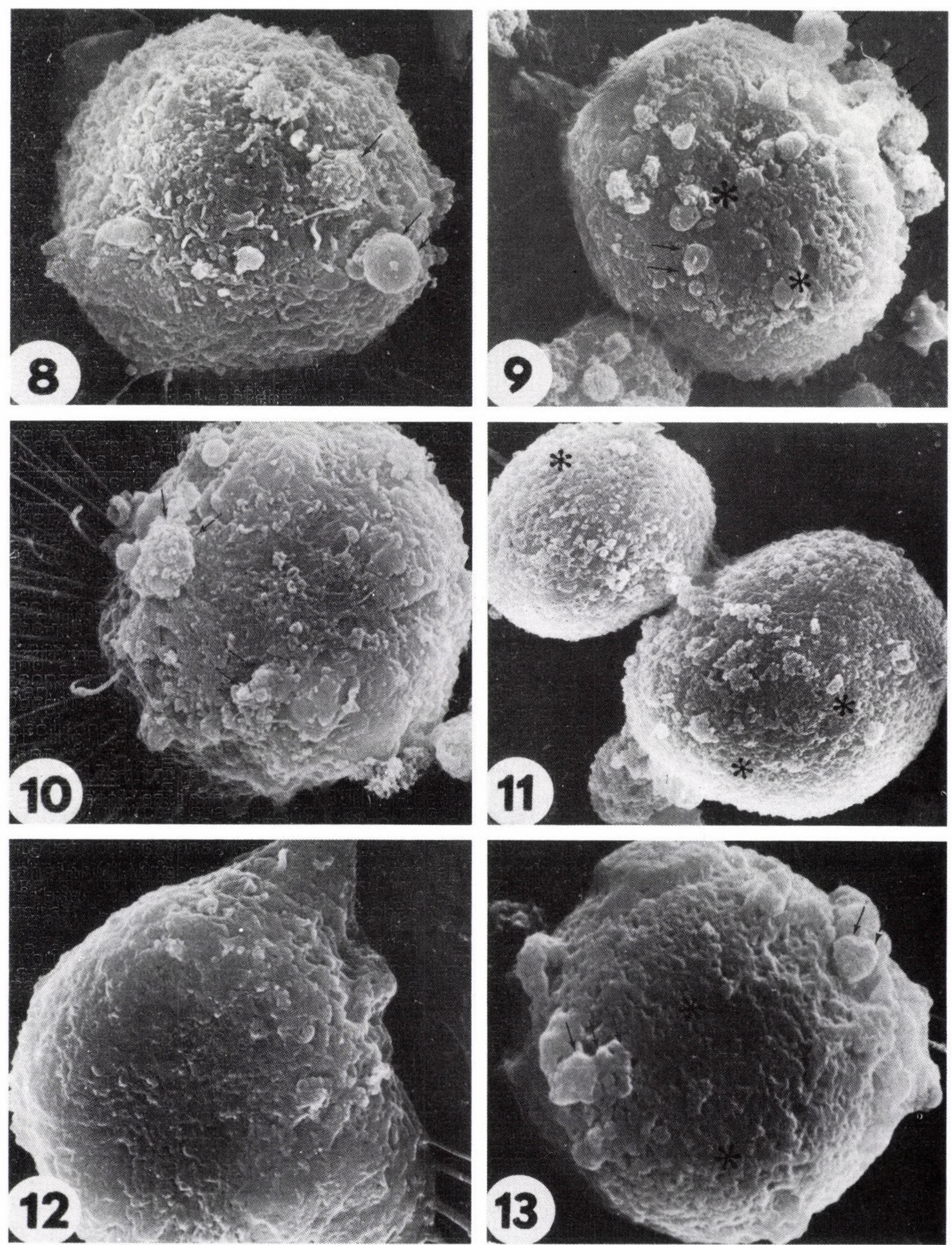
Fig. 3. The spread small cells of the lowest layer attach to the glass by extensions. Note the microblebs (arrows) and the micropit (asterisk). After the 1st h with PRL-treatment. X 3000

Fig. 4. The small control cell after the 3rd h of incubation possesses ruffled surface without microvilli. The delicate smoothness of the surface are seen. X 4500

Fig. 5. The small cell after the 3rd h of incubation with PRL. The surface of the cell are ruffled strongly. There are a lot of microblebs, nodular blebs (arrows) and micropits (asterisk) on the surface. X 4500

Fig. 6. The small cell after the 5th h of control incubation. Note the ruffled surface with some microblebs protruding from it. Very thin, thread-like adhesive filopodia are visible. X 4500

Fig. 7. Note the surface smoothness of PRL-treated cell after the 5th h of incubation. X 4500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flattened. Some micropits between microblebs were observed. The cells adhered to the glass (Fig. 9).

After the 3rd h of incubation the cells of the control group became oval and flattened and adhered to the glass more firmly. The number of thin adhesive cytoplasmatic processes increased. The numerous minute surface folds were more uniform in shape as compared to those seen after the 1st h of incubation (Fig. 10).

The cells treated with PRL were oval and the surface did not change as compared to that seen after the 1st h of incubation. The surface was ruffled strongly and formed rather the clusters of microblebs. The big nodular blebs disappeared, but a lot of micropits were observed on the surface. The cells were attached to the glass without any adhesive filopodia (Fig. 11).

After the 5th h of incubation, delicate smoothing of the surface of the cells in the control group was seen. Invariably, a reduced number of microblebs and microvesicles were seen. The cells were flattened and adhered to the glass firmly with numerous adhesive filopodia (Fig. 12).

In the experimental group, the surface of the cells became evenly smooth. The microfilaments, microblebs, as well as the surfaces of the

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Fig. 8. The large cell after 1st h of control incubation. Note the conglomerate of folds and furrows aut of which lobular blebs are protruding. The thread-like adhesive processes are visible. X 4500

Fig. 9. The corresponding cells after the 1st h of incubation with addition PRL. Numerous solid microblebs forming nodular tarsi on the surface and a lot of big nodular blebs (arrows) are visible. Some micropits between microblebs are visible too (asterisk). X 3000

Fig. 10. The large control cell after the 3rd h of incubation become lattened and the number of thin adhesive filopodia increased. The surface is very rich; nodular blebs (arrows), short microvilli and microblebs are seen. X 4500

Fig. 11. The corresponding cells after the 3rd h of PRL-incubation. Note the ruffled strongly surface aut of which the clusters of microblebes are protruding. A lot of micropits are visible (asterisk). X 3000

Fig. 12. The delicate smoothing of the surface is seen. The cell is very flattened and adhering to the glass with numerous firm filopodia. The ruffles and some microblebs are visible. The control incubation after the 5th h. X 4500

Fig. 13. The exposure to PRL during 5 h of incubation. The smoothness of the surface is visible. Smooth blebs (arrows) and a lot of micropits (asterisk) are seen. X 4500

nodular blebs looked as if they were "pressed and polished", like the surface of small cells after 5 h of experimental incubation. The shape of the cells was oval and they adhered to the glass without adhesive filopodia (Fig. 13).

Progesterone secretion

After isolation at the time "0" the luteal cells of the corpus luteum secreted 354.2 ng progesterone per 10^5 cells. With the time of incubation the accumulation of the hormone after 1, 2, and 5 h was 582.3; 1041.3; 1413.6 ng, respectively (Table 1).

Table 1

Accumulation of progesterone (ng/ml) in incubation media measured after 1, 3 and 5 h

Time of incubation	Control medium	PRL medium
1 h	582.3	1012.5
3 h	1041.3	2875.0
5 h	1413.0	2537.5

The treatment of the incubation medium with the PRL resulted in an increased progesterone secretion of 1012.5; 2875.0; 2537.5 ug after 1, 3, and 5 h of incubation, respectively. When ignoring the accumulation of progesterone and calculating secretion per/hour, a decrease of the hormone secretion was observed with the time of incubation (Table 2). Although declining secretory pattern was also maintained in prolactin treated cultures, this gonadotropin markedly stimulated progesterone secretion as compared to the control group (Table 2).

Table 2

Production of progesterone in ng/ml/1h

Time of incubation	Control medium	PRL medium
1 h	582.1	1012.5
2 h	347.1	958.3
3 h	282.0	507.4

The observed decline of progesterone secretion with the time of incubation was also manifested in the cell surface structure, which became smooth simultaneously with the decrease of progesterone secretion.

Discussion

Prolactin (PRL) is part of the luteotrophic complex necessary for the maintenance of the corpus luteum and the luteal phase progesterone secretion, not only in the rat /8, 17/ but also in a number of other species including the pig. It has been suggested /15/ that theca interna cells are the target tissue for prolactin, especially theca cells from large preovulatory follicles. In the theca cells PRL stimulated progesterone secretion as well as the aromatyse system. PRL is one of the hormones which is highly stimulatory for progesterone secretion by cells of an early corpus luteum of the pig /11, 14, 15/. PRL is though to induce synthesis of LH receptors in a fully developed midluteal phase of corpus luteum which at that time is maximally responsive to LH.

In this paper we have described hormonally induced changes of the surface ultrastructure of porcine early (days 1--3 of the luteal phase) corpus luteum cells. PRL-stimulated surface changes of luteal cells were time dependent. Surface changes in small cells were more rapid and dynamic than those of large cells. Maximal changes (stimulation) occurred 1 and 3 h after the exposure of the small cells to prolactin. After the 5th h the distinct smoothing of the surface of the small cells was seen, while the surface of "control" small cells was ruffled and short microvilli and little blebls were observed. Such a big difference between large cells after the 1st h of incubation in control and PRL medium was not observed. After the 3rd h surface of "control" cells was more rich in structures than that of PRL treated cells. Most of the large cells possessed smooth surface with numerous micropits after 5th h of PRL incubation. Delicate smoothing of the "control" cell surface was seen but no micropits were observed on it. Small and large cells showed different tendencies to adhere to the glass during the experiment. This result is in accordance with Lawrence and coworkers /13/ who observed a shape change of granulosa cells both time and dose dependent after FSH and LH administration. Centola /6/ observed that the amount of microvilli seen on the PRL stimulated granulosa cells from rat ovaries was greater than that observed in the control group. She also

observed incrased level of progesterone in cultured granulosa cells in the presence of prolactin and correlated the latter with cell surface features. In the present experiments we observed a similar relationship. The major changes on the surface of prolactin stimulated pig luteal cells after the 1st and 3rd h of incubation could be positively correlated with progesterone secretion. After the 5th h of incubation the decrease of progesterone secretion was correlated with the smoothness of cell surface.

Previous ultrastructural studies of luteal cells of the rat /5/, pig /4/ and human /2/ ovaries have shown the presence of membrane-bound granules in close proximity to the plasma membrane. Wilkinson and co-workers /18/ observed large numbers of microtubules and microfilaments associated with the inner aspect of the plasma membrane of rat luteal cells. One may therefore speculate that progesterone is secreted by diffusion of molecular size particles via the microtubular-microfilamentous system associated with the plasma membrane and microvilli protruding from the cell surface. Probably micropits observed on the cell surfaces after PRL-treatment are involved in that process.

On the other hand, during the experiment those two groups of cells may have changed their surfaces in two different ways. It is known that in young post-ovulatory corpus luteum two cell types are present not only in cell suspension prior to culture but also in monolayers of cultured cells /10/. Stoklosowa and Gregoraszczuk /15/ proved that the presence of theca cells in the forming corpus luteum and their interactions with the granulosa cells caused the sensitivity of the corpus luteum to PRL. According to Gregoraszczuk and Wojtusiak /10/ the small cells were of thecal origin. Hansel and Dowd /12/ have used highly specific labelled monoclonal antibodies to theca and granulosa cell surface antigens in an attempt to trace the contributions of the theca and granulosa to the bovine corpus luteum during the oestrus cycle and pregnancy. They proved that bovine luteal cells arised from two sources. The small luteal cells are all of theca cell origin: the large cells found early in the cycle (days 2—6) are mainly of granulosa cell origin. However, a population of large cells found after day 10 of the cycle are of the theca cell origin. These data and our findings throw a new light on the problem of the correlation of cell surface features concerning with progesterone secretion and cell derivation of the porcine corpus luteum.

Acknowledgements

The authors are grateful prof. S. Stoklosowa for her valuable comments, critical remarks and constant interest during this study. We wish to thank Dr. A. Wojtusiak for radioimmunological determinations of steroids hormones and Ms. J. Faber, assistant of the Scanning Electron Microscopy Laboratory of this Institute, for her help and maintenance of SEM facility.

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Acta Biologica Hungarica 40(1-2), pp. 157-160 (1989)

BOOK REVIEWS

TEMPORAL DISORDER IN HUMAN OSCILLATORY SYSTEMS

Proceedings of an International Symposium, University of Bremen, 8-13 September 1986.

L. Rensing, U. an der Heiden, M.C. Mackey (Eds)

Springer-Verlag Berlin-Heidelberg-New York-London-Paris-Tokyo, 1987.

This book of 259 pages contains 122 figures and 5 tables. There were 28 oral communications at the symposium. Four of them dealt with theoretical problems, 7 with neural and neuro-motor systems, 6 with circulation and respiration, 10 with circulation rhythms and 1 with ovarial rhythm. There are numerous references in each paper of the Proceedings. The participants came from the following countries: 24 from FRG 12 from USA, 3 from Belgium, 3 from Canada, 2 from Austria and 1 from the Netherlands. The book is very useful for all those who wish to be acquainted with mathematical and other methods of modelling suitable for the quantitative analysis of oscillatory phenomena in living organisms. In the theoretical part of the work the authors did not restrict themselves to the investigation of human oscillatory systems. On the other hand, all but a few of the other chapters deal with human systems and are mainly of clinical incitement. The only exceptions are the chapters discussing cellular circadian rhythms and identification of neural structures taking part in the regulation of circadian rhythmicity. A broad range of the applied mathematical methods is presented by the authors beginning from the classical spectral and correlation analysis through the correlated information content or coherence-analysis up to the partial or so-called state-dependent time delay differential equations. A remarkable feature of the book is that beside the precise mathematical description, the authors also put into plain words the essence of their methods. Due to this, the book is comprehensible and useful even to readers less or not familiar with mathematical methods.

L. Fedina (Budapest)

LIPOFUSCIN - 1987. STATE OF THE ART

Imre Zs.-Nagy (Ed.)

Proceedings of an International Symposium

Debrecen, Hungary, 26-30 August 1987.

Akadémiai Kiadó, Budapest 1988 pp 462 ISBN 936 05 4788 0

The Proceedings of the International Symposium on Lipofuscin have involved the contributions (including the discussions) of the participants in five scientific sessions. They are dealing with the following problems: theory and cellular mechanisms of lipofuscin formation; characterization of biological autofluorescent products; lipofuscin content in various animal

tissues, experimental manipulation of lipofuscin; as well as with disease-related problems of pigment accumulations. Besides, the contributions of the poster session have also been published (session VI). The texts of contributions are well documented by the tables and figures. At the end of the Proceedings there is a Keyword index and Author index.

An interesting "free radical" theory of aging and free radical diseases was reported by D. Harman. Aging is accompanied by accumulation of the waste products like lipofuscin in certain cell types. This theoretical background was explained by I. Zs.-Nagy. A proteinase inhibitor (leupeptin) model of lipofuscin formation was presented by Ivy and Gurd. Many among the contributions have a methodological character,describing laboratory procedures concerning lipofuscin presentation in various tissues.

This complex of the published data covers a multidisciplinary overview of the present situation in the lipopigment research. It is recommended for those who are interested in the topics of age- and disease-related formation of lipopigments.

Eva Reichertová (Bratislava)

ADVANCES IN LECTIN RESEARCH. Vol. 1.

H. Franz (Ed.)

VEB Verlag Volk und Gesundheit, Berlin, 1988 - pp 188, 44 Fig., 5 Tables, 2 Shem.

This book is the first volume of a three-volume monograph-like series to be published jointly by above publishing house and 4 co-editors. The other 2 volumes are to be published in 1989 and 1990, respectively. The book consists of 4 chapters. These are as follows:

1. Franz, H.: The Ricin Story; this introduces the reader into the history of lectin-research, formerly called ricin-research.
2. Rüdiger, H.: Preparation of Plant Lectins; this tells about the conditions of preparation such as extraction, purification, characterization, characterization of activity, different lectin types.
3. Driesche, E.: Structure and Function of Leguminosae Lectin; this tells about characteristic properties, different forms and mechanisms of action of particular lections of different legumes and their various organs.
4. Beurton, CR., Israel, R., Franz, H.: Illustrations of Lection-Producing Plants (I); this illustrates 24 angiospermous plant species with brief diagnoses and pictures. In these species lectins of different composition and action were found. At the same time the chapter points out that lectins were found in 80 families of phanerogamous plants and in about 100 species of algae, lichens and fungi. The widest variety of lectins occurs in higher phanerogamous families such as Leguminosae and Fabaceae.

By these review-like chapters the reader can be well acquainted with the present state of lectin research, starting from and remembering the 100 years of ricin-lectin research.

This work predicts future trends in this research field, such as the possible transfer of lectin-genes, elucidation of the mechanisms of the actions of immunoglobulines, infected conditions, drug specificity and a better knowledge of specific lectins in animals and plants.

The book will be of interest and use to specialists working in this field.

M. Maróti (Göd)

NEUROBIOLOGY OF INVERTEBRATES. TRANSMITTERS, MODULATORS AND RECEPTORS

Smyposia Biologica Hungarica Vol. 36.

J. Salánki and K. S-Rózsa (Eds)

Akadémiai Kiadó, Budapest (1988), pp 769+XV

The book contains the contributions of a satellite symposium of the 2nd IBRO Congress, held in Tihany, Hungary, last year. The meeting and accordingly the book represents the most recent event of a long series of symposia on invertebrate neurobiology, being regularly homed at the above place since 1968. The volume and its contributions offer a good overview, at least about a part, of the rapidly growing number of biologically active substances with chances to play an active role in different central and peripheral processes of neuronal regulation in invertebrates. Being the most voluminous among those published since 1968, the book covers several important and up-to-date questions emerging in connection to neuronal interaction and regulation at the transmitter, modulator and receptor levels and deals with them from physiological, morphological and biochemical aspects. Both the neurotransmitter and modulatory actions of bioactive substances are approached in a highly functional way, analyzing also their effects at the organism (behavioural) level. The major chapters of the book are the following: 1) Physiology, pharmacology and localization; 2) Peptidergic mechanisms; 3) Modulation, integration and learning; 4) Ion channels and intracellular mechanisms. In addition, poster presentations of the symposium are also contained by the book in the form of short communications. A subject index helps the reader to have a quick orientation in the details of the content.

With its glossy paper and generally good quality of figures, the appearence of the book is impressive. Some of the electron micrographs do not, however, correspond to international quality.

The book can be recommended to everybody working in any field of invertebrate neurobiology or being interested in following recent progress in comparative neurobiology.

K. Elekes (Tihany)

OXYGEN-FREE RADICALS AND TISSUE INJURY

Matkovics, D. Boda and H. Kalez (Eds)

Akadémiai Kiadó, Budapest 1988,pp 412

The volume contains the material of a two-day Symposium held in Hungary, January 1986. (I cannot resist to remark that it is unacceptable that

the publication of a book like this takes two years. Even in Hungary it can be achieved in six months.) This colloquium was preceeded two years earlier by another meeting at Pécs, 1984 on similar topics. It shows that there is a large number of scientists in Hungary with common interest working in a similar field of research.

There are 44 lectures in the book in alphabetical order of the first authors. It would have been desirable to create groups according to various aspects of research. Almost all of the papers were coming from Hungarian laboratories. One feels that it would be desirable in the future to put the results to international evaluation.

The papers have different formats, several of them can be looked upon more as extended abstracts than real publications in a journal (with referees) with detailed description of the methods. The topics of the lectures concerned cell protection to radiation with compounds having antioxidant properties, oxidative cell damage of newborns and the possibility of their protection. The applied experimental systems included not only animals but also plants.

Most of the papers are of applied nature, few of them treated fundamental questions.

The book is a good mirror of the research being done in Hungary on the biological significance of free radicals in human (and animal) physiology and pathology.

G. Szabó (Debrecen)

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Acta Biologica Hungarica 40(3), pp. 161—193 (1989)

THE ROLE OF GONADOTROPIN RELEASING HORMONE (Gn-RH) IN THE REGULATION OF GONADAL FUNCTIONS OF BIRDS
REVIEW ARTICLE

P. PÉCZELY

Agriculture University, Laboratory of Geese Biochemistry,
Gödöllő, Hungary

(Received: 1988—11—15)

Gonadotrop hormone secretion is regulated by the central nervous system through the hypothalamus. This neuro-hormonal regulation was first verified in birds by Follett /21/ who was able to increase the LH secretion of hypophysis in vitro by crude extract of quail hypothalamus. His results supported the indirect statements of earlier neuroendocrine studies and emphasized the importance of bird hypothalamus in the regulation of gonadal function /1, 62/.

A neurohormone fundamental in the central regulation of gonadic function, luteinizing hormone releasing hormone (abbreviated earlier as LH-RH, but recently, and, thus, hereinafter as Gn-RH) has first been isolated from porcine hypothalamus in Schally's Laboratory /41/, and, following the determination of its amino-acid sequence, it has been synthesized in the same year /42/.

It has been stated that this peptide, consisting of 10 amino acids (p Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), increases the LH and FSH secretion of the hypophysis both in vitro and in vivo. One year later, a decapeptide, similar in its structure to porcine Gn-RH was produced from sheep hypothalamus. Investigations of the two teams suggested that decapeptide containing arginine on place 8 was the physiological Gn-RH of mammals.

Keywords: Gonadotropin releasing hormone — hypothalamus — birds

1. Isolation and structural parameters of bird Gn-RH

Investigations concerning the isolation and structural problems of bird Gn-RH were started simultaneously by two different teams, Millar and King, in South-Africa, and Miyamoto, et al., in Japan, independently of each other. These studies were started with several ten thousand chicken

Send offprint requests to: Dr. P. Péczely, Agriculture University, Laboratory of Geese Biochemistry, H—2103 Gödöllő, Hungary

Akadémiai Kiadó, Budapest

hypothalamuses, and a few μg purified decapeptide was obtained in 1982. Their concordant results suggested that chicken Gn-RH differs from natural mammalian Gn-RH only in amino acid 8, which is arginine in mammals, and glutamic acid in chicken /33, 34, 49, 50/.

The two teams used similar methods for extracting isolating and purifying chicken Gn-RH. Fresh hypothalamus tissues were freeze-dried, degreased by petroleum ether, then extracted by acetic acid. After membrane filtration, the supernatant was freeze-dried again, then it was solved in acetic acid or ammonium acetate.

Miyamoto, et al. /49/ chromatographed the prepurified substance on Sephadex G-25, and tested the Gn-RH activity of each fraction by FSH and LH production of monolayer cell culture of rat hypophysis. After freeze-drying, the active fraction was taken to 10 mM ammonium formate, and was chromatographed on CM 52 cellulose by linear increase lo mM ⟶ 0.5 M concentration of the ammonium formate at pH = 4.6. The active raw eluate obtained from the column was further purified in 50 mM KH_2PO_4-H_3PO_4-H_3PO_4-CH_3CH linear system (pH = 2.0). The last purification was made on μ-Bondapack C-18 column in 10 mM ammonium formate CH_3CN linear gradient system (pH=4.0). This step was followed by the analysis of purified peptide amino acid.

In the system of King, Millar /33, 34/ the prepurified Gn-RH active substance was further purified first by immune affinity chromatography (by connecting rabbit gamma globulin (anti Gn-RH) to Sepharose 4B activated with cyanogenic bromide). Unbound peptides in the system were eluated by 0.5 M ammonium acetate, while immunoreactive Gn-RH by 1.5 N acetic acid. The Gn-RH activity of the fractions was tested by RIA, and the active fraction was freeze-dried.

Characteristics of Gn-RH active peptide, as well as its similarities and differences to the synthetic mammalian Gn-RH were clarified by means of various chromatographic systems (Sephadex G-25 superfine eluation with 2N acetic acid; by reverse phase HPLC with 0.01 M ammonium acetate and linear gradient (22-80%) acetonitril (pH = 4.0); by cation exchanging HPLC with 0.2 M ammonium acetate 10% ethanol (pH = 4.6); by CM 32 carboxymethylcellulose chromatography with 0.002 and 0.06 M ammonium acetate (pH = 4.5).

Finally, they compared the isoelectric focusing of chicken Gn-RH, and synthetic mammalian and synthetic Gln^8-Gn-RH.

At this time, the biological Gn-RH activity of the purified peptide was still not investigated by the South-African team.

The two teams had the following results concerning the purified bird (chicken) decapeptide:

1) On Sephadex G-25 and from Bio-Gel P-2 column, the immunoreactive Gn-RH of the chicken hypothalamus is eluated with synthetic mammalian decapeptide in one peak. This indicates that the molecular size of the two peptides is nearly similar.

2) On reverse phase HPLC, the immune reactive chicken Gn-RH appears in one peak, which precedes the mammalian Gn-RH. The peptide behaves similarly in cation exchange and cyrboxymethylcellulose chromatography. These observations refer to structural differences in the two Gn-RHs, and verify that chicken peptide has lesser positive charge.

3) Isoelectric focusing suggests that the chicken Gn-RH is less basic (pH = 7.3) than the mammalian one (pH = 9.1).

4) The use of different antisera showed that cross reactions with specific antibodies against p Glu (1), His (2), Pro (9) and Gly-NH_2 (10), further against p Glu (1) and Gly-NH_2 (10) (displacement curves parallel to those at synthetic mammalian LH-RH) were similar with chicken and synthetic mammalian Gn-RHs. On the other hand, cross reactions showed definite differences between chicken and synthetic mammalian Gn-RHs with antisera against Trp (3), Gly (6), Pro (9), and Trp (3), Gly (6), Leu (7), Arg (8), Pro (9), as well as Arg (8), Pro (9), Gly-NH_2 (10). Thus, the use of different antisera suggested that difference appears in amino acids 6-10 between the two releasing hormones.

5) The chromatographic and immunological comparison of natural chicken and synthetic Gln 8-Gn-RH showed perfectly identical behaviour of the peptides (reverse phase and cation exchange HPLC, different Gn-RH antisera, and isoelectric focusing). The results obviously suggested that chicken Gn-RH differs from (synthetic) mammalian decapeptide only in one amino acid, and this is the glutamic acid in position 8.

Later on, starting from a higher quantity (249 thousand) chicken hypothalamus, King and Millar /34/ produced 33.7 μg immunoreactive Gn-RH by the previously developed method, by obtaining a two millionfold purification from 7.5 g peptide. This decapeptide proved to be homogeneous during the control amino acid analyses, its chromatographic parameters (in cation exchanging and reverse phase HPLC) and its isoelectric point showed to be fully identical to the synthetic Gln8-Gn-RH. Thus, the control test has made it unambiguous that bird (chicken) Gn-RH differs from the synthetic mammalian releasing hormone only in amino acid 8, which is arginine in

mammals, and glutamic acid in chicken. Miyamoto, et al. /51/ have soon revealed that in the course of separating and purifying chicken Gn-RH — in the early phase of the process — another peptide of Gn-RH activity can also be separated. From the SP-Sephadex G-25 column applied, Gn-RH I was eluable with 2 M pyridine at pH = 5.0, while the later detected Gn-RH II was eluated with 2 M pyridin acetate buffer at the same pH value. In cation exchanging HPLC system, this peptide showed very intensive hydrophibic relation to the column. Final purification of Gn-RH II was performed on reverse phase HPLC.

The amino acid analysis of Gn-RH II verified that also this substance is a decapeptide. The accurate structure was determined on the basis of amino acid composition of peptide fragments created by chymotrypsin and thermolythic digestion through terminal analysis. Accordingly, in this decapeptide, amino acids differing from the mammalian Gn-RH can be found in places 5, 7 and 8 (Fig. 1).

Both teams have also produced chicken Gn-RH(s) synthetically, and thus, sufficient material was available for biological tests (in vitro and in vivo). King, Millar /33, 34/ used the solid phase method, and Miyamoto et al. /50/ the solution method for the synthetic production of chicken Gn-RH I. Chicken Gn-RH II was synthetized by the solid phase method by Miyamoto et al. /51/.

2. In vitro effect of bird and mammalian Gn-RH and of Gn-RH analogs

The biological effect of natural and synthetic Gn-RHs is controlled by two in vitro methods. In the course of purification and first syntheses of chicken decapeptides, the two teams regarded the degree of gonadotropic hormone release from isolated hypophysis cells as the biological measuring method of Gn-RH activity providing specific and quantitative information. Cells obtained from mammalian (rat and sheep) and bird adenohypophysis were used either in dispersed form or in cell cultures. LH adn FSH content of the incubation liquid was determined by a specific RIA method.

In rat hypophysis cell culture, chicken Gn-RH I caused dose-dependent stimulation to both LH and FSH production. The degree of stimulation was, however, only 1/25 rate of gonadotrophic hormone production increase elicitable with mammalian Gn-RH /49, 50/. The comparison of the two bird Gn-RH in rat hypophysis cells showed that the chicken Gn-RH II is about 8 times more active than chicken Gn-RH I, but the LH- and FSH-releasing

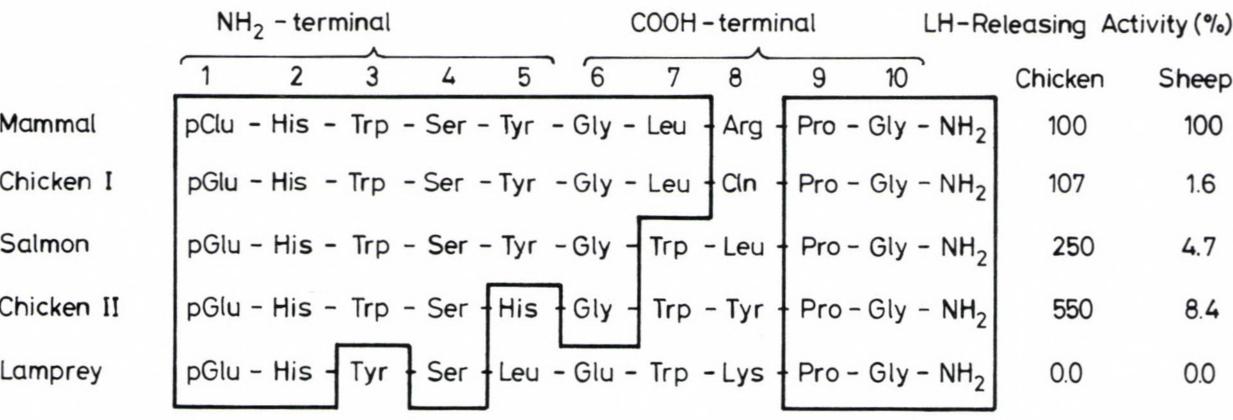

Fig. 1. Structure and LH-releasing activities of vertebrate Gn-RH's. (After Millar et al. /48/)

effect of this is still only one third of the similar effect of synthetic mammalian Gn-RH /51/.

The use of sheep hypophysis cell cultures gave the same results. The LH releasing activity of synthetic chicken Gn-RH was only the one hundredth of the mammalian Gn-RH /45/.

According to Johnson et al. /28/ the optimal effectivity of Gn-RH preparations is mostly the function of the concentration applied. With chicken hypophysis cell concentration less than 1 $\mu g/2x10^5$ cells, Gln^8-Gn-RH has significantly stronger LH-releasing effect than Arg^8-Gn-RH. on the other hand, in higher concentrations (10 $\mu g/2x10^5$ cells), the bird Gn-RH I and the mammalian Gn-RH have similar LH releasing effects.

These bioassays — aimed at the intensifying of gonadotropin secretion — have verified that bird Gn-RH results only in little release of gonadotropin from mammalian hypophysis, and it showed no obviously higher activity even in bird hypophysis cell culture (Gn-RH I was tested) than the mammalian Gn-RH.

Another method used for testing the biological activity of Gn-RH(-s) is the radioreceptor assay. Crude membrane pellet, obtained from rat and chicken adenohypophysis was used, which was saturated with 125-I labelled synthetic mammalian Gn-RH or with one of its agonists. Incubation was performed in the presence of increasing concentration of the test peptide (chicken Gn-RH I and II, and mammalian Gn-RH), and the degree of competition of the labelled ligand was determined.

The receptor binding activity of chicken Gn-RH I with rat hypophysis membrane preparation was only 1/500th part of the mammalian Gn-RH. Gn-RH II is significantly more effective than Gn-RH I, but even this value is only 1/8th part of the receptor binding activity of the mammalian Gn-RH /47/. Due to the weak binding capacity, a relatively high amount of chicken hypophyseal cell membranes was needed, and this resulted in a high increase of non-specific binding, and thereby the decrease of measuring accuracy. Nevertheless, results suggest that synthetic chicken Gn-RH I and synthetic mammalian Gn-RH are bound to the Gn-RH receptors of the chicken hypophysis with nearly similar affinity /45/.

As far as we know, no comparisons were still performed between the binding of the two chicken Gn-RHs to receptors in bird hypophysis membrane preparations.

The comparison of mammalian and bird Gn-RHs in receptor assays suggests that mammalian Gn-RH receptors respond sensitively and specifically

to the structure of amino acid 8. Maximal receptor binding occurs always when this amino acid is arginine.

In the background of this phenomenon we can see that between His^2, Tyr^5 and Arg^8 a combined stabilizing hydrogen bond develops which ensures molecular stabilization required for biological activity /61/. According to data available up to now, such structural stability is not needed in birds /46/, i.e. the Arg can be substituted with two amino acids without the decrease of LH-releasing activity (Gln^8 or Phe^8-Gn-RH). With the use of other substituents, however, the LH secretion of test chicken hypophysis cells decreased (Met^8, His^8, Leu^8: to 30%; Ser^8, Trp^8, Cit^8: to 10-20%), and the Asn^8 and Glu^8-Gn-RH had even negligible LH-releasing activity.

According to the recent results of Millar et al. /47/ the mammalian Gn-RH receptor is highly discriminatory while the avian one is more promiscuous. In position 8 any basic or neutral residue is acceptable for chicken receptor. His^5 substitution in mammalian Gn-RH stabilize its capacity but leads to poor activity in avian releasing hormon. Trp^7 substitution is acceptable to both receptors.

It has become obvious during in vitro testing of the biological activity of Gn-RH preparations that the determination of gonadotrop releasing and receptor binding activity leads to different results in quantitative respects. Chicken Gn-RH I provides only 2% of mammalian Gn-RH releasing activity if dispersed sheep hypophysis cells are applied. At the same time, its receptor binding activity is only 0.2% of the mammalian Gn-RH /47/. Very similar results were obtained in rat hypophysis cell cultures: the releasing effect was 2.5%, while the receptor binding activity was 0.5% /25/. It must be, however, emphasized that chicken Gn-RH II, which is significantly more active than Gn-RH I, has almost double-sized receptor activity than the LH-releasing activity measured in sheep hypophysis culture /47/.

The cause of differences in results obtained by the two in vitro methods is not to be attributed to the relatively short biological half-life of Gn-RH, because 95% of the decapeptide was still regainable from the medium at the end of the incubation period. Probably, the cause for this difference is that receptor binding has a competitive character, while the increase of releasing potential has not /46/.

The gonadotropin releasing potential of Gn-RH results in both FSH and LH release. According to Hattori, et al. /26/, chicken Gn-RH I and mammalian Gn-RH (exerting similar intensity) elicit lesser degree FSH and

higher degree LH release in in vitro incubated Japanese quail adenohypophysis fragments. Measurements with dispersed hypophysis cells and cell cultures generally showed that mammalian Gn-RH and their agonists caused FSH and LH relase increases of similar intensity. The same effect was elicited by chicken Gn-RH I /47/. However, in vitro measurements of the same authors with chicken Gn-RH II showed that this decapeptide has 2.5 times higher FSH than LH-releasing potency. According to Miyamoto et al. /51/ the FSH releasing activity is higher by only 28% than the LH releasing effect.

The comparison of the biological effect of Gn-RH from various vertebrates showed that changes in position 7 lead to effectivity increase. Perhaps this explains for the fact that Trp^7-Gn-RH in salmons shows a 2.5 times higher LH-releasing potential, and 1.8 times higher FSH-releasing potential than chicken Gn-RH I, in chicken hypophysis cell cultures /46/. It seems probable that Trp^7 forms a "stabilization bridge" at Leu^8 in salmons, and at Tyr^8 in chicken Gn-RH II.

Similar, but less effective activity increase occurs when His substitution is applied in position 5 of chicken Gn-RH II. At the same time, with other Gn-RHs, His^5 substitution caused a decrease in the gonadotropic releasing activity of the decapeptide in chicken hypophysis cell cultures /46/.

The biological half-life of gonadotropic releasing hormones is very short, it can be measured in minutes. The biological half-life of chicken Gn-RH I and II was determined (in vivo) by Sharp et al. /59/ in domestic hen and cock. Their data suggest that the biological half-life of the two bird Gn-RH is similar, approximately 3 minutes, and is not modified by the sex of the birds.

To inhibit quick elimination, it is common to make three types of structural changes in mammalian and synthetic Gn-RHs: D amino acid substitution to position 6, N-methyl-leucin build-up to position 7, and transformation of Gly^{10} into ethylamide. These transformations lead to the synthesis of "superactive" analogs, which have longer biological half life, and higher receptor binding capacity, too. These analogs result in two or three times longer (prolonged) LH release in in vitro systems.

The effect of several such "superactive" Gn-RH analogs were also studied in birds. Most of such investigations, however, were focused on the modification of some physiological function, i.e. in vivo data provide only little information about the relation between the chemical structure and the LH releasing or receptor binding affinity. Therefore, we will deal with

these in vivo studies in connection with the physiological regulating role of Gn-RH.

There are relatively few Gn-RH analogs, the (releasing or receptor binding) effects of which were studied in vitro. Bonney, Cunningham /3/ compared the LH-releasing activity of Gn-RH vs. des-Gly^{10}-Gn-RH and Phe^{5}-Gn-RH in isolated chicken hypophysis cells. The des-Gly^{10}-Gn-RH proved to be 1.5 times more active, and Phe^{5}-Gn-RH 2.37 times more active than the mammalian Gn-RH. The LH-releasing activity was dramatically decreased by tyrosin connected to the Phe^{5}-Gn-RH in position 11 (0.9%), thereby indicating the importance of the decapeptide structure.

Millar and King /46/ compared mammalian and chicken Gn-RH I activity to their D-Trp^{6} analogs in chicken and rat hypophysis systems. As compared to mammalian Gn-RH, LH-releasing activity had increased to 26 times in chicken system, and to 36 times in rat system (with both analogs). In the rat receptor binding assay, the chicken Gn-RH I AD-Trp^{6} analog proved to be 2.3 times more active, while the mammalian analog 28 times more active than the mammalian Gn-RH.

Millar et al. /47/ studied the activity of chicken Gn-RH II active analog (D-Arg^{6}-Gn-RH II) in chicken, sheep and rat hypophysis systems. The agonist has 18.3 times higher LH, and 26.4 times higher FSH-releasing effect in chicken system than the mammalian or chicken Gn-RH I releasing hormone. With sheep hypophysis cells, the LH-releasing potency is only 71% of the mammalian one, but this is 8.5 times higher than that of the basic molecule (chicken Gn-RH II). Concerning receptor binding activity, D-Arg^{6}-Gn-RH II proved to be 6 times more active in rat system than the mammalian Gn-RH, and 46 times more active than the chicken Gn-RH II. With D-Leu^{6}, des-Gly-NH_2^{10}-Gn-RH mammalian hypophysis cells it was 26 times more active than the mammalian Gn-RH, but it was hardly more effective than that in chicken in vitro system (Hasegawa et al. /25/). Similar results were reported by Millar and King /46/ who found D-His^{6}(Im-Bzl)-Gn-RH 50 times more active in mammalian system, and only 5 times more active in chicken in vitro hypophysis culture than the natural mammalian Gn-RH.

These data predict that Gn-RH analogs substituted with D-amino acids in position 6 will have superactive properties in mammalian and bird hypophysis cells. Results of these experiments also suggest that we can expect different degrees of responses in different species. Gn-RH antagonists are synthetic analogs which are bound with high affinity to the receptors of gonadotropic cells, but they have little or no biological

activity. Millar and King /46/ also tested five Gn-RH analogs of antagonist effect of these analogs on spontaneous LH release, and their effect on the modification of LH secretion stimulated with chicken Gn-RH I previously used in 10^{-9}M concentration. The concomitant evaluation of the two effects showed the following effectivity precedence: The most effective inhibition was exerted by (D-pGlu^{1}. D-Phe^{2}, D-Trp^{3,6})-Gn-RH, which showed 50% inhibition on the LH release in $5 \cdot 10^{-8}$M concentration, and its spontaneous LH releasing effect was negligible. (Ac-D-p-Cl-Phe^{1,2}, D-Trp^{3,6}, D-Ala^{10})-Gn-RH, (Ac-D-Phe^{1}, D-pcl-Phe^{2}, D-Trp^{3,6}, D-Ala^{10})-Gn-RH, (Ac-D-pcl-Phe^{1,2}, D-Trp^{3}, D-Phe^{6}, D-Ala^{10})-Gn-RH showed ever less inhibiting effects. These showed an antagonist effect that was in several cases different than that observed in the rat hypophysis cell culture, which again suggests the difference of Gn-RH receptors in birds as compared to mammals.

3. In vivo effect of natural mammalian and bird Gn-RHs, synthetic agonists and antagonists

Many tests were performed in birds to determine the effect of Gn-RHs analogs on LH, FSH and sexual steroid secretion. Most of these studies were focused on the LH-releasing effects of Gn-RH compounds. The results suggest that the LH plasma level elevates significantly within 5 to 10 minutes on the effect of natural or synthetic Gn-RHs. This high LH level shows a remarkable decrease within 15 to 30 minutes, and returns to the initial value within 1—2 1/2 hours /8, 10, 23, 24, 26, 66, 74/. Most of the experimental results apply to the domestic hen, but studies were also performed in Japanese quail, turkey and white crowned sparrow.

In domestic hens the effective dose is between 0.5 and 20 µg/kg. Most of the data suggest that a specific dose-and-effect relationship can be seen in male birds, both in the increase of LH and sexual secretion (in domestic cock: Furr et al. /23/, Sterling and Sharp /66/, Péczely and Gombos, unpublished data; in Zonotrichia: Wingfield et al. /74/).

In female birds, the first reports also argue for a dose-dependent ovulation inducing effect of Gn-RH /69/. Recent data suggest, however, that the premature ovulation inducing effect of Gn-RH depends not as much on the dose, but rather on the actual physiological state of the ovaries /19, 20/. The application of 10-100 µg/kg and 100 µg/kg doses of synthetic Gn-RH and an agonist, LD-Ser-But^{6}-Az-Gly^{10}-Gn-RH proved that there is no dose-and-

effect relationship in the domestic hen concerning the increase of plasma LH, progesterone or 17-β-estradiol level /24/.

Most of the experiments were focused on the comparison of the effectivity of mammalian and chicken Gn-RHs vs. synthetic analogs. In these studies the dose-and-effect relationship was investigated in male and female birds, and Sterling and Sharp /66/ compared the LH-releasing effect of porcine Gn-RH, superactive Buserelin (D-Ser-But^{6}-des-Gly^{10}-Gn-RH) vs. chicken Gn-RH I. Similar LH release was elicited by porcine and chicken Gn-RH I. Similar LH release was elicited by porcine and chicken Gn-RH, but Buserelin was twice as effective in cocks. In laying hens, higher effectivity could be detected only with Buserelin, and no difference was seen in the effect of avian and mammalian Gn-RH. Similar findings were seen in male Japanese quails by Hattori et al. /26/: concerning LH and FSH release, chicken Gn-RH proved not to be more effective than the synthetic mammalian Gn-RH. The dose-and-effect relationship was more marked when the mammalian Gn-RH was used. LH and FSH maximums appear in the plasma within 5 minutes, and this is 20 to 30 times higher than the basic value in the LH, and only 2 to 5 times higher in the FSH.

In vivo comparative studies of the two bird Gn-RH were made by Chou et al. /10/ in cocks, and by Sharp et al. /59/ in cocks and hens. Chou et al. /10/ found that chicken Gn-RH I and II have similar LH-releasing effect in subcutaneous doses of 1 μM and 10 μM. According to the same authors, Gn-RH II proved to be 4.7 times more active under in vitro conditions. Differences between the in vitro and the in vivo effects are presumably due to the higher receptor activity and quicker enzymatic degradation of Gn-RH II. Similar results were reported by Sharp et al. /59/, who found that the two chicken Gn-RH had similar LH-releasing effect in cocks also with intravenous administration. Gn-RH II, however, caused 36.5 times higher LH release in hens than Gn-RH I. According to the above authors, the two Gn-RHs have similar biological half-life, therefore, the sexual difference in the mechanism of effect is presumably due to the different Gn-RH receptor affinity of gonadotropic cells.

Present observations of Sharp et al. /60/ show that the hypothalamic Gn-RH I content of cockerels significantly increased at 15 weeks of age, but did not alter the Gn-RH II content. Similarly, castration increased only the Gn-RH I, and not the Gn-RH II content of hypothalamus. In laying hens the immunization against Gn-RH I stopped the laying, but the animals immu-

nized against Gn-RH II continued to lay. These results suggest that Gn-RH II is not a physiological gonadotropin releasing hormone.

Gn-RH effects can be remarkably modified also by the age and actual physiological condition of the bird in addition to its sex. In infantile or premature chicken (8 to 10 weeks before starting laying), the plasma LH level is increased to 6-7 times higher by 10 µg/kg mammalian Gn-RH. At the start of maturation (when the LH level starts to decrease), only 1.5—2 times higher LH concentration increase occurs on Gn-RH stimulation. In the rapid growth phase of follicles (1 week before the start of egg laying), the LH response to Gn-RH shows further decrease /72/.

The Gn-RH stimulation test used at the time of egg laying showed that there is no difference in the degree of LH release between young hens of higher laying capacity and elderly hens of lower capacity /70/. Mature domestic hens /58/ and turkeys in longer pause of egg laying, however, respond with higher LH release to single intravenous injection of synthetic Gn-RH than the egg-laying birds.

Breeding and nestling quiding bantam hens also show higher LH level elevation after Gn-RH stimulation than the egg-laying birds /58/. These results suggest that in mature female birds the hypophysis is less sensitive to exogenous Gn-RHs in the egg-laying period than in the interval when there is no periodic (preovulational) release of endogenous Gn-RH. It is probable that the Gn-RH sensitivity of the hypophysis is decreased by the high estrogen level in egg-laying birds. The degree of Gn-RH stimulated LH release is decreased by estrogen treatment in ovariectomized hens /71/ and in cocks /38/. In male birds the LH plasma level is lower in still unmatured ones than in adults, and this shows higher (4—5 times) elevation to Gn-RH than in sexually active animals where this increase is only 2—3 times higher /11, 59/.

LH release, is, however, not increased, but dramatically decreased by drastic reduction of the androgen level after Gn-RH stimulation.

The degree of LH level elevation is decreased to one third after castration in cocks /23/, and blocks the elevated Gn-RH sensitivity following photostimulation in Japanese quails /15/.

Photorefractivity does not affect the Gn-RH sensitivity of the hypophysis in male birds. Sexually active and photorefractory Zonotrichia /74/ and ganders /53/ show similar response to exogenous Gn-RH. This observations, however, suggest that also a seasonally minimal androgen level is sufficient to maintain the Gn-RH sensitivity of the hypophysis.

The effect of Gn-RH on the sexual steroid plasma level was studied mainly in male birds. Gn-RH analog administered in 0.5 to 20 μg/kg does ($D\text{-}Lys^{6}$-Gn-RH-EA), and synthetic mammalian Gn-RH in the same dose starts plasma testosterone level elevation within 10 to 15 minutes, which reaches its maximum within one or two hours. This is generally 3 to 5 times higher than the initial value /11, 56, 64/, but also extreme increases were reported (14 times increase in infantile guinea hen /40/). The androgen level returns to the initial value within 4 to 6 hours /56/.

The comparison of testosterone secretion modifying effect of 10 Gn-RH agonist and 13 antagonist was performed vs. synthetic mammalian Gn-RH in mature cockerel. (Péczely, Seprődi, Gombos, Muray, unpublished data.) Gn-RH and all analogs were injected i.v. in a single does (0.5 μg/kg). Plasma testosterone concentrations were determined in minutes 0, 10, 30, 60 and 120, and were expressed in the percentage of the "0-minute control". The results suggest that the synthetic mammalian Gn-RH, the $D\text{-}Phe^{6}$-des-Gly^{10}-Gn-RH-EH, D-parachlor-Phe^{6}-Gn-RH and the $D\text{-}Phe^{6}$-Gn-RH had the most intensive stimulating effect. These cause a 2.8 to 3.4 times plasma testosterone level elevation within 30 to 60 minutes after the injection. $D\text{-}Asp^{6}$-Gn-RH-EA has a specific prolonged effect, and shows a weak initial stimulating effect, then a 4.3 times testosterone concentration elevation within 60 to 120 minutes. Two Gn-RH tetramers, and the D-$thyroxin^{6}$-Gn-RH showed, however, negligible testosterone plasma level elevating effect (Fig. 2).

Compounds showing previous Gn-RH antagonist effect in rat (inhibition of ovulation) /37/ did not prove to be uniformly inhibitors under in vivo conditions in cockerels. The following compounds had intensive plasma testosteron decreasing effect: Ac-D-Lys^{1}-D-Cpa^{2}, D-$Trp^{3,6}$, D-Ala^{10}-Gn-RH, and Ac-D-Trp^{1}, D- pa^{2} (L-β-Asp (1-Ind) $)^{3}$D-Phe^{6}, D-Ala^{10}-Gn-RH, and Ac-D-$Cpa^{1,2}$, D-Trp^{3}, D-Phe^{6}, D-Ser^{10}-Gn-RH, which decreased the androgen concentration by min 50% within 60—120 minutes after the injection. Compounds Ac-Sar^{1}, D-Cpa^{2}, D-Trp^{3}, D-Phe^{6}, D-Ala^{10}-Gn-RH, Ac-Gly^{1}, D-Cpa^{2}, D-Trp^{3}, D-Phe^{6}, D-Ala^{10}-Gn-RH, Ac-D-$Cpa^{1,2}$, D-$Trp^{3,6}$, D-Lys^{10}-Gn-RH, For-D-Trp^{1}, D-Cpa^{2}, D-Trp^{3}, D-Phe^{6}, D-Ala^{10}-Gn-RH and p-Glu^{1}, D-Phe^{2}, D-Trp^{3}, D-Cpa^{6}-Gn-RH had a weak inhibiting effect. Instead of the expected inhibiting effect, certain antagonists proved to be of stimulating effect, and increased the plasma testosterone level (Ac-5F-DL-Trp^{1}, D-Cpa^{2}, D-Trp^{3}-D-Phe^{6}. D-Phe^{6}, D-Ala^{10}-Isophtaloul dimer of Ac-D-$Cpa^{1,2}$, D-Trp^{3}, D-Lys^{6}, D-Ala^{10}-Gn-RH, H-D-Phe^{1}, D-Phe^{2} (Asp (Ind)$)^{3}$-D-Phe^{6}-Gn-RH). These compounds

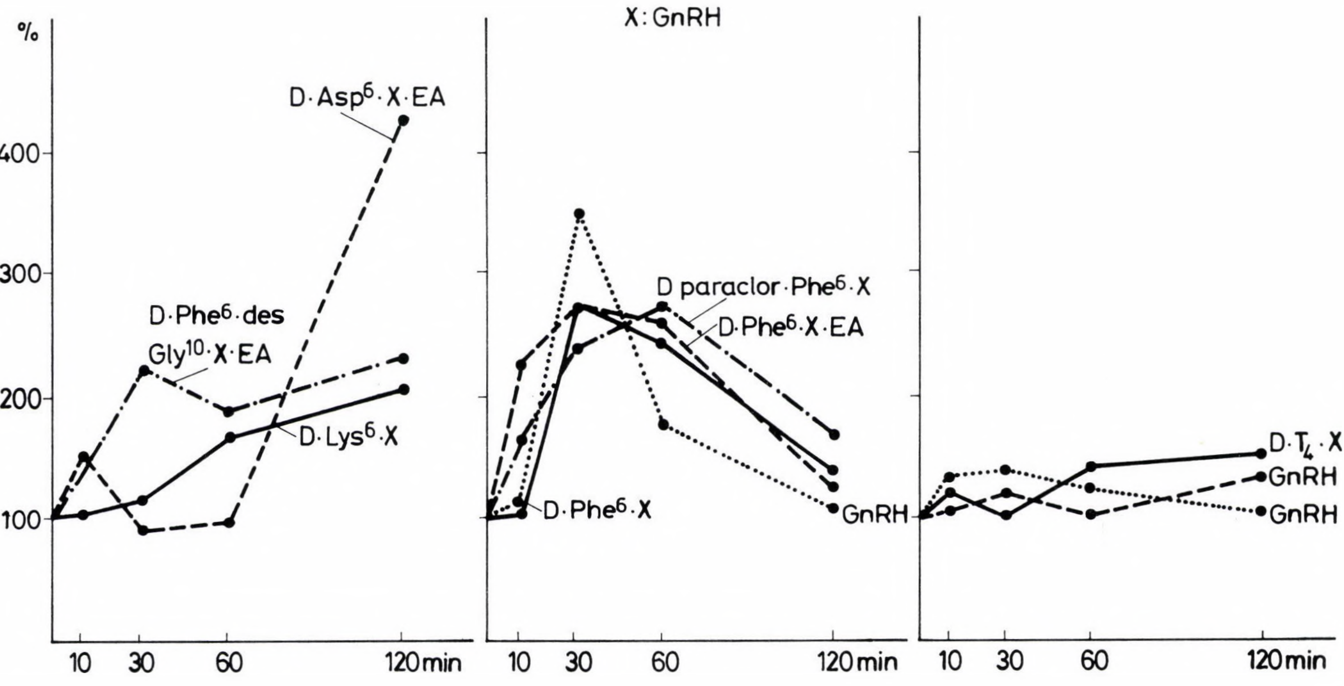

Fig. 2. Effect of Gn-RH agonists on the testosterone plasma level in adult cocks (0.5 μg/kg, i.v.) (n = 5)

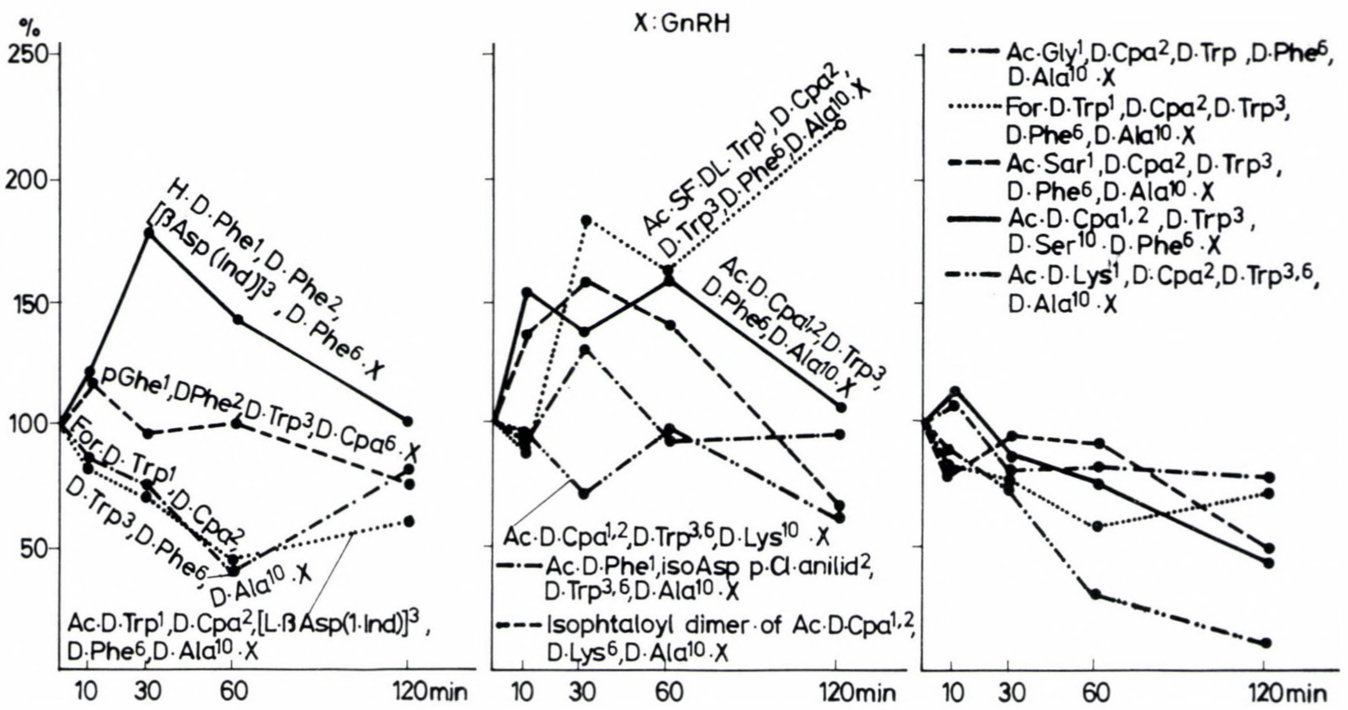

Fig. 3. Effect of Gn-RH antagonists on the testosterone plasma level in adult cocks (0.5 μg/kg, i.v.) (n = 5)

generally increased the plasma testosterone level by 50—80% within 30 minutes (Fig. 3).

In female birds, we know only one in vivo data which suggests that plasma progesterone and 17-β-estradiol levels are changed on the effect of synthetic Gn-RH and an agonist (D-Ser, But^6-Az, Gly^{10}-Gn-RH) /24/. The response of the two steroid hormones is relatively little, and not as systematic or dose-dependent which the LH (thus, e.g. progesteron level is significantly increased by 50 μg/kg, but 20 and 100 μg/kg are uneffective). The progesterone response is more marked, and this gives a maximum between 40 and 100 minutes (with 50 ug/kg Gn-RH and 10, 20, 100 μg/kg analog. The increase is 2—2.5 times higher than the initial level. Due to the big scatter of 17 β-estradiol, the results are rather difficult to evaluate, and, therefore, we can speak only about a tendency of increase within 40 minutes.

4. Localization of Gn-RH neurons in the bird brain

The site of the origination of Gn-RH, and the route of the neuro-hormone transporting axons were determined by immunocytochemical methods and by the measurement of Gn-RH activity of circumscribed cerebral areas. Immunocytochemical studies in the seventies were still not unambiguous enough, and, possibly, this was due to the properties of the antiserum used at that time. Calas et al. /9/ were the first to describe the "LH-RH-immuno-reactive" fibres in the median eminence of the bird (duck), but they were not able to detect Gn-RH cell bodies by the antiserum and technique used at the time. Later, Bons et al. /4, 5/, and Hoffman et al. /27/ described Gn-RH immunopositive cells in the infundibular region of duck, chicken and pheasant hypothalamus. The existence of this "posterior-hypothalamic" Gn-RH cell population, however, could not be verified later. As it has turned out /12/, the "LH-RH-antiserum used by Hoffman et al. /27/ gives significant cross reactions with ACTH". The infundibular region has remarkable ACTH activity /52/ and also has immunopositive cell population /2/, which may explain for the "infundibular Gn-RH area".

Sterling and Sharp /65/, as well as Józsa and Mess /30/ were able to detect immunopositive cell bodies in the olfactory bulb, septal area, and in the anterior and medial hypothalamus by antisera produced against synthetic mammalian Gn-RH.

Fibres of Gn-RH cells in the olfactory bulb and the septum (mainly in the lateral septum) mostly irradiate to the teleencephalon along the basis of the lateral ventricle, and, thus, their direct hypophyseotropic function is not probable. A minor fraction of axons originating from the Gn-RH cells of the septal area, and also certain fibres from the bulbs run to the commissure pallii, and encricle the nucleus of the pallial commissure. Other fibres of this bundle are curved ventrally, and — as mixed with certain Gn-RH axons of the preoptic area — they form the periventricular Gn-RH fibre system of the third ventricle. Thus, functionally, these tracts also take part in the formation of the hypothalamo-hypophyseal Gn-RH system.

Most Gn-RH cells of the hypothalamus are located in the ventromedial preoptic area, around the organum vasculosum laminae terminalis (OVLT). A large mass of immunopositive perikarya can be observed in the magnocellular paraventricular preoptic nucleus and in the stratum cellulare internum, in the subependymal zone of third ventricle. Most fibres of these cells are united in bundle running medially on the dorsal surface of the chiasma to the median eminence. More caudally, the Gn-RH cells can be observed in an ever more dorsal position: they form a large population in the paraventricular nucleus and further in the dorsomedial nucleus of the thalamus. Fibres of these cells participate in the formation of the periventricular fibre network. On the one hand, this fibre system projects ventrally, and its elements take part in the formation of the ventro-basal bundle running to the median eminence. On the other hand, periventricular and septal fibres caudally irradiate to the substantia grisea centralis along the strio-medullar tract, and certain Gn-RH axons can be recognized on the boundary of the pons and the mesencephalon.

Most of the periventricular Gn-RH fibres enter the multilayer ependyma of the OVLT and th SCO, and terminate among the cells. Also a strict contact can be seen between the Gn-RH terminals and the internal layer of the ventricular ependyma. These morphological data suggest that a part of Gn-RH transport is directed towards the liquor.

Extended observations of Sterling and Sharp /65/ and Józsa and Mess /30/ in domestic hens confirm those first results (Calas et al. /9/ in ducks; De Reviers and Dubois /16/ in cockerels; Sharp et al. /57/ in greenfinch), according to which a large number of Gn-RH fibres and terminals can be found in the anterior and posterior median eminence.

Gn-RH fibres can be seen in both the inner and the outer zones of

the median eminence. Fibres in the inner zone can be followed up to the pars nervosa, but they do not enter the posterior lobe. A large mass of Gn-RH fibres is to be observed in the external zone of the median eminence. The radial fibres of the external zone contain a large number of granules, which occasionally accumulate along the portal capillaries of the "Mantle plexus". These data suggest that the larger part of Gn-RH secretion is directed towards the cephalic lobe of the pars distalis.

According to Sterling and Sharp /65/, a close connection can be presumed between the pars tuberalis covering the median eminence and the peripheral Gn-RH terminals. The functional-morphological investigation of Gn-RH immunoreactive neurons was performed in starlings exposed to different photoperiods /22/. The diencephalon was examined with an antiserum developed against synthetic mammalian Gn-RH giving definite cross reaction to chicken Gn-RH I. Highly stainable Gn-RH immunopositive neurons were found in two areas in sexually active male starlings kept in 11L-13D system. There are cells in the subcommissural region of the OVLT, which extends dorsally along the third ventricle to the commissure anterior, the second cell bundle goes to the habenular area along the septomesencephalic tract from the precomissural part of the OVLT, while it penetrates the septum. In photosensitive 8L-16D, sexually inactive birds the staining of the perikarya of these two areas is similar to that in active starlings, but less fibres can be seen in the periventricular area, and in the median eminence. In 18L-6D photorefractory, male birds the Gn-RH perikarya are faintly stained, and their diameter significantly decreases. The fibres are difficult to observe, and there is practically no Gn-RH immunopositive substance in the median eminence. The semiquantitative evaluation of the changes suggests that intensive Gn-RH synthesis and release takes place in the hypothalamus of sexually active male starlings, and it significantly decreases in photorefractory birds.

Mikami et al. /43/ compared the localization of Gn-RH immunopositive cells investigated by means of using antisera against chicken Gn-RH I and II in chicken and quail hypothalamus. The localization of chicken Gn-RH I immunopositive neurons was very similar as were demonstrated by antisera against mammalian Gn-RH. The chicken Gn-RH II cells were observed in the rostro-medial part of mesencephalon but never in the hypothalamus. No Gn-RH II immunoreactive fibers were found in the zona externa of median eminence. These results suggest that Gn-RH II is not released from the hypothalamus into the adenohypophysis to control the gonadotropic functions (Fig. 4).

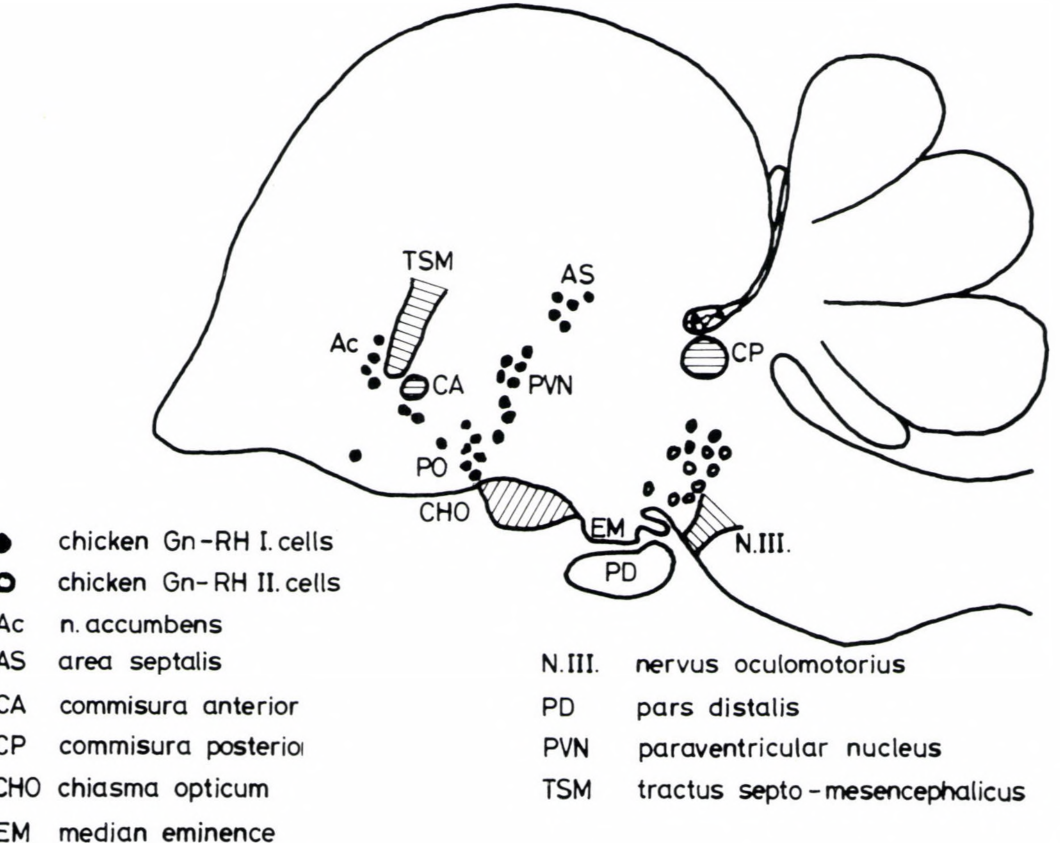

Fig. 4. Distribution of chicken Gn-RH I and Gn-RH II in the brain of chicken. (After Mikami et al. /43/, modified)

Another method for the morphofunctional study of the Gn-RH system is the RIA determination of the Gn-RH content of well circumscribed small brain areas. On the base of quantitative differences, this method also allows of drawing functional consequences. Knight et al. /35/ found the highest Gn-RH concentration (6.55 $\pm$ 1.86 pg/µg protein) in the cockerels in the medio-basal hypothalamus (which also contains the median eminence, and they found significantly lower concentration in the medial preoptic area (0.95 $\pm$ 0.07 pg/µg protein).

Significant increase of Gn-RH level was obtained after castration in five hypothalamic areas the anterior preoptic area, the dorsal preoptic area, the paraventricular nucleus and the posterior medial and lateral hypothalamic nuclei. Gn-RH concentration elevation was inhibited by testosteron replacement treatment in castrated birds.

Johnson and Advis /29/ determined the Gn-RH concentration during the ovulation cycles in anterior (medial preoptic area) and posterior hypothalamus (infundibulum) in domestic hens. According to their observations, the Gn-RH activity of the two areas can change independently of each other, which suggests a certain independence of peptide hormone synthesis in the anterior hypothalamus, and release from the median eminence.

5. Long acting effect of Gn-RH and its agonists in birds

Gn-RH, and mainly its superactive agonists may have gonado-inhibiting and gonadostimulating effect in mammals in the case of repeated dosage depending on the dose and the mode of application. Daily one or two repeated applications of higher doses can elicit the block of the gonadal function (functional gonadectomy), while pulsative administration of low doses for weeks and months (as administered many times a day) may induce puberty, i.e. may stimulate gonadal function /17, 32, 39, 63/.

The LH-releasing and ovulation inducing effect of repeated administration of synthetic porcine Gn-RH and superactive Buserelin was studied by Sterling and Sharp /65/ in female birds. The decapeptides were injected in 1 and 10 µg doses once daily for 12 days to laying hens. Synthetic Gn-RH and the Buserelin caused no change in the egg laying sequence of the treated animals, i.e. they induced neither ovulation inhibiting, nor ovulation stimulating effect in this dose. The LH-releasing effect of Gn-RHs was not less, but remarkably higher on day 12 than on day 1, i.e. the repeated

dosage caused no desensitizing effect at the hypophyseal level. This means that domestic hens respond dissimilarly to mammals, where daily one or two doses of Buserelin causes the decrease of the LH secretion (desensitization) and inhibition of ovulation. Authors data suggest that this prolonged, low-dose administration of Gn-RH causes permanent supernormal LH release in female birds.

Similar results were obtained (Péczely, Győrvári, unpublished data) by D-Phe^6-Gn-RH treatment (0.5 μg/kg) daily in male turkeys. Though the plasma testosterone level starts to decrease from day 6 on, it was still higher than the initial value even on day 12. The volume of sperm yielded from the animals by massage technic was higher than the initial volume on day 6, but even higher on day 12.

Further on, we tested the effect of Gn-RH analogs administered intermittantly in low doses in male turkeys, ganders and cocks on their sperm production, sexual activity, plasma LH and testosterone levels (Péczely, Seprődi, Győrvári, Teplán, Muray, Érchegyi, Vadász, unpublished data). On the effect of a single injection of (β-Asp-DEA)^6-Gn-RH-EA, the plasma LH level of turkeys showed a maximal value within 20 minutes, but also a second maximum was observed within 100 to 120 minutes. Testosterone peak develops within 80 to 120 minutes (Fig. 5). The injection of this analog 3 times in every three days results in a decreasing-tendency increase of plasma LH (slight desensitizing effect) and the ever intensive increase of the testosterone level (sensitization effect) (Fig. 6).

After the third treatment, both the LH and the testosterone levels returned to the initial value. The volume of sperm yielded form the animals by massage was significantly higher at the end of the week after the treatment than in the control goup. A 30-day follow-up study of sperm volume of treated turkeys showed permanently higher production than in the control group.

We performed large-scale (including 180 animals) sperm production studies after the use of two Gn-RH analogs in male turkeys. Following an application three times on every other day, D-Phe^6-Gn-RH resulted in 33% sperm production increase, which lasted for 30 days. (β-Asp-anilide)^6-Gn-RH administered three times 60 days after the first treatment (also after 10—18 days of latency period), an increased sperm production could be obtained extending the whole production cycle (further 65 days), which was on the average twice as much as in the control group (Fig. 7).

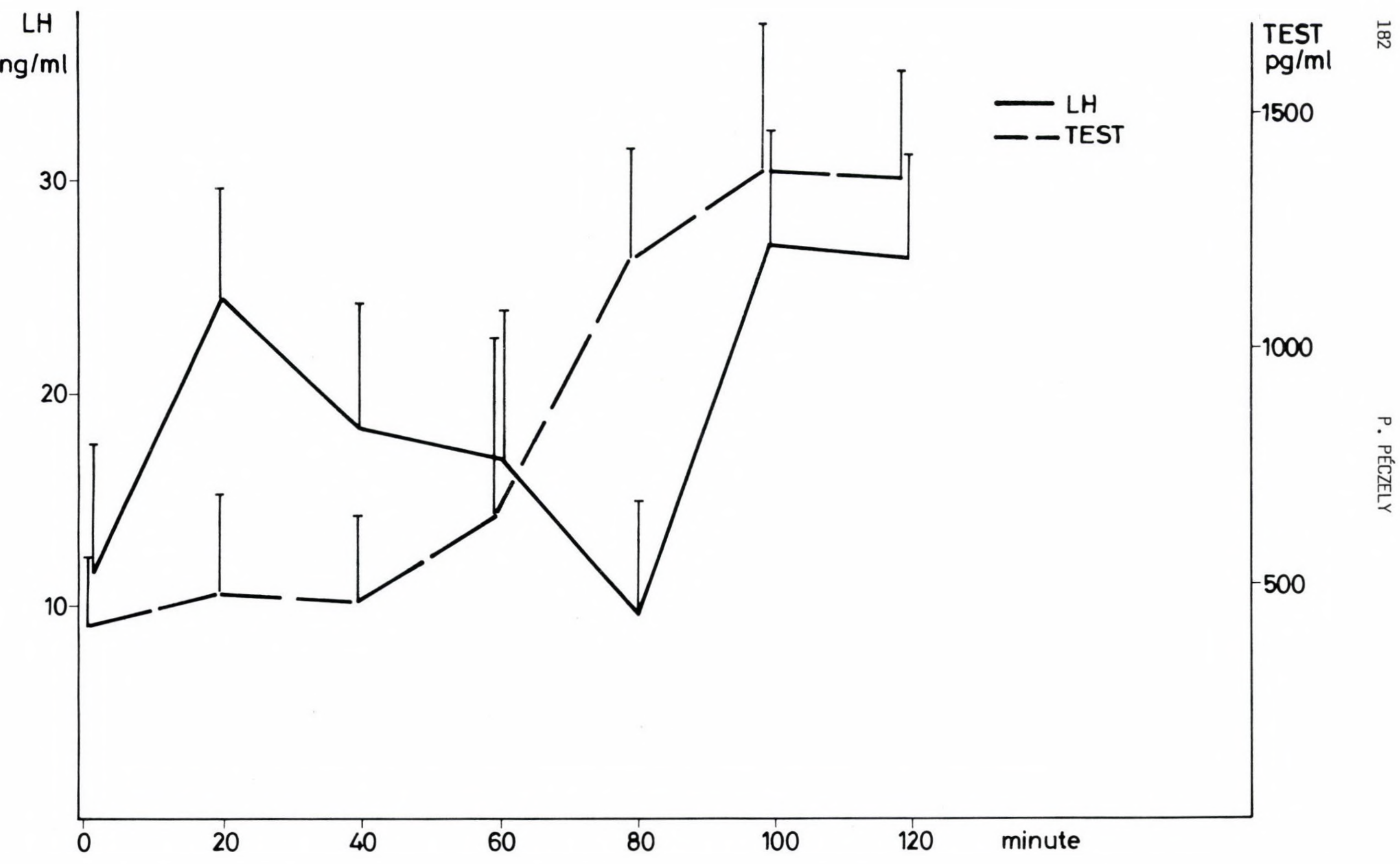

Fig. 5. Acute effect of (β-Asp-DEA) [6]-Gn-RH-EA on the LH and testosterone plasma level in male domestic turkeys

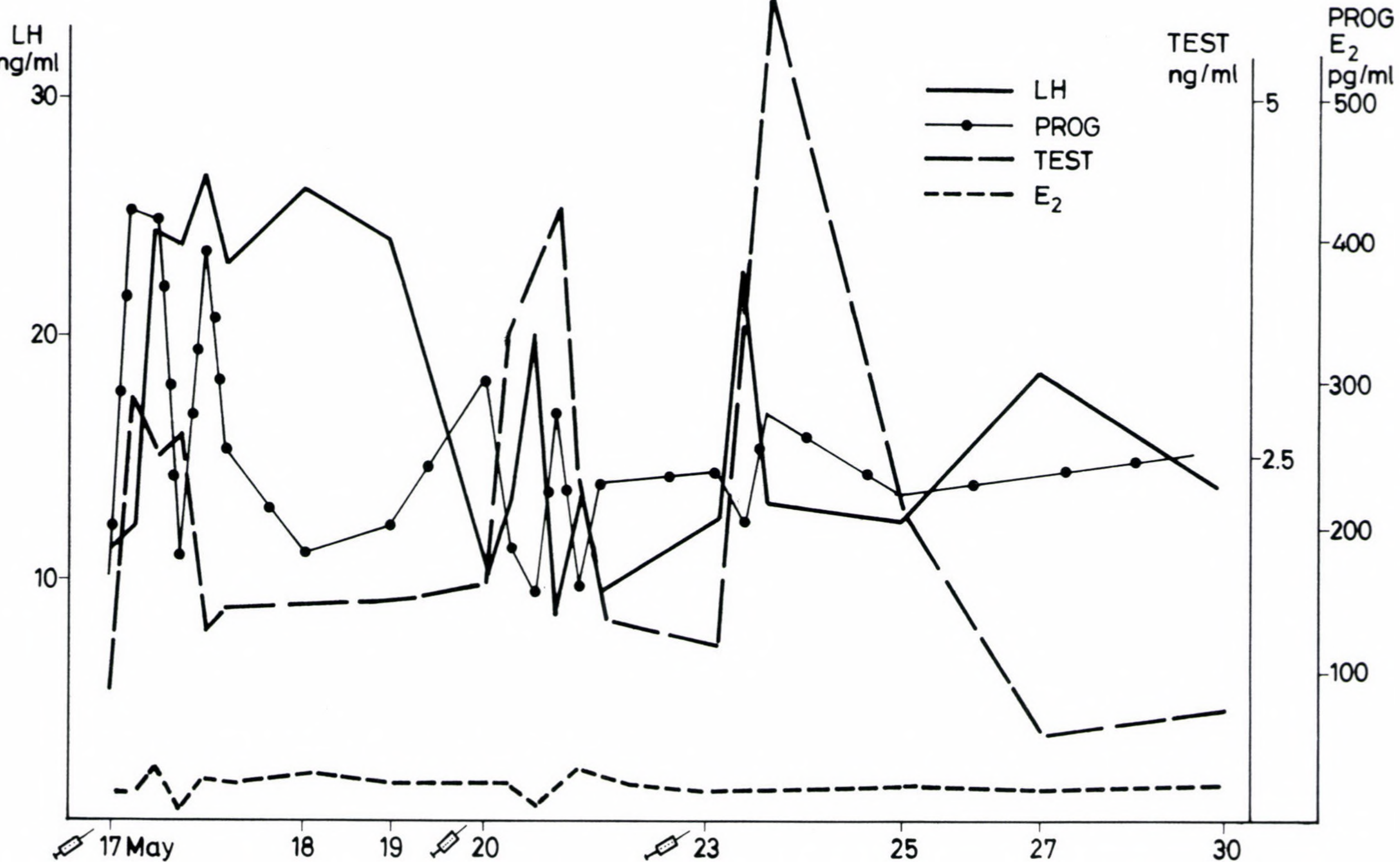

Fig. 6. Long-time effect of three injections of $(\beta\text{-Asp-DEA})^{6}$-Gn-RH-EA on the function of hypophysis-gonad axis in male domestic turkeys

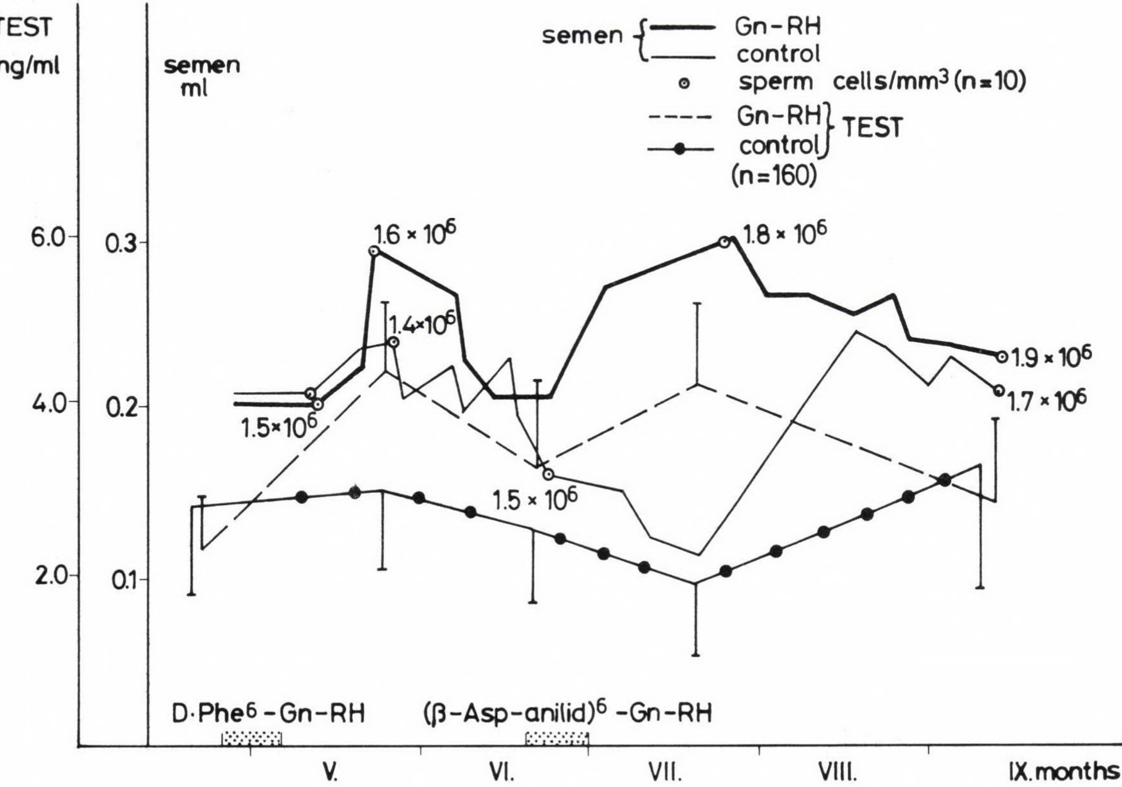

Fig. 7. Effect of repeated injections of Gn-RH agonists on the semen production and testosteron plasma level in male domestic turkeys

In ganders, a three-times, every-other-day treatment with D-Phe^{6}-Gn-RH-EA (Ovurelin) administered at the beginning of the spring production cycle (early January) in a dose of 1 μg/kg resulted in a significant sperm production increase lasting for two months. Sperm was obtainable from the treated ganders as long as until mid-July, thus, their sexual activity was nearly one months longer than that of the control animals. Considering the spontaneous sexual reactivization in the autumn, it is obvious that sperm production of treated ganders starts about two weeks earlier, and is more marked than that of the controls during the whole cycle. These results also suggest that Gn-RH stimulation applied intermittantly causes long-lasting changes in the whole seasonal sexual cycle of ganders, which also manifests itself in the shortening of the photorefractory period in the summer.

In domestic cockerel, the volume of sperm yieldable by massage significantly increased in the whole period of the production cycle (6 months) by the administration of D-Phe^{6}-Gn-RH-EA ("Ovurelin") three times, every other days (0.5 μg/kg).

"Ovurelin" treatment of cockerels of natural breeding (harem-type management) resulted in the increase of their sexual activity (libido). Therefore we compared the fertilizing capacity of treated and control cockerel by the use of "traditional" sex rates of 1:10, 1:15 and 1:20. The fertilizing capacity of animals treated with the Gn-RH analog was significantly better in the whole test period (4 months) than that of the controls /55/.

Our investigations with Gn-RH analogs in male birds show that low-dose treatments administered 3—4 times intermittantly causes gonad function increase lasting for weeks or months. This long lasting effect is the most stable if the treatment is started 1 to 3 weeks before the end of sexual maturation. It is presumable that in the background of the phenomenon we can find the gonadotropic function returning effect of repeated stimulation (which means sensitization at the testis level).

Our observations /54/ suggest that Gn-RH treatment applied in the early infantile age may modify the time of sexual maturation of male birds. We treated ganders with D-Phe^{6}-Gn-RH-EA ("Ovurelin") (in a dose of 1 μg/kg/animal) when they were a few days old, one, two and three months old. Sexual maturation of a few days old and one month old treated animals occurred earlier: the plasma testosterone level was higher the phallos was longer and sperm production started 3 weeks earlier. Treatments in the age of two and three months did not lead to significant acceleration of maturation. In the

background of the phenomenon we can suppose the increased sensitivity to Gn-RH stimulus in the early infantile age ("Sexual imprinting").

6. Regulation of Gn-RH secretion in birds

The hypothalamic secretion of Gn-RH is regulated primarily by the feedback effect of sexual-steroid hormones, and the same hormones also exert their effects at the adenohypophysis level, and modulate the effect of Gn-RH for the production of the gonadotropic hormones.

Castration increases the Gn-RH content of the hypothalamus in cocks, and the testosterone replacement treatment inhibits this increase /35/. This observation obviously verifies that the androgens regulate Gn-RH synthesis and release in the hypothalamus by negative feedback. In vitro studies of isolated hypophysis cells of sexually mature Japanese quails showed that their preincubation with testosterone and 5 α-dihydrotestosteron caused remarkable decrease in LH release on the effect of chicken Gn-RH I. In the same experiment, 5 β-dihydrotestosteron and 17 β-estradiol had no effect on the degree of LH release on Gn-RH stimulus. That is, the testosterone and its active 5 α-metabolite are the inhibiting modulatory factors of Gn-RH exerting their effect at the adenohypophyseal level. In hypophysis cells of infantile quails, even the testosterone and the 5 α-dihydrotestosteron caused no similar inhibiting effect which could indicate the dependence of this regulating system on the maturation /13/. Experiments in photostimulated male Japanese quails verify that the change of Gn-RH content of the hypothalamus shows no such quick and dramatic changes than plasma LH and testosteron levels, and the mass of the testis. The arrangement of castrated quails in a 20L : 4D system leads to a decrease of the LH level in the hypophysis, and an increase of 5 to 8 times of the plasma LH level (the changes appear within 19 to 24 h as from the start of the dark period). In this period, the Gn-RH content of the hypothalamus shows no change, its slight increase occurs only within 48 h /14/. The authors presume that the difference between the LH and the Gn-RH is only apparent, because the increasing synthesis compensates the increased release in the first hours.

Similar results were obtained in a chronic experiment. Gn-RH content of the hypothalamus of intact and castrated Japanese quails showed increase only between days 10 and 21 of the photostimulation /44/.

Concerning female birds, the Gn-RH secretion regulating effect of

sexual steroids was studied mainly in domestic hens. Kawashima et al. /31/ reported that the Gn-RH activity of the hypothalamus shows remarkable elevation one hour after the administration of progesteron injection. That is, progesteron increases Gn-RH secretion by feedback effect in birds. Estradiol treatment administered 4 h earlier caused further increase of Gn-RH increase elicitable by progesteron. Wilson and Cunningham /73/ also underlines the importance of the estrogen priming, manifested at the hypothalamic level, in the regulation of Gn-RH secretion in female birds.

Relationship between changes in the Gn-RH content of the hypothalamus and the preovulation LH peak and ovulation was also studied in domestic hens. Knight et al. /36/ found no relationship between changes of hypothalamic Gn-RH level and the appearance of the preovulational peak. On the contrary, Johnson and Advis /29/ found that the Gn-RH content of the hypothalamus is higher in egg laying hens than in moulting hens. Gn-RH content of the medial preoptic area increases during the preovulation peak, but no changes can be seen in the infundibulum. If the preovulation peak was increased by progesterone treatment 8 h before this, then the Gn-RH content decreased both in the medial preoptic area and the infundibulum. These data unambiguously prove that progesterone has a stimulatory effect on LH through intensifying Gn-RH secretion (synthesis and release), and elicits ovulation.

Biogenic amine control of Gn-RH secretion was studied by Millam et al. /44/ in a superfusion system. The level of Gn-RH produced by Japanese quail basal hypothalamus showed remarkable increase on the effect of epinephrin, norepinephrin and β-adrenerg agonist isopropanol. Their data confirm those earlier assumptions which suggested an adrenergic regulation of gonadotropic hormone /6, 18/ and make it probable that the adrenergic stimulations play a role in the maintenance of physiological release of Gn-RH.

Presumably, endogenous opioids tonically inhibit the Gn-RH secretion of the bird hypothalamus. According to the in vitro experiments of Stansfield and Cunningham /67, 68/ (in superfusional system), Gn-RH basic secretion was significantly increased by naloxon. The Gn-RH release increasing effect of K^+ depolarization was remarkably decreased by Met-enkephalin and by D-Ala^2-Met-enkephalin. Also the depolarization stimulus inhibiting effect of the two enkephalins was blocked by naloxone. The authors have also detected that neither Leu-enkephalin, nor β-endorphin caused effect on either the Gn-RH basic secretion or the Gn-RH secretion

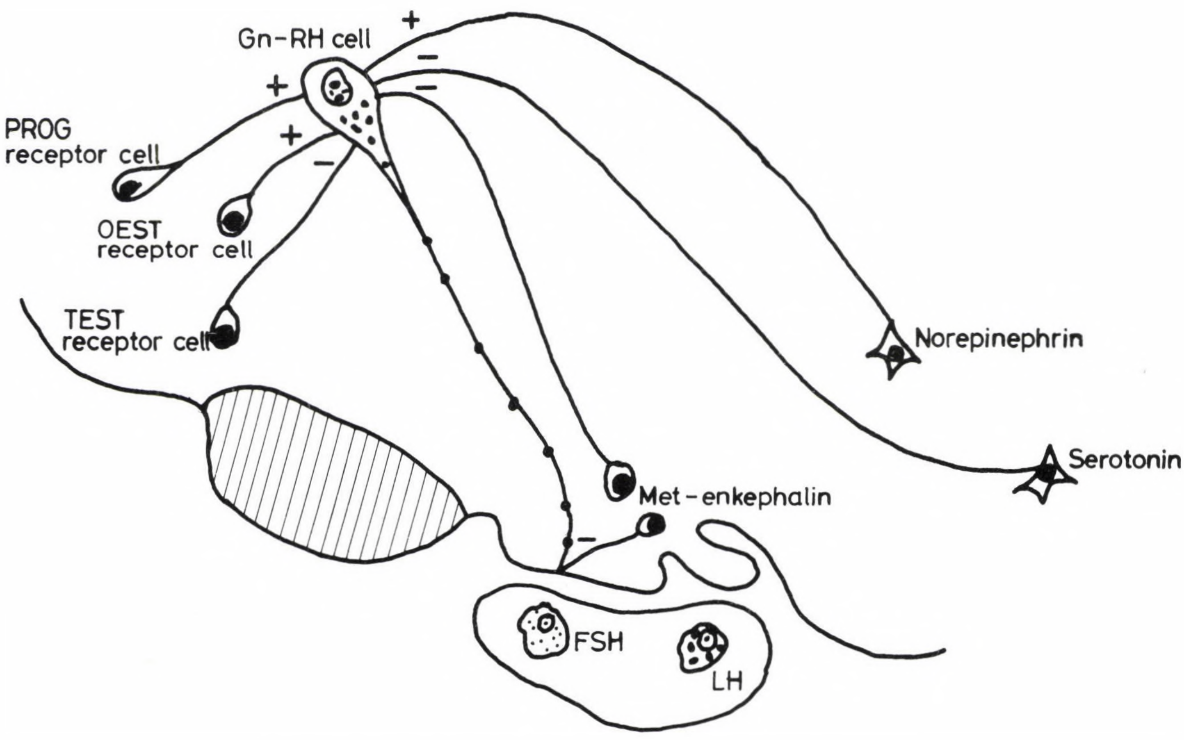

Fig. 8. Possible regulation of Gn-RH secretion in birds

stimulated by K^+ depolarization. Presumably, the basic secretion is inhibited by opioids exerting their effect through μ-receptors, white opioids effecting through "δ", and at lesser degree, through "μ"-receptors may play a role under the conditions of increased Gn-RH secretion (Fig. 8).

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THE INFLUENCE OF OLFACTION ON EXPLORATORY BEHAVIOUR IN THE PARADISE FISH (MACROPODUS OPERCULARIS L.)

A. MIKLÓSI and V. CSÁNYI

Department of Behaviour Genetics, Eötvös Loránd University of Budapest, Göd, Hungary

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We studied the role of olfaction on the exploratory behaviour of the paradise fish. Intact animals showed habituation of exploratory behaviour toward a heterospecific fish after five consecutive encounters. Fish with olfactory nerves destroyed spent significantly longer time with exploration compared to the control even at the fifth encounter. We suggest that olfactory inputs have a strong influence on exploratory behaviour.

Keywords: Olfaction — exploratory behaviour — paradise fish

Introduction

For fish living in murky water, where visibility is poor, it is important to receive chemical cues from the environment. Thus it is not surprising that various behavioural forms of fish are controlled entirely or partially by olfaction. For example Davis et al. /6/ demonstrated that the olfactory bulbectomy resulted in an increased egg cannibalism in the paradise fish (Macropodus opercularis L.). In another study they reported that isolated paradise fish showed a well pronounced approach response and high approach frequency to water inflow from a tank containing another conspecific fish /5/.

When investigating predator recognition authors concentrated mostly on the role of visual stimuli carried by the predator /7, 9/ although it is

Send offprint requests: A. Miklósi, Department of Behaviour Genetics, Eötvös University of Budapest, Jávorka S. u. 14, H–2131 Göd, Hungary

Akadémiai Kiadó, Budapest

reasonable to suppose that olfaction may also play role in recognizing heterospecific fish, especially predators.

Recently we found /1/ that a five minute encounter of paradise fish with an unfamiliar goldfish resulted in a significant decrease of the exploratory behaviour at a second encounter with the same fish. Repeated exposures to a living pike or goldfish led to lower approach tendency in both cases as a result of habituation but the approach reaction to the goldfish diminished faster. Dishabituation of the approach reaction was observed during encounters of the paradise fish with unfamiliar companions of other species. The latter indicates that some kind of memory develops during the encounters with heterospecific fish. Csányi et al. /4/ demonstrated that a five minute encounter with a heterospecific fish results in a memory trace which lasts for at least 3 months.

In this experiment we examined the possible role of olfaction in the approach reaction of the paradise fish encountering with heterospecific companions.

Materials and Methods

Subjects

An outbred population of Macropodus opercularis served as subjects. The fish were bred in our laboratory. All fish were between 150-180 days old at the time of the experiments, and were housed in groups of 15 in 80 l glass aquaria. The aquaria were well filtered and the temperature (28 ^{o}C) was held constant: each unit contained waterplants (Hygrophyla polisperma) planted in clay pots. The animals were fed daily on special fish food.

The same individual of the wild type of the common goldfish (Carassius auratus) was used throughout the experiments (18 cm long, 100 g in weight).

Surgery

After two days of adaptation to the laboratory conditions, fish of certain groups underwent surgical treatment. The fish were put into cold water (4 ^{o}C) until they became immobile. After cooling a 2 mm diameter hole was bored in the skull by a dental drill and the olfactory nerves were cut at the tip of the olfactory bulbs. For fish in the sham operation group after making the hole the olfactory nerves were left intact. After surgery the wound in the skull was filled with gel foam and the animals were put in individual tanks and were not fed for two days.

After completing the behavioural experiments the fish were killed in ice water and the skull was opened under a stereomicroscope and the removal of the olfactory nerve was checked.

Behaviour units

During the experiment the following behavioural units were observed (for details see (3));

Escape (ESC): forceful swimming perpendicular to the glass side; Swim (SWI): fast locomotion;

Air-gulping (A-G): Being an anabantoid fish, M. opercularis swims from time to time to the surface and gulps air as an oxygen source;

Approach (APR): It is shown in the presence of other living fish and characterized by slow locomotion, orientation toward the approached object and it is clearly expressed by ocular movements.

Floating (FLO): The immobile animal is floating under the surface not deeper thań 1-2 cm, with the body axis parallel to the surface;

Hanging in midwater (HIM): This is like FLO but in the middle range of the test aquarium;

Resting (RES): This is like FLO or HIM but with the fish staying on the ground: The anal or caudal fin touches the bottom of the tank.

The latter three units are called as passive units. The mean percentage of time of the behavioural units during the observation periods were calculated for statistical analysis.

Apparatus

The experiment was carried out in a large 100x50x50 cm glass tank which contained small pebbles and some waterplant that did not disturbed the observation of the fish.

Procedure

The paradise fish were divided into three groups: 25 animals served as sham-operated controls (SHAM), 18 fish received olfactory deafferentation prior to the experiment(OLF1), and 10 fish after the first session (OLF2).

Following surgery and isolation, the fish were kept in community aquaria during the whole course of the experiment.

On the first experimental day the paradise fish, one by one, were made familiar with the empty experimental tank for 5 min. On the second day the fish met a goldfish in the experimental tank (first habituation session). This encounter was repeated 4 times for each paradise fish. The intersession interval was 9 days. An encounter lasted for 5 min and the behaviour of the paradise fish was observed and recorded by means of a small computer (Commodore 64) which was programmed to accept keyboard inputs as code for behaviour units. Details of this method had been published earlier /10/. The observer was unaware of the surgical pretreatment of the fish, and the members of the three groups were randomized for the observations on each habituation session.

Results

Exploratory behaviour

The means of APR of the various experimental groups are shown on Figure 1. An unbalanced two-way ANOVA was calculated and we found a significant effect for days ($F(4.201)=26.45$, $p < 0.001$), and for treatments ($F(2.201)=56.38$, $p < 0.001$) but interactions were not significant ($F(4.201)= =1.97$, $p < 0.08$).

In the case of controls a rapid decrease of approach behaviour during the habituation sessions was observed. There is a significant decrease in the mean time spent with approach even between the first and the second encounters ($t=5.97$, $p < 0.001$). In the OLF1 group significant differences were found in the mean time spent with approach only between the first and the fourth sessions ($t=4.5$, $p < 0.001$). In OLF2 group means of APR differed significantly only between the first and the fifth sessions.

Comparing the mean time of approach behaviour in all groups at the first session we found that the OLF1 group spent significantly more time with exploration than controls ($t=-2.08$, $p < 0.04$). (The OLF2 group had not been operated at that time.)

The means of APR were significantly different between the SHAM and both operated groups even in the fifth session ($t=2.3$, $p < 0.05$ for the OLF1 group, and ($t=-2.3$, $p < 0.02$) for the OLF2 group).

Other behavioural observations

On the course of the experiment it was observed that the means of ESC unit increased significantly in the SHAM group over the five habituation sessions ($F(4.123)=7.8$, $p < 0.001$).

This was not the case for either operated groups in which the mean percentage of time spent with escape had much lower values during the whole experiment compared to the controls and did not increase significantly during the habituation trials ($F(4.87=0.94$, $p < 0.45$ for OLF1 and $F(3.32)= =0.8$, $p < 0.5$ for OLF2).

The means of SWI were very similar for all groups and during all sessions. The ANOVA showed no significant differences either among the means of groups nor among the means of sessions.

The frequency of air-gulping (A-G) increased significantly during the habituation sessions in all groups ($F(4.121)/2.18$, $p < 0.01$ for the

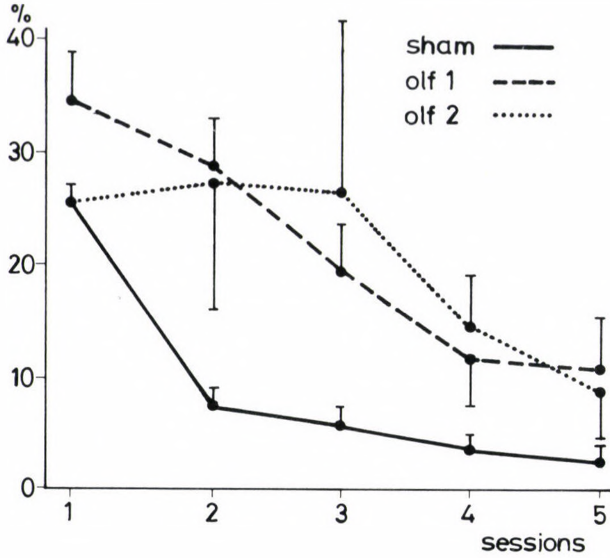

Fig. 1. The percentage of time spent with approach toward the goldfish in each of the five sessions (mean±SD)

SHAM, $F(4.87)=2.5$, $p < 0.05$ for the OLF1, and $F(3.32)=4.0$, $p < 0.02$ for the OLF2 group), but were significantly lower in the two operated groups ($F(4.201)=43.2$, $p < 0.001$ for the effects of the treatment).

There were not observable differences between the SHAM and both operated groups concerning the total time they spent with passive behaviours (RES, HIM, FLO) but in spite of that the SHAM and the operated groups spent equal time with hanging in midwater (HIM) there was a tendency in the case of the controls to spend more time near to the water surface (FLO) than operated animals, whereas the latter rested more on the bottom of the experimental tank (RES). ($F(2.201)=13.3$, $p < 0.001$ for means of FLO; $F(2.201)=62.8$, $p < 0.001$ for means of RES).

Discussion

We found that animals with olfactory nerve cut were different from the sham-operated controls in the build-up of habituation to the presence of a heterospecific fish. In the experimental groups (OLF1 and OLF2) the inten-

Table 1

The behaviour units in mean (+SE) percentage of time recorded during the five habituation sessions

Sessions		1	2	3	4	5
Behav. elem.	Groups					
ESC	SHAM	15.7±3.9	27.0±4.2	34.7±4.2	35.5±4.2	42.6±4.6
	OLF1	4.2±5.0	12.2±4.9	12.7±4.9	18.6±5.0	16.0±4.9
	OLF2	14.7±3.8	6.2±6.5	17.5±6.9	18.4±6.9	18.1±6.9
SWI	SHAM	28.8±1.7	24.3±1.9	22.8±1.9	23.4±1.9	21.9±2.0
	OLF1	24.9±2.6	25.4±2.5	21.7±2.5	24.3±2.6	30.0±2.5
	OLF2	22.5±2.3	24.4±4.6	26.0±4.9	20.0±4.9	30.4±4.9
A-G	SHAM	5.1±0.6	5.8±0.6	6.8±0.6	7.5±0.6	8.5±0.7
	OLF1	3.5±0.5	3.3±0.5	4.4±0.5	5.3±0.5	5.2±0.7
	OLF2	5.0±0.5	2.2±0.7	4.6±0.7	4.0±0.7	5.8±0.7
APR	SHAM	25.3±1.4	7.7±1.5	5.1±1.5	3.4±1.5	2.0±1.6
	OLF1	34.9±3.9	28.8±3.2	19.1±3.8	11.1±3.2	10.4±3.8
	OLF2	26.0±2.3	27.3±7.7	26.7±9.9	14.1±4.5	8.3±4.0
FLO	SHAM	11.8±2.3	16.5±2.5	16.6±2.5	18.6±2.5	15.1±2.5
	OLF1	5.7±3.0	7.0±3.0	13.2±3.0	8.7±3.0	12.8±3.0
	OLF2	10.5±2.5	3.9±3.5	10.7±3.7	9.6±3.7	22.0±3.7
HIM	SHAM	12.4±1.9	14.6±2.1	13.9±2.1	11.6±2.1	13.0±2.2
	OLF1	14.4±2.4	11.4±2.4	14.0±2.4	15.1±2.4	15.1±2.4
	OLF2	15.0±2.3	23.2±4.9	12.3±5.2	15.0±5.2	14.0±5.2
RES	SHAM	2.9±0.9	2.6±1.0	3.9±1.0	2.4±1.0	2.3±1.1
	OLF1	7.4±2.8	13.3±2.7	16.4±2.7	12.1±1.8	8.8±2.7
	OLF2	6.5±2.0	12.5±3.3	4.7±3.5	9.8±3.5	3.9±3.5

sity of approach behaviour diminished more slowly over the sessions than in the control group (SHAM).

Klein et al. (8) found that anosmia in rats led to increased exploration behaviour and on basis of these results he suggested that the brain retains information about the odours of the explored object whose visual stimuli repeatedly elicited approach behaviour On the other hand in intact animals the olfactory bulb may send inhibitory stimuli to the brain which might result in a decreased exploratory behaviour in the next session. We assume that the presence of an olfactory memory trace is necessary for this inhibition.

This explanation could also be valid in our case. The continuously moving goldfish, whose behaviour generates not only visual but perhaps acoustical stimuli as well, appeares to repeatedly trigger off approach behaviour in intact paradise fish. The results of the OLF2 groups indicate that one eoncounter with the goldfish is not sufficient to produce a long lasting inhibition of approach in intact animals because after surgery these animals behaved just like the fish in the OLF1 group. The comparison of the other behavioural elements revealed that the olfactomy changed not only the pattern of exploration but other behaviour of the fish as well. The marked difference of the escape (ESC) behaviour between the SHAM and the two operated groups may indicate that the control animals became "bored" with the experimental procedure and tried to leave the aquarium. But this was not the case with the operated animals perhaps partly because they found in each session a "new" object to approach.

On the other hand this higher exploratory activity in the OLF1 and OLF2 group is accompanied by a partially suppressed active behaviour in so far as they show a lower air-gulping (A-G) frequency and spend an increased time with resting (RES).

Continuous exploration of the environment and learning its characteristics are basic features of the behaviour of the paradise fish. Based upon previous findings we assumed that a dynamic model of the environment is existing in the paradise fish brain /2/ and this model organizes its behaviour. Results of the present experiments show that representation of the olfactory world around the animal is also part of this environmental model.

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EFFECT OF THE COMPONENTS OF THE PHYSICAL ENVIRONMENT ON ESCAPE BEHAVIOUR OF THE PARADISE FISH (MACROPODUS OPERCULARIS)

P. TÓTH and V. CSÁNYI

Department of Behaviour Genetics, Eötvös Loránd University of Budapest, Göd, Hungary

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Physical features of a seminatural environment on provoking various defence behaviour elements of the paradise fish were studied. It was found that most escape behaviour forms were under the control of visual stimuli. Transparent physical space induced escape and inhibited jumping. While the latter was induced by the presence of vertical untransparent surfaces. Dark places usually were avoided by the paradise fish. Placed into an unfamiliar environment in the presence of different escape routes the paradise fish seemed to make decisions after careful comparison of alternatives.

Keywords: Defense behaviour — escape — physical environment — paradise fish

Introduction

Losing visual or other perceptual connection with their conspecifics is a strong and frightening stimulus for social animals, as being alone involves increased danger of predation. In such a case animals usually react with some active or passive forms of species-specific flight behaviour /1/. By leaving the dangerous place and re-establishing connection with its conspecifics, active flight behaviour may diminish the menace for the animal /10/. Passive defensive reactions, e.g. freezing make it more difficult for the predator to notice the lonely animal /19/.

It has already been observed in our laboratory that if paradise fish loose the connection with their conspecifics they strive to re-establish it

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Send offprint requests to: P. Tóth, Department of Behaviour Genetics, Eötvös Loránd University of Budapest, Jávorka S. u. 14, H–2131 Göd, Hungary

Akadémiai Kiadó, Budapest

with a movement characterized by constant swimming along the glass side of the aquarium trying to get through it again and again /6/. This behaviour element, which we called "escape" (ESC), has been thoroughly studied /7,8/. A similar behaviour of the bat locked up in a glass case was observed by Chase /3/.

In our present experiments we studied how the visual components of the physical environment influence escape behaviour of the paradise fish.

Materials and Methods

Animals

Females of Macropodus opercularis bred in our laboratory were used in the experiments. At the time of the experiments they were between 150 and 180 days old and were kept in groups of 30 (15 males and 15 females) in 80 l aquaria. Each unit contained a clay pot of Hygrophyla polisperma, the water was continuously filtered and the temperature was held constant (28 ^{o}C). The aquaria were lit by two 40W fluorescent tubes for 16 h a day. The fish were fed twice a day by specially made fish food. The observations started at least one hour after feeding. Each animal was used only once in the experiments.

Experimental methods

The experimental aquaria, which we are going to describe in each case in turn, were placed in a separate room. The observations were made from an outer room through a closed TV circuit. The aquaria were filled with fresh water after each subject. Behaviour units of the paradise fish were recorded by a computer method described earlier /17/.

Behaviour units

Behaviour repertoire of the paradise fish was described earlier /7/ in this experiments the following behaviour units were used:
Escape (ESC): rapid movement by all fins, swimming to and fro along the side of the aquarium;
Jump (J): after a slow backward movement, a jump towards the surface, carried out by a strong slap of the caudal fin;
Freezing (FRZ): the animal is completely motionless.
Apart from the elements mentioned above wer measured step-out latency (SL) in some experiments in which the test aquarium was divided into two compartments.

Results

Experiment 1

In this experiment we were looking for the environmental stimuli necessary to provoke escape (ESC).

Equipment and method

The inner surface of some of its sides of a 20x20x20 cm glass aquarium were wholly or partially covered by sand embedded in silicon rubber, the outer surfaces of the glass sides were painted by a non-transparent dark green. Aquaria were different as far as the number of the sand-covered sides were concerned, the number of animals used in the experiment with the given aquarium is given in brackets.

"A": all sides and also the bottom of the aquarium were covered, (N=14).

"B": there was a 6 mm wide uncovered surface in the sand layer on the inner-middle part of one of the side walls from the bottom to the rim. The outer coat of paint was complete, the fish could not see out of the aquarium, (N=10).

"C": in one of the walls, there was a 6 mm wide uncovered part in the cover on both the inner and the outer surfaces in the middle, where the animal could see out of the aquarium, (N=12).

"D": At 15 mm height from the bottom in the middle there was a round shaped surface of 35 mm in diameter where the cover was missing on both sides, (N=10).

"E": one of the side walls was completely uncovered, it could be seen through, (N=10).

The experimental animals were put in the aquarium one after the other, and the aquarium was covered with a glass panel to prevent the animals from jumping out. We recorded ESC for 300 s started from the time the animal were placed in the aquarium.

Results

ESC could only be observed with fish in the C, D and E aquaria (Fig. 1). In the A and B aquaria the animals were intensively swimming to and fro along the walls and up and down at the corners without continuously touching them, as they did with transparent glass walls. Also the attempts to swim through the glass wall which are characteristic features of ESC were

missing. The one-way ANOVA analysis of the results showed significant differences among the group means of the time of escaping (t%) in the C, D and E aquaria ($F/2.29/=38.75$, $p < 0.05$). The group means in each aquarium were also significantly different respectively (Duncan range test, $p < 0.05$).

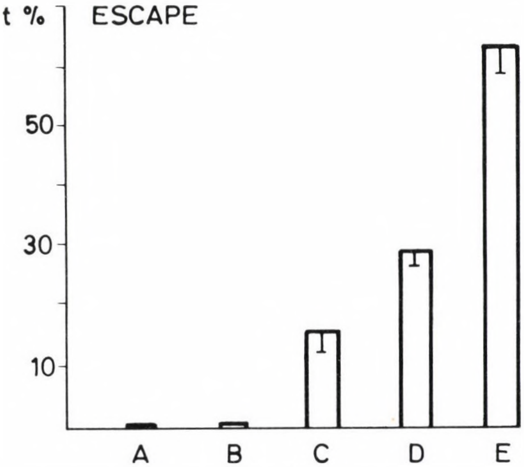

Fig. 1. Percentage of time spent in ESC ±SE in aquaria of different covering

Experiment 2

Experiment 1 showed that the space seen behind the glass wall of the aquarium provokes the escape behaviour of the paradise fish. In Experiment 2 we studied the influence of the size of the surface of transparent (uncovered) area on the wall of the aquaria was exerted on escape behaviour.

Equipment and method

We had 20x20x20 cm test aquaria in the way described in Experiment 1 group C, but the width of the transparent stripe was variable. Width is given below (mm) and the number of animals tested in each case are shown in brackets: 1(10), 2(12), 3(12), 4(12), 6(12), 7(12), 8(10), 12(15), 20(11), 35(14), 90(12), 200(15) (i.e. the whole side wall was uncovered).

The observation were made in the way described in Experiment 1.

Results

The correlation between the time percentage of ESC and the width of the uncovered stripe on the glass wall is shown in Fig. 2. In the case of the very narrow transparent stripes (1 and 2 mm) we could generally observe explorative movement around the stripe and resting just in front of the stripe. In case of stripes above 2 mm we observed that the animals moved up and down along the uncovered glass surface. One-way ANOVA showed significant differences among time percentages of the escape behaviour element ($F/11.140/=62.06$, $p < 0.01$). The Duncan test indicated five independent, non-significant ranges. So the occurrence of ESC increased with the increasing width of the transparent stripe. The most significant change took place in the 0—2 mm range.

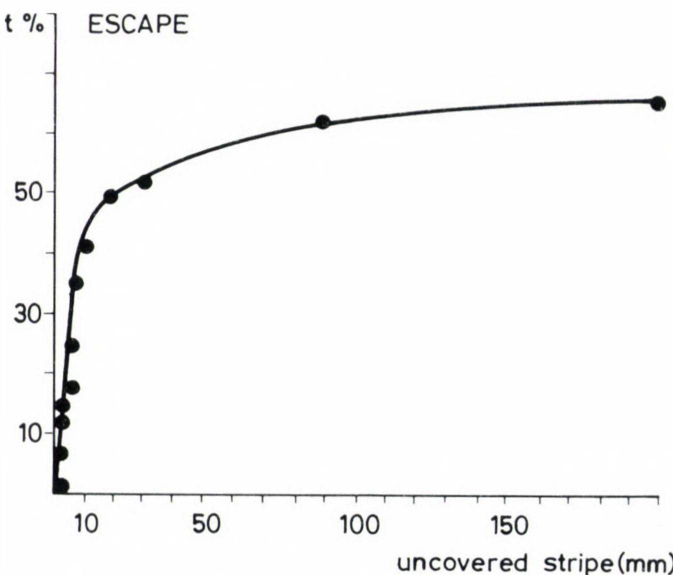

Fig. 2. Percentage of time spent in ESC in aquaria with an uncovered part of different widths

Experiment 3

In this experiment we placed a sand covered glass panel at various distances from the outer part of a transparent side of the aquarium, thus changing the dimensions of the visible space, and we studied the effect of the change on the duration of ESC.

Equipment and method

The bottom and all sides of the 20x40x20 cm glass aquarium were covered in the way described in Experiment 1. The aquarium was partitioned by a 1 mm thick glass panel into two identical sections, and both were filled with water to the same level. An other glass panel covered with sand was placed behind the partitioning glass panel at various distances. The distances between the transparent and the covered glass panels were the following: 1, 2, 4, 6, 8, 11, 16, 21, 26, 31, 41, 51, 101 and 650 mm respectively. 650 mm is the distance between the 1 mm glass panel which was partitioned the aquarium and the wall of the experimental room. The occurrence of ESC were recorded with 10—13 individuals in each case with the method described in Experiment 1.

Results

The relationship between the duration of ESC (t%) and the natural logarithm of the distance of the covered side behind the glass wall is shown in Fig. 3. The differences among the group means were analyzed by one-way ANOVA ($F/15.152/=70.86$, $p < 0.01$) and Duncan test (Table 1).

Table 1

The effect of the distance of the covered side behind the glass wall on ESC

	Group means of ESC
Non-significant ranges	3.5 — 8.1
	5.0 — 12.2
	8.1 — 14.0
	12.2 — 18.7
	14.0 — 20.3
	18.7 — 24.5
	53.2 — 57.3
	57.3 — 63.5

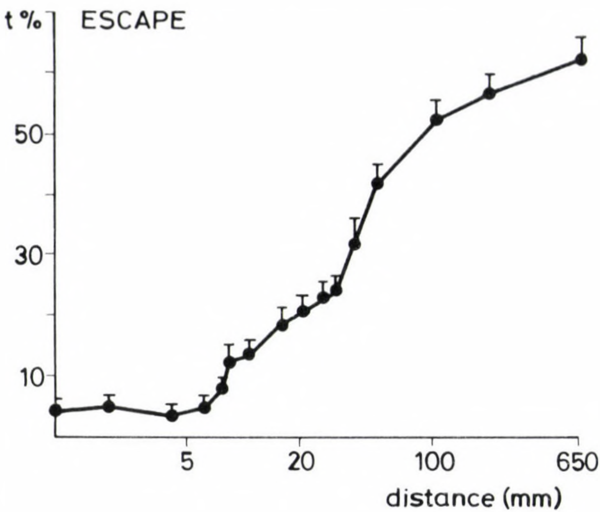

Fig. 3. Percentage of time spent in ESC taken as a function of the distance between the 1 mm glass wall and the covered other side wall behind it

The occurrence of the escape did not change up to a distance of 7 mm above this value there were significant differences in the group means between every pair of groups belonging to subsequent increasing distances.

Experiment 4

In this experiment we studied the influence of the number of the transparent side walls on ESC, FRZ and on the number of the jumping attempts (J).

Equipment and method

Wholly, partially or uncovered aquaria were placed and the behaviour elements were recorded in the way described in Experiment 1.

The proportions of the covering in the experiment was the following:
"A": all the side walls and the bottom were uncovered, transparent (N=15).
"B": all the side walls were uncovered, except for the bottom (N=11).
"C": the bottom and one side wall were covered, only one was transparent (N=10).
"D": the bottom and two neighbouring side walls were covered (N=13).

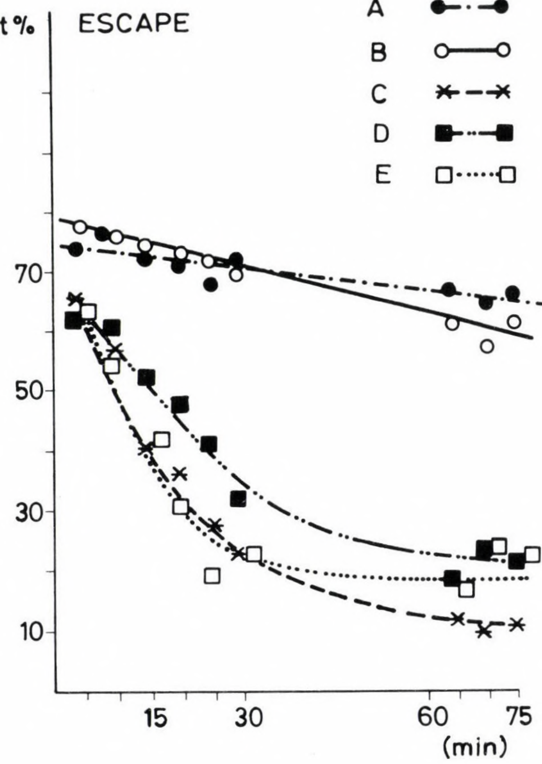

Fig. 4. Percentage of time spent in ESC in the subsequent 5 min intervals, when the number of the covered side walls was increased

"E": the bottom and three side walls were covered, only one was transparent (N=10).

"F": the bottom and all the side walls were covered (N=14).

Observation time was 75 min. The behaviour elements were recorded within two intervals, between 0-30 and 60-75 min. In the analysis, just as above, the time percentages of ESC in every five minutes, and the percentage of the individuals performing the given element in case of FRZ and J were calculated during the two periods.

Results

No escape was observed with the fish in the F aquarium during the experiment, Fig. 4 shows how the time percentage of escape changed with the groups in the other aquaria (A—E). Applying two-way ANOVA with repeated

Table 2

The effect of the extent of covering on ESC

	Group means of ESC (t%)				
Type of the aquarium	A	B	C	D	E
Non-significant	68.7-77.4	70.3-79.2	36.2-41.0	48.0-52.8	54.5-63.2
ranges	63.7-73.9	57.1-59.4	21.7-27.7	41.8-51.8	41.7-54.5
			9.1-11.5	31.8-48.0	30.9-41.7
				17.6-31.8	16.1-30.9

measurments in the analysis (The proportion of covering X the change of time) there were significant difference both in the effect of the covering (F/4.544/=42.32, $p < 0.01$) and that of the time (F/32.544/=96.76, $p < 0.01$). Interaction was also significant (F/32.544/=6.8, $p < 0.01$). Groups A, B and C, D, E which were clustered differently in Fig. 4 were checked with two-way ANOVA (with repeated measurments). There is no significant difference between groups A and B (F/1.192/=1.33. However, the change of time causes significant differences in both groups (F/8.192/=16.79, $p < 0.01$), interaction is not significant (F/8.192/=1.98).

There is no significant difference in the effect of the covering in the C, D and E groups (F/2.352/=2.62), whereas the change of time is significant (F/8.352/=78.42, $p < 0.05$) and so is interaction (F/16.352/= =1.99, p 0.05). The Duncan test results of the various group's means are given in Table 2.

Jump-out attempts were observed only with the animals in the aquaria with the covered side walls (C—F). The percentage of the individuals making jump-out attempts in the first and second quarters of an hour is given in Fig. 5. As a control, 12-h observation was made with five individuals in the A and B aquaria respectively, but no jump-out attempts were observed. The proportion of individuals making jump-out attempts was significantly different within the two intervals in all the aquaria with covered side walls (Cochram Q test, C: $p < 0.01$, D: $p < 0.01$, E: $p < 0.01$, F: $p < 0.05$). The F aquarium was different from all the other aquaria in the first interval (Fischer exact test, from C: $p < 0.009$, from D: $p < 0.0008$, from E: $p < 0.021$), there were no significant differences among the other aquaria and in the second interval.

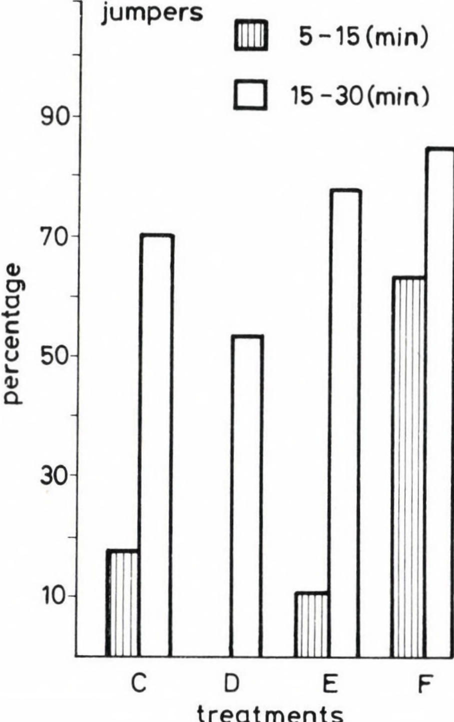

Fig. 5. The proportion of the paradise fish attempting to jump-out in per cent in the first and second 15 min, when the number of the covered side walls was increased

As for FRZ (Fig. 6) only the animals in the D aquarium showed a significant difference within the two intervals (Cochran Q test, $p < 0.02$). We found a significant difference between the B and C groups during the first 15 min (Fischer exact test, $p < 0.018$), the B and D ($p < 0.043$) and the D and E ($p < 0.038$) groups. In the second quarter of an hour the A and E ($p < 0.025$), the C and D ($p < 0.004$) groups showed significant differences.

Experiment 5

In this experiment we studied fish in a two-partitioned aquarium separated by a green plastic wall with a round hole in its middle that makes movements between the two sections possible. We measured how the first

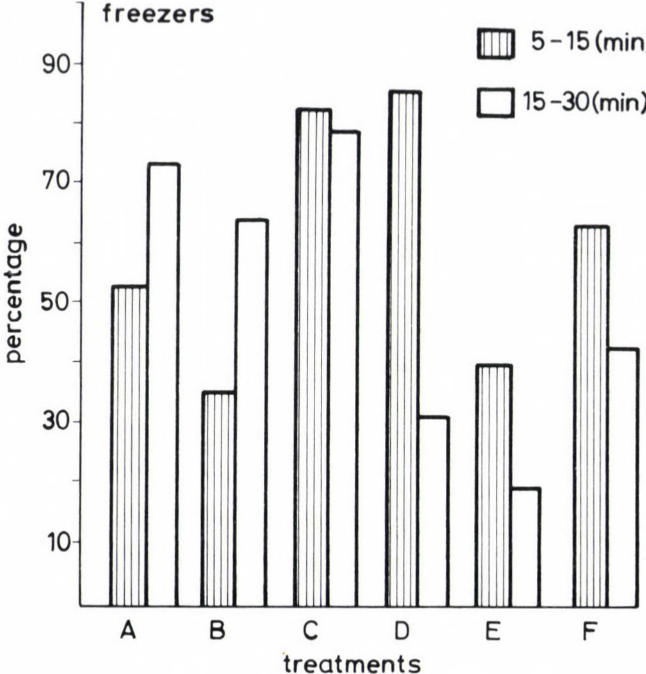

Fig. 6. The proportion of the paradise fish performing freezing in per cent in the first and second 15 min, when the number of the covered side walls was increased

swim through of the paradise fish were affected by the diameter of the hole on the separating wall, the covering and the lighting of the starting chamber.

Equipment and method

The experimental equipment was a 20x40x20 cm glass aquarium, which was divided into two identical compartments by a green plastic panel. There was a round hole, of different diamters in each case, in the middle of this panel. The lower edge of the hole was placed 1.5 cm from the bottom of the aquarium. We used three variations of covering of the compartments. The way of covering was the same as in Experiment 1.

"O": a compartment with uncovered, transparent side walls (open);

"C": the side walls are covered, the compartment is lit from above (closed);

"CD": the side walls are covered, the top is also covered, the compartment is dark (closed-dark).

The variations are as follows: (type of the start compartment is the first) 0/0, 0/C, 0/CD, C/0, C/C, C/CD. The diameter of the round hole was 2.5, 3.5, 6, 9, 11, 16 cm, respectively.

The aquaria were placed in an isolated room. The observations were made from behind a green plastic screen.

The measurement started after the animals were placed into the start compartments and ended when the fish swam through the hole. The maximum duration of the experiment was 1800 s. If the step-out had not occurred up to that time, the measurement was terminated.

Results

In Table 3 we gave the number of the individuals that swam through the hole of different diameters during the 1800 s and the number of the animals that remained in the start compartment. It occurred only in the open start compartment that some animals did not swim through the gate. Even in these cases the number of the animals was significant only in the 0/CD euqipment. Comparing the pairs: 0/CD-C/0: x^2=10.76; 0/CD-C/C: x^2=21.14; 0/CD-C/CD: 13.77; and $p < 0.001$ in each case.

Table 3

Shuttling activity of the paradise fish

Start compartment		Number of fish remaining in the starting compartment number of fish left the starting compartment — Diameter of the hole (mm) 2.5	3.5	6	9	11	16
N	0	3(17)	0(21)	0(23)	2(30)	1(35)	0(15)
	C	3(20)	5(21)	1(24)	0(23)	0(21)	0(20)
	CD	7(13)	9(23)	5(20)	3(19)	1(19)	0(20)
Z	0	0(10)	0(10)	0(10)	0(10)	0(10)	0(10)
	C	0(20)	0(36)	0(20)	0(10)	0(20)	0(10)
	CD	0(11)	0(24)	0(10)	0(10)	0(11)	0(10)

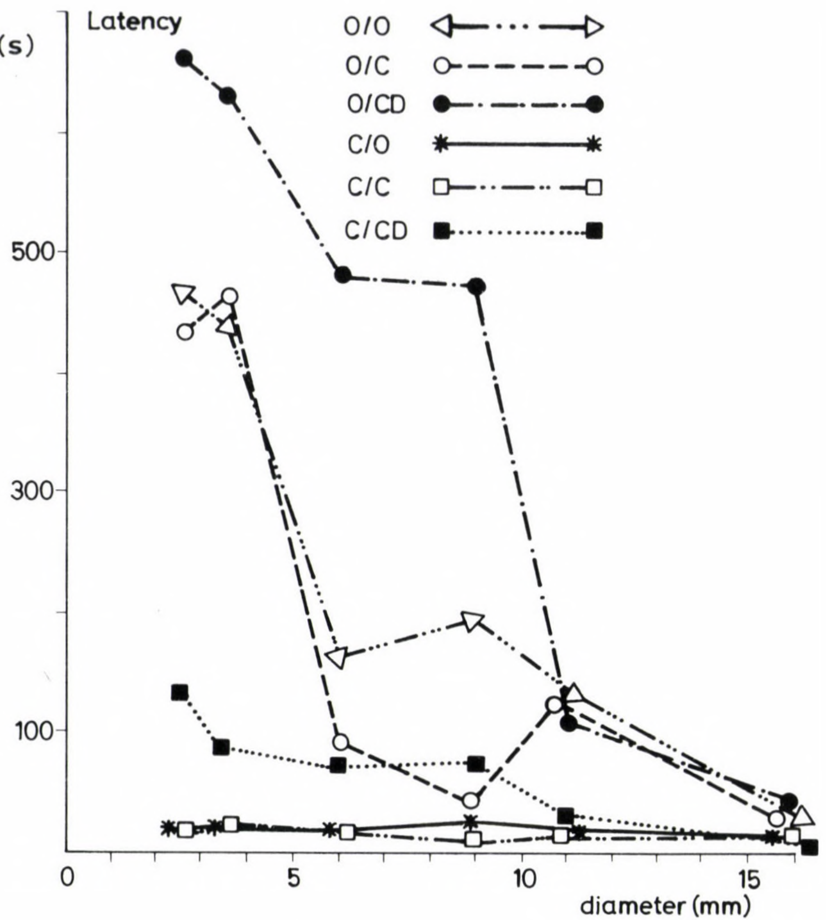

Fig. 7. The means of the step-out latencies in seconds through gates of different diameters in the two compartments aquaria (O Open, uncovered; C closed, covered and lit; CD closed and dark, covered and unlit compartment)

In Fig. 7 we gave the means of the step-out latencies according to the type of the experimental equipment and the diameter of the space. The step-out latencies were analyzed by four-way ANOVA (the covering of the start compartment X the covering of the second compartment X the lightning of the latter X the diameter of the gate). The analysis has got a defective lay-out as we did not study the O/OD and C/OD type equipment where OD is for the uncovered, dark compartment, in transformations were made on the data.

We found significant differences with all the four main effects. The covering of the start compartment: $F/1,600/=142.02$, $p < 0.0001$. The covering of the second compartment: $F/1,600/=5.42$, $p < 0.002$. The lighting of the second compartment: $F/1,600/=68.29$, $p < 0.0001$. The diameter of the hole: $F/5,600/=25.87$, $p < 0.0001$. We found significant interactions between the covering of the start compartment and the diameter of the hole: $F/5,600/=$ $=4.97$, $p < 0.0002$, the covering of the start compartment and the diameter of the hole: $F/5,600/=5.13$, $p < 0.0001$. So darkness and the narrow space are aversive for the paradise fish and thus they are increasing the latency, and the transparent glass wall by provoking ESC, delays the step-out between the two compartments.

Discussion

The animal's behaviour is affected not only by its inner state, but its animate and inanimate environment as well. For example analyzing the proportion of food consumption we have to consider not only the strength of hunger as the inner motivation but the lay-out of the food and the other objects of the environment and the presence of predators as well /13, 14/. Not only the predator but also the environment have an influence on which components become active of the escape repertory of a given species.

In the first three experiments we were looking for the environmental stimuli provoking the defence behaviour element, ESC.

In Experiment 1 non-transparent aquarium wall prevented ESC, (group A) even if the cover of the inner side of the wall was partially removed (Group B). Transparent surface on the wall induced it. The narrow but transparent stripe or round spot (groups C and D) make the paradise fish try and get through it, quite irrespective of the fact whether the attempts are successful or not. The surface of the uncovered area influenced the ESC reaction in a limited range between 0—150 mm^2, (Fig. 2).

In the third experiment we could observe the finer visual control of ESC. A transparent layer of water up to 7 mm thick has no influence on the escape behaviour but a distance longer than 7 mm makes the animal try, and got it in an increased degree (Fig. 3). From this we can draw the conclusion that a visual space of a certain size induce the fish to swim through in spite of the tactile cues of the obstacle. A similar observation was made by Chase /3/ on bats.

Part of the results of Experiment 4 concerned also ESC. Fish staying in a completely open space (Fig. 4A and B) were not able to habituate their escape reaction completely, only to a slight degree. Complete covering inhibits ESC entirely (Group F).

It is well known that open spaces are aversive to various fishes. Fraser and Cerri /9/ observed that minnows tend to avoid an open space in the presence of a predator. The aversive effect of the open space are indicated not only by prolonged ESC but also that freezing occurs with more fish in the open (A-B) aquaria in the second period while some covering diminish the freezing tendency (groups D, E, and F).

It was also observed that fish try to escape from an aversive environment of from a predator by jumping out of water as well /12/. In our experiments the covered vertical side wall provokes this form of escape in the paradise fish (groups C, D, E, F). Visual stimulus is absolutely essential for this, mere tactile, mechanical stimuli are not enough to induce it. The paradise fish frequently lives in temporary waters, which could dry out occasionally, jumping out to find open water supposed to be an adaptive behaviour.

Judging from the duration of ESC and frequency of FRZ and J, we can consider D, E and F aquaria which are more covered as less aversive environment than A and B or C. This is indicated by the quick habituation of escape, the low frequency of freezing. Paradise fish need at least two days to habituate for a new environment /6/ this explain constant low escape tendency of the animal and also shown by the number of jump-outs, which is increasing at the beginning and still remains high after a longer period of time.

The results in the last experiment show that a closed, covered space does not offer a safe hiding place for the paradise fish. The dark compartment is aversive for the animals, which is indicated by the fact that even with 9 cm there is a high latency to enter. The light intensity is a decisive factor in the distance from which the predator and the prey are able to discover each other /16/. The daily activity of many predator and prey fish is connected with the change in light intensity, which influences the risk of the prey /11/. The danger of predation is generally less in the sunny periods of the day /2, 15/. This may be the reason for the aversion of the paradise fish to darkness.

Not only dark or light characters of the various places are influencing its behaviour, but other finer details too. It prefers swimming into

a hole to jump out, however if we offer the possibility of escape, it will choose this behaviour as long as the hole separating the two compartments is smaller than 6 cm.

There are two main motives influencing the behaviour of the fish staying alone. They are the potential predator effect on the one hand /4, 5/ and the intention to get back to the familiar environment to the conspecific on the other /6/. The actual way of escape depends on the animate and inanimate environment so that the presence or absence of certain stimuli influences the appearance of the alternative answers in a decision process. When choosing the experimental equipment in a laboratory, it is essential to explore and take into consideration these stimuli.

Acknowledgements

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INHIBITION BY SODIUM BROMIDE OF SYNAPTIC POTENTIATION AND CALCIUM ACCUMULATION IN THE SUPERIOR CERVICAL GANGLION OF RAT

Á. PÁRDUCZ[1], J. TOLDI[2], F. JOÓ[1] and J.-R. WOLFF[3]

[1] Laboratory of Molecular Neurobiology, Institute of Biophysics, Biological Research Center, Szeged, Hungary; [2] Department of Comparative Physiology, József Attila University, Szeged, Hungary; [3] Department of Anatomy, University of Göttingen, Göttingen, FRG

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Electrophysiological data show that sodium bromide (NaBr) inhibits the development of the post-tetanic potentiation in superior cervical ganglia of rat. NaBr treatment also prevented the accumulation of calcium in synaptic vesicles and dendritic vacuoles otherwise seen for some minutes after a tetanizing stimulation. These observations support the notion that the calcium ions entering into pre- and postsynaptic elements play an important role in the genesis of synaptic potentiation.

Keywords: Post tetanic potentation — sodium bromide — superiod cervical ganglion — rat

Introduction

Long-term potentiation (LTP) is a long lasting increase in synapse efficacy following a very short train of repetitive stimulation /3/. This phenomenon was noted in a variety of different nervous system regions /2, 8, 13, 18/ and also in the rat suprior cervical ganglion (SCG) /6, 9/. In their *in vitro* study Briggs *et al*. /5/ demonstrated that in the case of SCG the post tetanic enhancement has two distinct phases. There is an early, rapidly decaying component, termed post-tetanic potentiation (PTP) and a slower one, with time constants of 2.5 min and 105 min, respectively. In our experiments the potentiation was elicited *in vivo* and in these circumstances we could observe the first phase only.

Send offprint requests to: Dr. Á. Párducz, Laboratory of Molecular Neurobiology, Institute of Biophysics, Biological Research Center, P.O.Box 521, H–6701 Szeged, Hungary

Akadémiai Kiadó, Budapest

It is generally accepted that calcium ions play an important role in the formation of LTP /5, 10/. It could be demonstrated that the inward Ca^{2+}-fluxes and the resulting increase in intracellular calcium serve as a trigger signal for the induction of the activity dependent changes of synaptic transmission and neuronal excitability /1, 17/. Due to technical difficulties, however, very few data are available on the fine structural localization of the cation, especially its change in distribution in relation to the development of the enhancement of synaptic efficacy has not been fully explored. Recently we demonstrated /15/ that in the SCG the potentiation effect was accompanied by an increase of calcium deposits in synaptic vesciles and dendritic vacuoles. In the present study experiments were performed in rats treated with NaBr, a compound with known hyperpolarizing effect in the SCG /14/. As in these circumstances the neuronal excitability was also influenced it was tempting to study whether the NaBr treatment causes and change in the PTP formation and the distribution of calcium.

Materials and Methods

Twenty adult Sprague-Dawley rats of CFY strain were used under chloralose urethan anaesthesia (110 mg/kg body wt. chloralose, 770 mg/kg urethane). The animals were drinking NaBr in tap water (280 mg/100 ml) for 5 days. This treatment raises the bromide concentration in blood to 11.58 ± 0.24 mEq/l /16/. After tracheal cannulation, the SCG was exposed and freed from connective tissue, a small window was made in the capsule at the rostral pole of the SCG for electrophysiological recordings. Compound ganglionic potentials were monopolarly recorded with a ball-tipped silver wire electrode from the rostral pole of the ganglia *in situ*. Details of the *in situ* recording from the SCG have been published earlier /7, 16/. To elicit the enhanced synaptic efficacy submaximal stimulation of the presynaptic fibres was carried out, as described by Brown and McAfee /6/. To cope with the variance of amplitudes we used the averaging technique in the following manner: the control and test stimuli were given with low frequency (0.2 Hz) before and after the tetanizing preganglionic stimulation which was carried out at 20 Hz for 20 s. The pulse duration was 0.3 ms. The recorded signals were stored on an FM tape recorded (Hewlett-Packard) or were averaged on line in the blocks of 10 potentials with a MOTOROLA computer (MC 6800). During the control period 5 blocks of 10 potentials were evaluated (Mean $\pm$ S.D.). Potential blocks were taken at 1; 5; 10; 20; 25; 30; 35 and in some cases at 60 min after the tetanizing train. Because of the low stimulating frequency and the short time course of PTP we could get only 1 block of potentials in each testing point. The results presented below are based on amplitude measurements as used in other laboratories.

For electron microscopy, the ganglia were perfused via a cannula inserted into the carotid artery with a solution of 3% glutaraldehyde and 90 mM potassium oxalate (pH 7.4, 4 ^{o}C) for 5 min. Distribution of calcium was studied by the method of Borgers *et al.* /4/. Morphometric studies were

carried out on photographs at a magnification of 67.000:1 in double blind fashion. The number of synaptic vasicles/μm^2 of synaptic profiles with or without calciumpyroantimonate granules was assessed in 20 to 40 synapses per survival time using an electronic pen and a digitalizing table (MOP AM2, KONTRON, München, FRG) connected to a microprocessor system. As the Kolmogorov tests proved the existence of normal distribution of data, the Student's t test was used for statistical analysis.

Results

After tetanizing stimulation, the enhancement of ganglionic response was observed in all ganglia not treated with NaBr. The largest increase (180—200% compared to the pretetanic controls) was always detected between the 1st and 5th min after tetanizing stimulation. The amplitudes then decreased gradually and usually returned to the control level about 15—20 min after tetanizing train (Fig. 1).

In contrast, no enhancement of the compound ganglionic potential was seen after tetanizing stimulation in ganglia pretreated with NaBr. Instead, the amplitudes of ganlgionic potentials seemed to decrease slightly within the first 10 min before returning to the control level (Fig. 2).

As a result of oxalate-pyroantimonate fixation, electron-dense granules, known to be composed of Ca-pyroantimonate /11/, were detected in different cell organelles (nuclei, mitochondria, vacuoles, etc.) and occasionally freely in the cytoplasm. The number of synaptic vesicles did not change in any experimental situation. All values were found between 193 ± 46 and $168 \pm 38/\mu m^2$ of synaptic profiles, not differring significantly. The presence of calcium could be detected in the majority of synaptic vesicles, this was not influenced by the NaBr treatment (Fig. 3). Following stimulation , however, we could find an obvious difference between the control and NaBr treated groups. As reported earlier /15/ in control ganglia the number of calcium containing deposits localized in pre- and postsynaptic organelles was temporarily increased after 20 s stimulation at 20 Hz. In the presence of NaBr this was not observed, i.e. contrary to the untreated ganglia the tetanizing train did not result in a higher labelling of synaptic vesicles and dendritic vacuoles (Fig. 4).

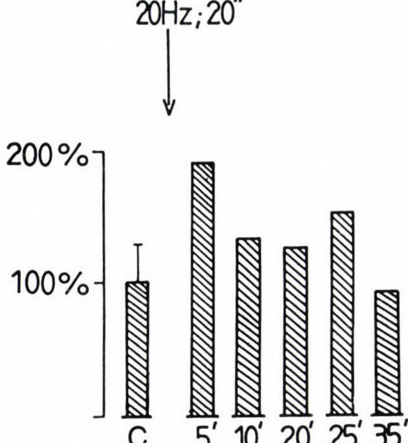

Fig. 1. Amplitudes of evoked responses before and after 20 s of tetanizing stimulation at 20 Hz from control (non-treated) ganglia, expressed as percentage of amplitudes recorded before the train. Each column represents the averages of 10 potentials, the control value and S.D. (C) were calculated from 5 blocks of 10 potentials

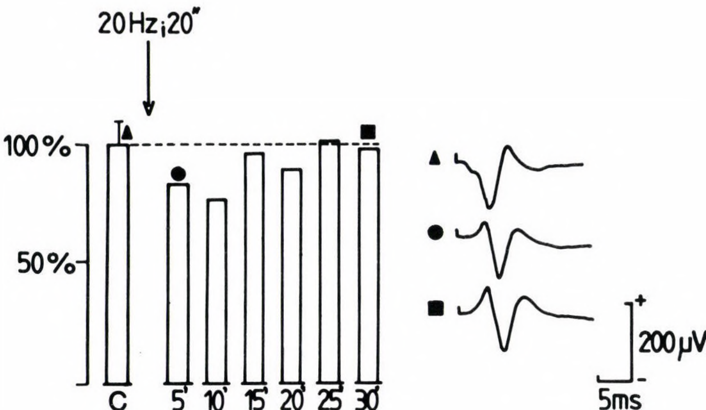

Fig. 2. In animals pretreated with NaBr the tetanizing stimulation did not result in potentiation. Representative ganglionic evoked potentials are shown on the right before (triangle), 5 and 30 min (circle, square) after the tetanizing stimulation

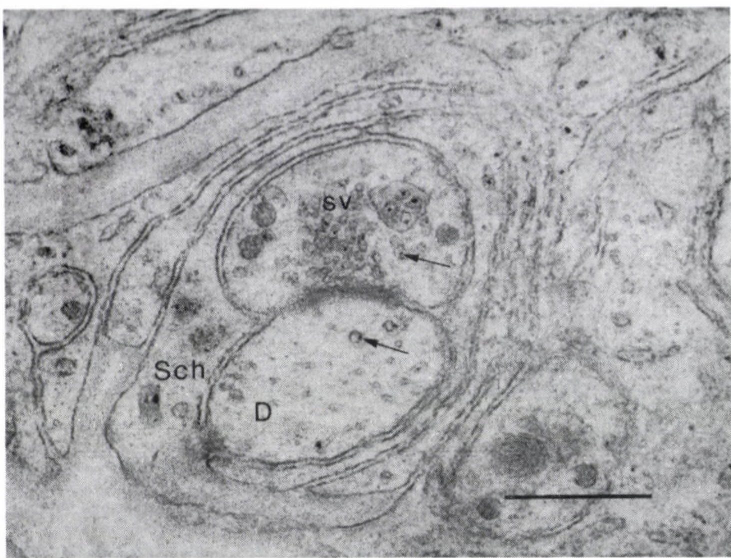

Fig. 3. Ganglionic synapse from NaBr treated rat fixed 1 min after the tetanizing train. Note the presence of electron-dense deposits in the synaptic vesicles and dendritic vacuoles (arrows). D: dendrite, Sch: Schwann cell process, sv: synaptic vesicles. Scale bar 0.5 μm

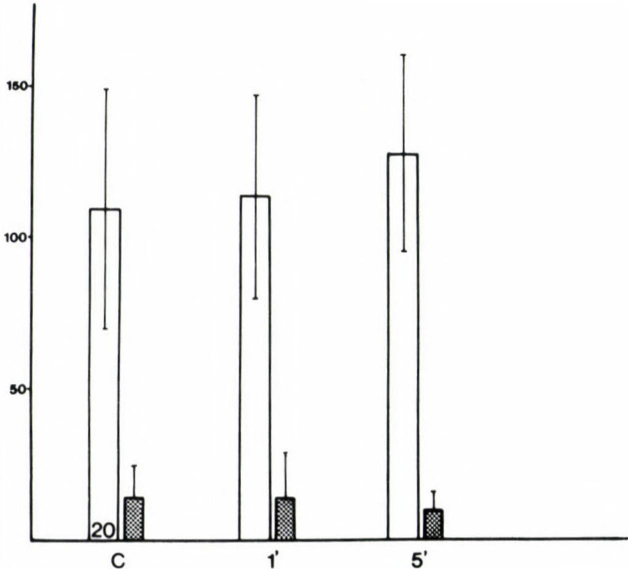

Fig. 4. The density of Ca-pyroantimonate containing synaptic vesicles (open columns) and dendritic vacuoles (dotted columns) in NaBr treated ganglia before and after the tetanization. The values are expressed as number/μm^2 ± S.D. of pre- and post-synaptic profiles

Discussion

Two conclusions can be drawn from the results presented here: i) the development of PTP in the SCG of rat can be prevented by NaBr treatment, ii) the mechanism of potentiation apparently is calcium dependent because the increase in the number of calcium containing pre- and postsynaptic organelles observed in ganglia with PTP, did not take place after NaBR pre-treatment.

Our knowledge about the mechanism of action of NaBr on neurons is rather limited. Montoya and Riker /14/ demonstrated that this substance has a hyperpolarizing effect on sympathetic ganglion cells. On the other hand, both LTP and PTP need a certain amount of excitation to become expressed. Drugs, like NaBr, which depress excitation can block facilitation of synapses indicating the importance of presynaptic events in the triggering of PTP. In a previous study /12/, we showed that NaBr inhibits the acetylcholine release from presynaptic endings. The observation that NaBr prevented the PTP formation in the SCG may, therefore, be related to its inhibitory effect on transmitter release.

The present experiments provide further support for the hypothesis that calcium ions play an important role in the genesis of PTP. We reported previously /15/ that the change in number of calcium containing synaptic vesicles and dendritic vacuoles coincides well with the appearance and disappearance of potentiation. This relationship is further supported by our present experiments, because in ganglia, where PTP could not be induced we did not find any increase in labelled pre- and postsynaptic structures. Naturally, the temporal coincidence of electrophysiological and morphological findings does not mean a causal relationsip. In our opinion, the presence of calcium containing reaction product in certain cell organelles indicates role in the sequestration and transport of the excess intracellular calcium which has accumulated during activity.

The lack of increased labelling in NaBr treated ganglia indicates that less calcium is present in the nerve terminals, i.e. the inward Ca^{2+} movement was somehow inhibited during the tetanizing train. By interfering with the depolarization secretion coupling, the lower intraterminal calcium concentration may result in a decreased transmitter release and this is why the PTP cannot be induced under these circumstances. Although the way of action is not known and needs further investigations, this effect of NaBr may offer a relatively simple and new experimental approach for studying the molecular mechanisms underlying LTP formation.

Acknowledgement

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EFFECTS OF ISCHEMIA ON OPIOID RECEPTORS IN NEWBORN PIG LUMBAR SPINAL CORD

K. GULYA, P. TEKULICS* and P. KÁSA

Central Research Laboratory and *Pediatric Clinic,
Szent-Györgyi Albert Medical University, Szeged, Hungary

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The characteristics of opioid receptors were studied by the binding of (^{3}H)naloxone in ischemic lumbar spinal cord segments of newborn pigs. Ischemia was elicited by ligating the aorta for 30 min. The number of mu opioid receptors decreased, from 117 ± 18 to 89 ± 11 fmol/mg protein, while the K_d value was not significantly altered. It is concluded that even a relatively brief interruption of the oxygen supply may cause severe damage in the lumbar spinal cord of the newborn pig, affecting the opioid neurotransmission. The animal model employed here might be suitable for studying the effects of hypoxia in newborns and children during chest operations involving the decending aorta.

Keywords: Ischemia — opioid receptors — spinal cord — pig

Introduction

The presence of endogenous opioid peptides in the spinal cord has already been well documented: biochemical and immunochemical /4, 23, 26, 28/ studies have mapped the cytoarchitecture of the opioid system in this area. Multiple opioid receptor subtypes are widely distributed throughout the central nervous system. Behavioural /25/, receptor binding /19/ and autoradiographic /1, 11, 13/ studies have revealed their presence in the spinal cord.

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*Send offprint requests to: Dr. Károly Gulya, Central Research Laboratory, Szent-Györgyi Albert Medical University, Somogyi u. 4, H-6720 Szeged, Hungary

Akadémiai Kiadó, Budapest

The binding characteristics of these receptors can be affected by several endogenous (ions, nucleotides, other endogenous peptides, among others) and exogenous (shock, hypoxia, hemorrhagic shock, etc.) factors. The possibility that endogenous opioid peptides and receptors might be involved in spinal cord injury has also been hypothesized in several reports /5, 6, 10/.

Although spinal cord injury followed by paraplegia is the most dreaded complication of operative procedures on the descending aorta, various principal causes have been theorized /2, 3, 15, 17, 20, 22, 29, 31/. Wadouh et al. /31/ concluded that spinal cord injury must be caused by an inadequate oxygen supply. This would explain the obvious neurodegenerative changes seen in spinal cord injuries caused by ischemia. Accordingly, it appeared that a study of the mu opioid receptors in the lower spinal cord would facilitate a better understanding of the basic neurochemical changes involved in the development of paraplegia.

Materials and Methods

Chemicals

The chemicals used and their sources were as follows: (N-allyl-2,3-^{3}H) naloxone (9.25 MBq; Amersham, U.K.); naltrexone-HCl (Endo Labs., Du Pont, de Nemours, Belgium); bovine serum albumin (RIA grade, Sigma, St. Louis, Mo); toluene, 2,3-di-2-(phenyloxazolyl)benzene (POPOP), 2,5-diphenyloxazole (PPO) and Triton X-100 (Reanal Fine Chemicals, Budapest, Hungary). All other reagents were of analytical grade.

Surgical procedure

Ten newborn pigs of either sex, weighing 1000 to 1700 g, were acquired from a local breeding farm. Under ketamine-HCl anesthesia, the aorta was isolated (5-6 mm distal from the origin of the subclavian artery) and ligated for 30 min. During this time, all clinical and electromyographic signs of paralysis were evident in all cases. Sham-operated animals served as controls. Removal of the ligature was followed by a 4 h autoreperfusion period, during which time the blood supply of the ischemic territory was fully reestablished. After completion of the reperfusion, animals were killed by the intracardial injection of 20 ml of air, and segments L_{2-6} of the lumbar spinal cord were removed.

Receptor binding

Pig spinal cord membranes were prepared as follows. Lumbar sections (500-800 mg) of spinal cord were rapidly removed and placed in cold glass dishes. Regions of interest were separated and homogenized in ice-cold

50 mM Na/K-PO_4 buffer (pH 7.4 at 4 °C) with a polytron homogenizer. The homogenates (5% w/c) were centrifuged twice at 43.000 g for 10 min, the final pellets were reconstituted in 50 mM Tris-HCl buffer (pH 7.4 at 25 °C) and 0.2 ml was used for (^{3}H)naloxone binding. The saturation of (^{3}H) naloxone binding was performed as described by Gulya et al. /14/ with slight modifications. Aliquots of membranes were incubated with (^{3}H)naloxone (0.02-4.0 nM) in the absence or presence of 10^{-6} M naltrexone in a final volume of 1 ml in 50 mM Tris-HCl buffer (pH 7.4 at 25 °C) containing 5 mM $MgCl_2$ and 2 mg/ml bovine serum albumin (RIA grade) for 120 min at 25 °C. All incubations were performed in duplicate. Bound and free ligand fractions were separated and the reaction was terminated by rapid vacuum filtration (Brandel Cell Harvester, Gaithersburg, Md) through Whatman GF/C glass fiber filters presoaked in 0.1% polyethylenimine. After filtration the filters were rinsed twice with 5 ml of ice-cold physiological saline and then placed in scintillation vials. Six ml of scintillation cocktail (1000 ml toluene, 150 mg POPOP, 4 g PPO, 100 g Triton X-100) was added and the samples were counted at least 8 h later in an LKB Rackbeta 1215 liquid scintillation counter with 44 per cent efficiency.

Protein measurement

Protein contents of samples were measured by the method of Lowry et al. /18/, bovine serum albumin being used as standard.

Data analysis

Binding data were analyzed with a non-linear least squares regression program (Biosoft, Elsevier, U.K.). The results given for B_{max} and K_d the arithmetic means ± S.D. and geometric means (with the range of values) of n experiments, respectively. Student's t-test was used for statistical analysis. A p value of 0.05 was taken as indicating a significant difference in the mean values being compared.

Results

The saturation of (^{3}H)naloxone to newborn pig lumbar spinal cord membranes revealed that the 30-min aorta occlusion significantly decreased the maximal number of opioid receptors (B_{max}, arithmetic mean ± S.D.), while the dissociation constant (K_d) was unaffected (Table 1). Non-linear regression analyses gave apparent dissociation constants (K_d, geometric mean with the range of values) of 1.25 nM (0.90-1.67) and 0.90 nM (0.7-1.51) for the control (sham-operated) and ischemic animals, respectively. The B_{max} value in the controls was found to be 117 ± 18 fmol/mg protein, while in the ischemic animals B_{max} was 89 ± 11 fmol/mg protein, differing from the control value significantly ($p < 0.05$).

Table 1

Alterations in opioid receptors during ischemia in spinal cord segments L_{2-6} of newborn pig

	K_d(nM)	B_{max} (fmol/mg protein)	n
Sham-operated animals	1.25 (0.90 - 1.67)	117 ± 18	5
Ischemic animals	0.90 (0.70 - 1.51)	89 ± 11*	5

Saturation of (^{3}H)naloxone binding was carried out at 25 °C in 50 mM Tris-HCl buffer (pH 7.4 at 25 °C) containing 5 mM $MgCl_2$ and 2 mg/ml bovine serum albumin (RIA grade) for 120 min. Specific binding was defined as the difference between the total and nonspecific binding (in the presence of 10^{-6} M naltrexone). The K_d and B_{max} values are the geometric mean (with the range of values) and the arithmetic mean ± S.D., respectively. n = number of animals.

*$p < 0.05$ (Student's *t*-test)

Discussion

The complications of surgical procedures (coarctation resection, repair of traumatic injury and resection of an aneurysm) on the abdominal or thoracic aorta include paraplegia of the lower extremities due to ischemic hypoxic damage to the distal part of the spinal cord. Various explanations of the mechanism of the injury have been offered, but none has been definitely established. Since the most feared complication of surgical intervention affecting the thoracic or abdominal aorta is paraplegia, an animal model in which the mu opioid receptors were studied in 24-hour-old pigs was employed to study the extent of damage caused by ischemia in the lumbar spinal cord.

The sensitivity of nervous tissue to hypoxia is well known /9, 21, 27/. Neurotransmitter systems are affected by anaerobiosis /21, 24, 30/ and some of them are more easily damaged than either the energy metabolism or ion transport /24/.

Endogenous opioids and their receptors are hypothesized to be involved in spinal cord injuries. For example, Faden et al. /7/ reported that WIN 44.441-3, a compound with a substantial selectivity towards kappa

opioid receptors, improved neurologic functions. The involvement of these receptors was reinforced by reports of selective increases in kappa opioid receptor binding capacity /16/, and of increased concentrations of the putative endogenous kappa agonist dynorphin A_{1-7} /8/. On the other hand, naloxone, which binds mostly to mu opioid receptors, improves spinal cord blood flow and neurologic recovery following both traumatic cervical /5, 6/ and thoracic /10/ spinal cord injury in cat, indicating the possible role of mu opioid receptors in spinal cord injury. The slight but significant decrease in (^{3}H)naloxone binding observed in our experiments (either having a biological significance or not) shows that even a short interruption of the adequate oxygen supply can affect the opioid system.

The exact cellular basis for the altered receptor number as a consequence of ischemia is not yet known. However, since a reduction in protein synthesis /12/ probably occurs very early during ischemia, the decrease in receptor number reported here may reflect a reduced protein synthetic activity rather than a direct receptor molecule-oriented effect of ischemia.

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MERCURY AND CADMIUM INDUCED STRUCTURAL ALTERATIONS IN THE TASTE BUDS OF THE FISH *ALBURNUS ALBURNUS*

V. BOROVYAGIN*, L. HERNÁDI, J. SALÁNKI

*Institute of Biophysics, USSR Academy of Sciences,
Puschino joka, USSR

Balaton Limnological Research Institute of the Hungarian Academy of Sciences, Tihany, Hungary

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The ultrastructural damages of the taste buds of the fish, Alburnus alburnus were studied after applying 0.05 µM and 0.5 µM mercury chloride as well as 0.1 µM and 1 µM cadmium chloride. The most conspicuous alterations were induced during the first week of heavy metal exposition. The main structural alterations are: 1) the swelling of sensory microvilli and cilia; 2) the extreme dilation of the rER tubules and nuclear membranes, which is most expressed after cadmium exposition; 3) the increase in the number of lysosomes and dens bodies, which is more expressed after mercury exposition; 4) the swelling of the innervating nerve fibres at the synaptic areas of the taste buds, especially after mercury exposition. The damaging processes induced by the applied dose of heavy metals did not increase after the first week of exposition. The taste buds showed regenerated structural appearance after two weeks of exposition to 1 µM $CdCl_2$, while the evoked structural alterations could be detected even after two weeks of exposition to 0.5 µM $HgCl_2$.

Keywords: Taste buds – receptor cells – Cd^{2+}–Hg^{2+} – fish – Alburnus alburnus

Introduction

Heavy metal compounds are considered to be among those agents most dangerous to organisms living in surface waters. At low concentrations heavy metals change the life functions of aquatic animals, while at high concentration they evoke structural and functional damage of the circulatory, respiratory /8/ excretory, and the nervous systems. For fish, chemo-

Send offprint requests to: Dr. L. Hernádi, Balaton Limnological Research Institute of Hungarian Academy of Sciences, H–8237 Tihany, Hungary

Akadémiai Kiadó, Budapest

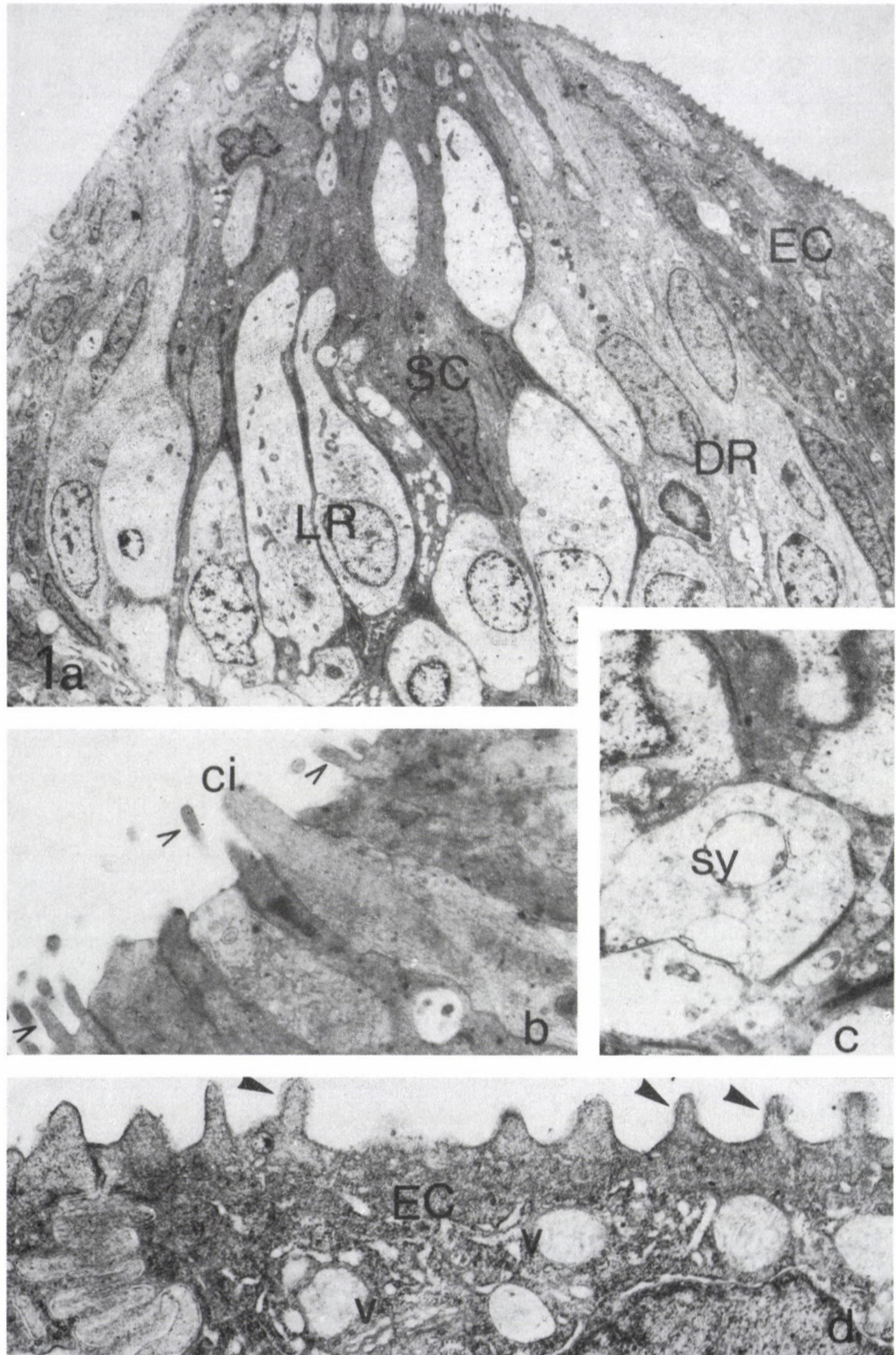
reception plays the most important role in evoking different behavioural patterns /4/. Since they are not protected by external barrier, the receptor membranes that contain the receptor molecules in chemoreceptor organs are directly exposed to the chemical environment /5, 6, 12/. The pollutant chemical compounds may also bind directly to the receptor membranes and may evoke structural alterations both in the receptor membrane and in the whole receptor organ. One type of the chemoreceptor modality is the taste sensation transmitted by secondary taste receptor neurones, which are grouped into taste buds or papillae in fish. It was recently demonstrated that mercury in a high, but sublethal concentration (300 $\mu g/l$ Hg^{++}) (1.5 μM $HgCl_2$) evokes time dependent alteration in the scanning microscopic appearance of the taste buds of the bleak Alburnus alburnus /10/. The present study was undertaken to investigate what type of ultrastructural alterations are induced by sublethal mercury and cadmium pollution in the chemosensory taste buds of the fish Alburnus alburnus.

Materials and Methods

Adult fish specimens of Alburnus alburnus, 7—9 cm in length and 6-8 g in weight, collected in Lake Balaton between June and August were used for the experiments. The fish were kept in aquaria until use. Groups consisting of 12 fish were separated into different aquaria (120 l water), which contained: 1) lake water as control; 2) 10 $\mu g/l$ Hg^{++} (0.05 μM $HgCl_2$); 3) 100 $\mu g/l$ Hg^{++} (0.5 μM $HgCl_2$); 4) 10 $\mu g/l$ Cd^{++} (0.1 μM $CdCl_2$); 5) 100 $\mu g/l$ Cd (1 μM $CdCl_2$) diluted in lake water.

Samples were taken following 1, 2, 3, 7 and 14 days exposure. After decapitation the upper jaw with the palatal organ as well as the lower jaw with the tongue and lip were dissected in cold 2.5% glutaraldehyde buffered with 0.1 M Na-cacodylate (pH 7.4). The dissected samples were kept in fresh fixative for 3 h at 4 ^{o}C. After a short wash in buffer, the samples were post fixed in 2% OsO_4 buffered with 0.1 M s-collidine (pH 7.4) for 2 h at 4 ^{o}C. The samples were dehydrated through increasing concentrations of ethanol; they were block-contrasted in 70% ethanol saturated with uranyl-acetate and embedded into Spurr medium. After polimerization the samples were cut, and semi-thin sections were used to identify taste buds. The ultra-thin sections were contrasted with lead citrate and investigated under a TESLA BS 500 electron microscope.

Fig. 1. a) The taste buds are built up by epithelial cells (EC), light and dark receptor cells (LR, DR), supporting cells (SC); b) The light receptor cells have a cone-like cilia on their apical end (ci), while the dark receptor cells end in microvilli (open arrowhead) on their apical end; c) at the basal region of the taste buds synaptical contacts (sy) can be observed between the receptor cells and the innervating nerve fibres; d) in the cytoplasm of the epithelial cells numerous vacuolae (v), on whose surface a microridge system (arrowhead) can be observed. a x 3.000; b x 10.000; c x 10.000; d x 15.000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v
d

Results

Control samples

Taste buds (Tbs) were observed to be most dense on the palatal organ in a horseshoe shape arrangement. The taste buds are built up by the following cell types (Fig. 1):

a) undifferentiated epithelial cells that possess microridge systems on their surface and having dense cytoplasm with tonofilaments;

b) receptor cells, which are grouped in the apical area of the taste buds, and their cytoplasmic density show light or dark appearance.

The light sensory neurons are dominant in the middle of the TBs (Fig. 1). In their cytoplasm, especially in the apical part, the ER elements are often present in vacuolated or vesicular forms. Microfilaments are frequently seen in the cytoplasm. In the upper region of the cytoplasm large, dense granules can be detected (Fig. 2). The light sensory cells possess a large thick cytoplasmic protrusion, the cilia, which frequently have numerous tubular structures on their tips (Fig. 1b).

The dark sensory cells have a dense cytoplasm with neurotubules, especially in their apical part (Fig. 2b), and numerous ER tubules can be observed around the nucleus (Fig. 1a). In the supranuclear region a well developed Golgi complex and numerous dense granules are present. Their cytoplasm ends in several thin microvilly (Figs 1b, 4d). The dark sensory cells can be observed most frequently at the marginal areas of the TBs (Fig. 1a). They have a lobulated basal area where the innervating axonal processes make synaptic contacts with them (Fig. 3).

c) Basal cells can be observed at the bases of the TBs at the innervating neural processes; they have a dense cytoplasm. Numerous free robosomes and glycogen granules can be frequently observed in their cytoplasm (Fig. 3). The taste buds are innervated by myelinated and unmyelinated nerve fibres that reach the TBs through the corium papillae (Fig. 3). The nerve fibres make synaptic contacts with the sensory cells at their base and at their lateral part (Figs 1a, 3). Typical and regular synaptic structures can be rarely observed (Fig. 1c).

Effect of mercury on the ultrastructure of the taste buds

The application of 0.05 μM $HgCl_2$ does not evoke detectable fine structural alteration within two weeks. After applying 0.5 μM mercury, the

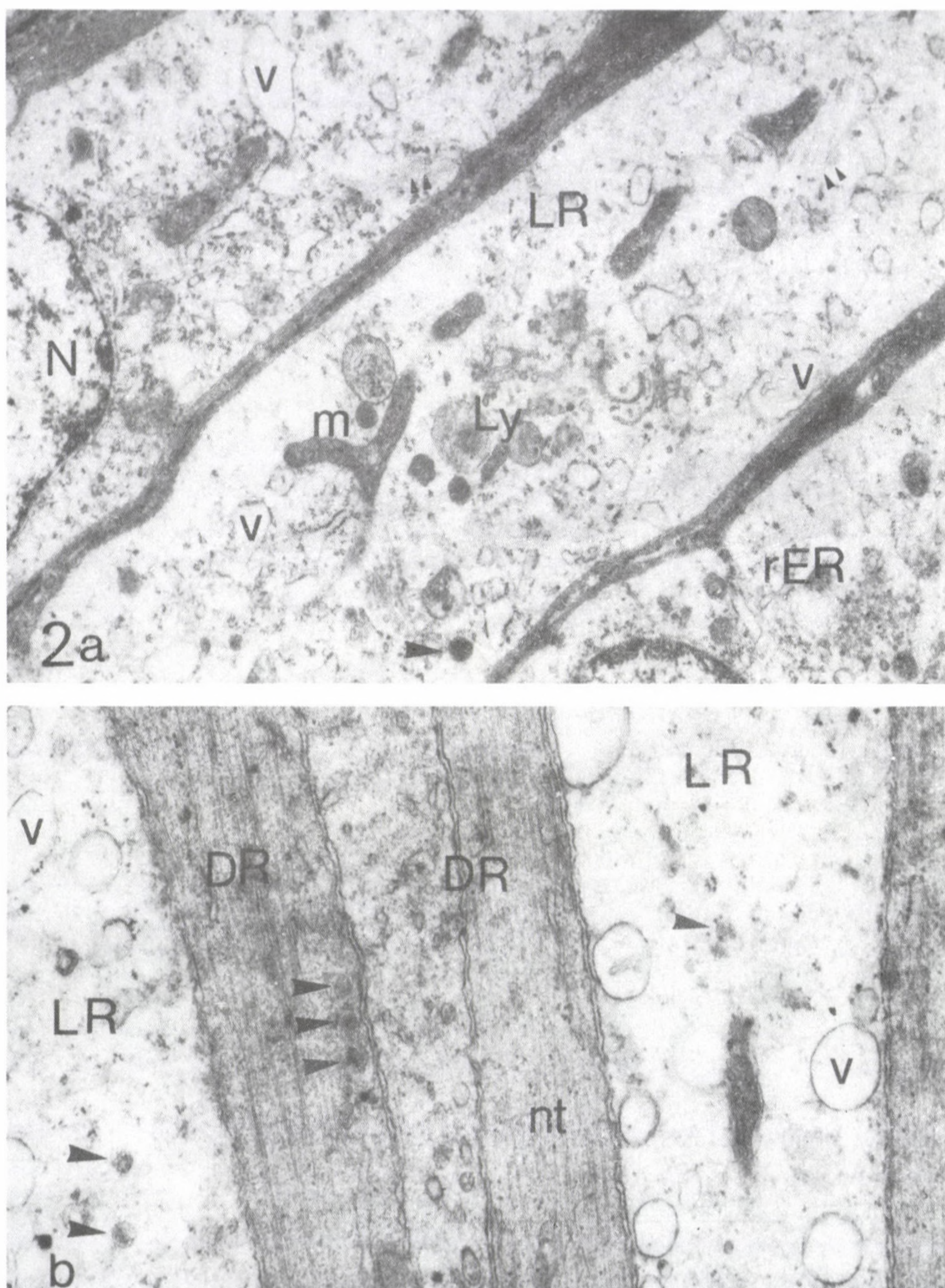


Fig. 2. The light receptor neurons (LR) have numerous rER elements around the nucleus (N), and they have numerous neurofilaments (small arrowhead) and rER vacuolae (v) in the cytoplasm. The dark receptor cells (DR) have numerous neurotubules (nt) in their cytoplasm. In the apical part of the cytoplasm granular vesicles (arrowhead) can be observed in both types of receptor cells. m = mitochondria, Ly = lysosome. a x 16.000; b x 28.000

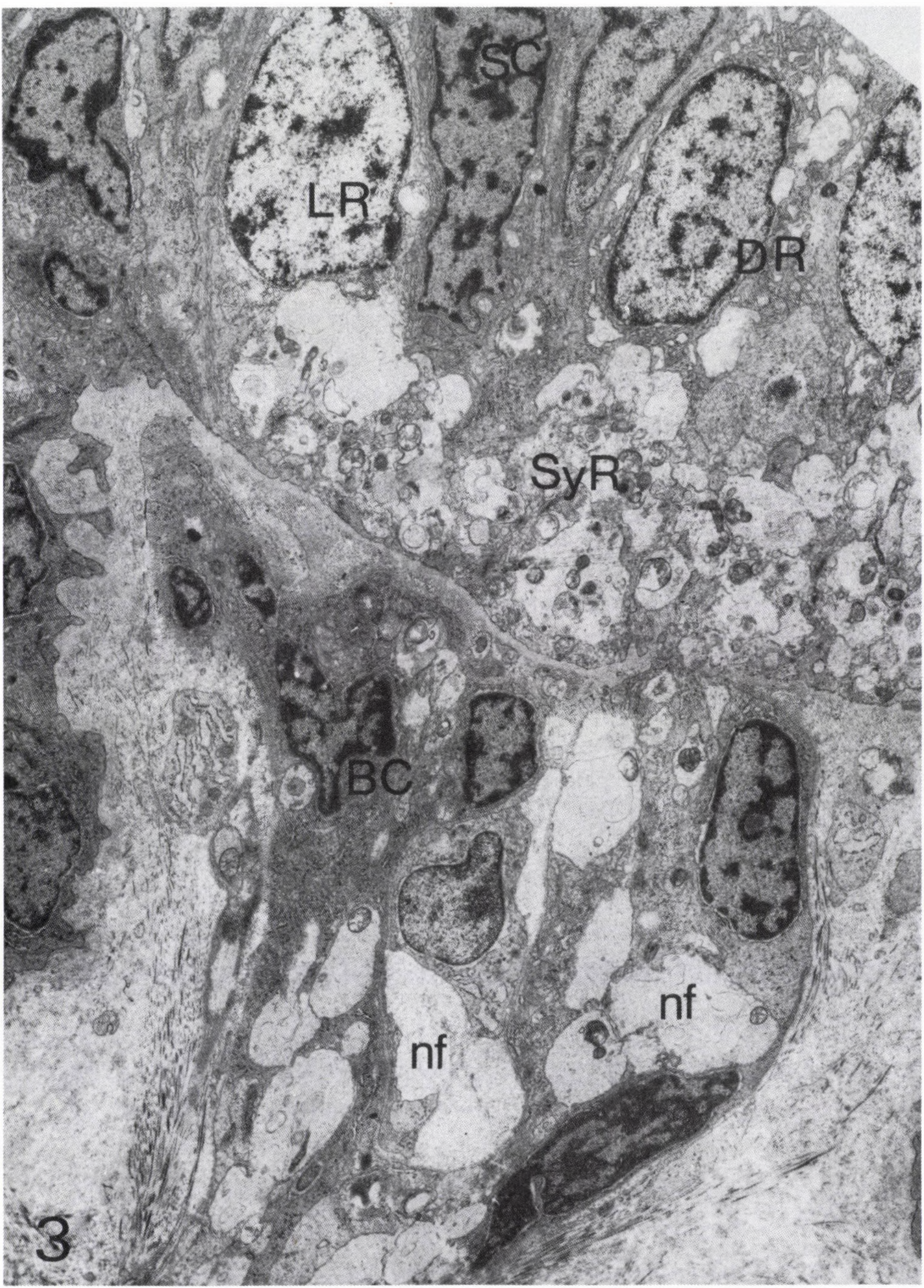


Fig. 3. The innervating nerve fibres (nf) reach the taste buds through the corium papillae penetrating the basal lamina (BL) and establish a synaptic region (SR) at the base of the light (LR) and dark receptor cells (DR). SC = supporting cell, BC = basal cells. x 6.500

first detectable alteration is the increase of mucosal secretion over the sensory pits of the TBs on the first day. By the second day of exposition the thick cilia of the light sensory cells show a slightly swolllen appearance with an irregular outer membrane (Fig. 4d). The neurotubules are frequently disorganized and disappear from the apical parts. Parallel yet different alterations can be observed in the perinuclear regions of the light sensory cells. The number of rER vesicles increases, and the surfaces of ER tubules are frequently free of ribosomes. From the third day of exposition, phagosomes, in addition to lysosome-like structures, can be detected in the perinuclear region. Multivesicular bodies and whorled up lamellar structures can also be detected in sensory cell cytoplasm (Fig. 4a-c). The number of dense particles increases in the lysosomes and phagosomes in the course of treatment (Fig. 5b). The nuclear membrane frequently shows numerous large swellings. The epithelial cells around the sensory pits become swollen (Figs 5a, 11a) and therefore emerge over the sensory pits. At the basal end of the light sensory cells in the synaptic regions, mitochondria show signs of swelling from the first day of exposition. However, after a longer exposition the basal end of the sensory cells appears clear and almost empty (Fig. 6a). After three days of exposition mitochondira are usually swollen in both the sensory cell processes and in the axonal processes; dense membranous bodies can frequently be observed in the innervating axonal processes (Figs 6a, b; 11A).

The dark sensory cells showed less sensitivity to mercury exposition. Structural alteration — i.e. the presence of lamellar or dense bodies which are usually in the cytoplasm in the apical part of the cells could be observed only after the fifth day. The alteration of undifferentiated epithelial cells occurs in varying degrees (Fig. 5a). They usually appear swollen, with celar type degeneration, and numerous vacuolae can be detected in their cytoplasm. Their nuclear membranes are swollen (Figs 5a; 11A). Beyond seven days exposition the evoked structural alterations in the different cell types did not increase further; this condition remained unchanged even after 14 days of exposition.

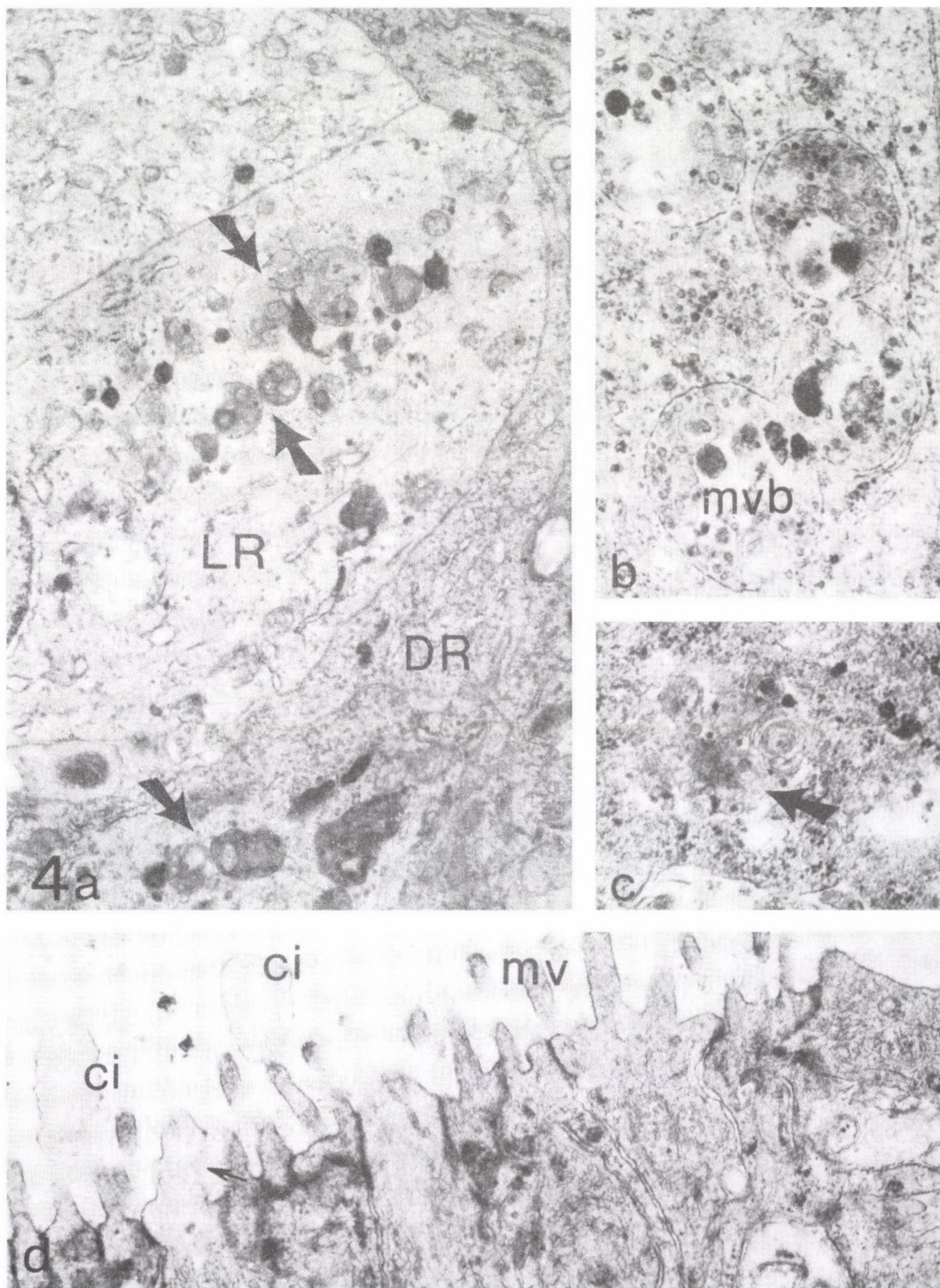


Fig. 4. In the first day of mercury exposition the cilia (ci) and microvilli (mv) of the receptor cells are slightly swollen, and their outer membrane becomes irregular (small arrow) (Fig. 4d). After 3 days of the mercury administration (Fig. 4a, c) the receptor cells (LR, DR) show structural alterations. Numerous dense-bodies with different size (arrow) (Fig. 4.a, c) and multivesicular bodies (mvb) can be observed (Fig. 4c) in their cytoplasm. a x 16.000; B x 30.000; c x 30.000; d x 24.000

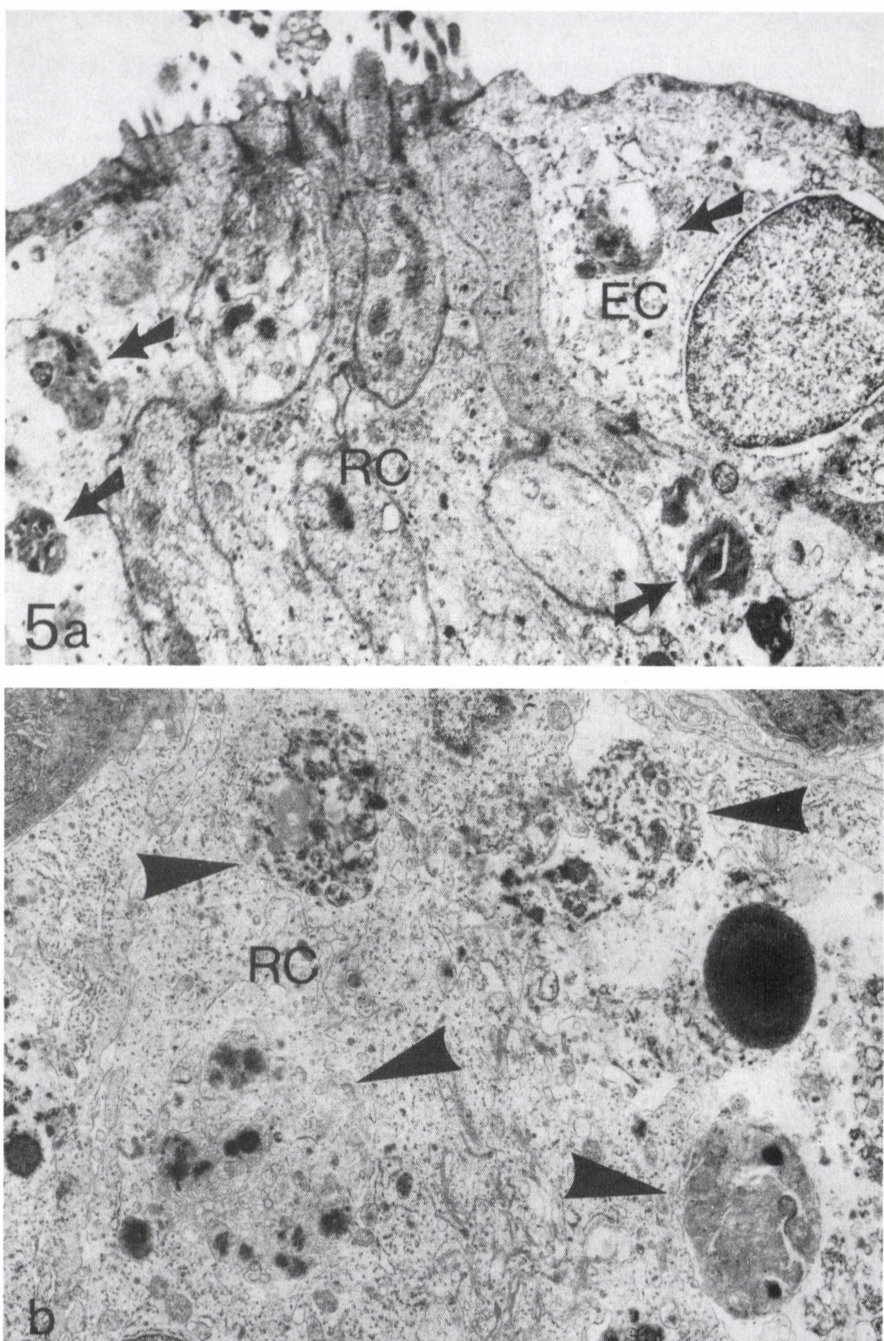
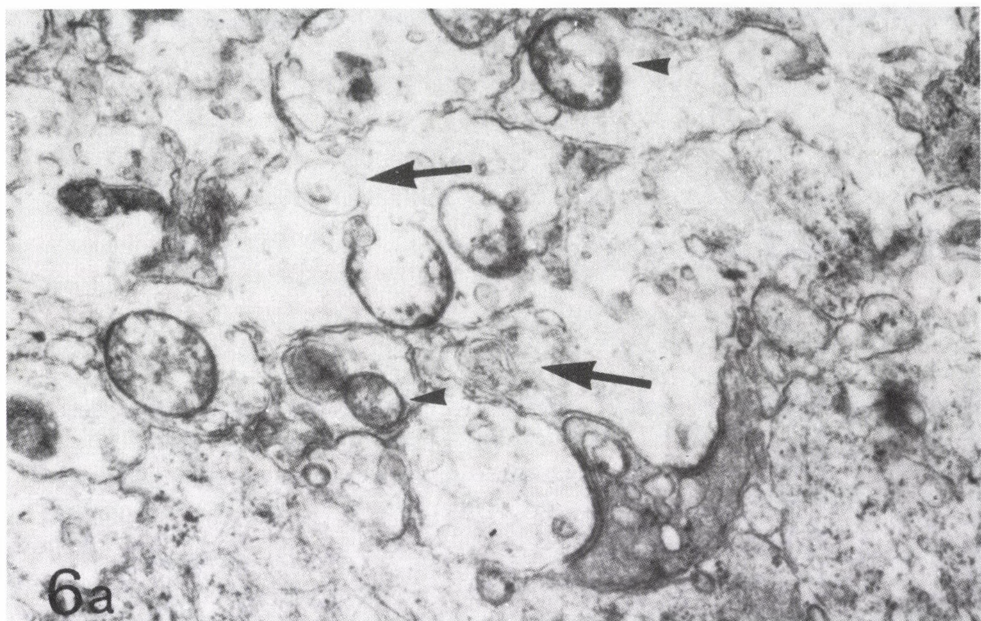


Fig. 5. After seven days of mercury exposition all the receptor cells (RC) and epithelial cells (EC) show remarkable structural alterations (Fig. 5a). The receptor cells and epithelial cells have a disorganized cytoplasm and have numerous lysosomes and dense bodies (arrow) (Fig. 5a) as well as phagosomes (large arrowhead) (Fig. 5b). a x 12.000; b x 9.000



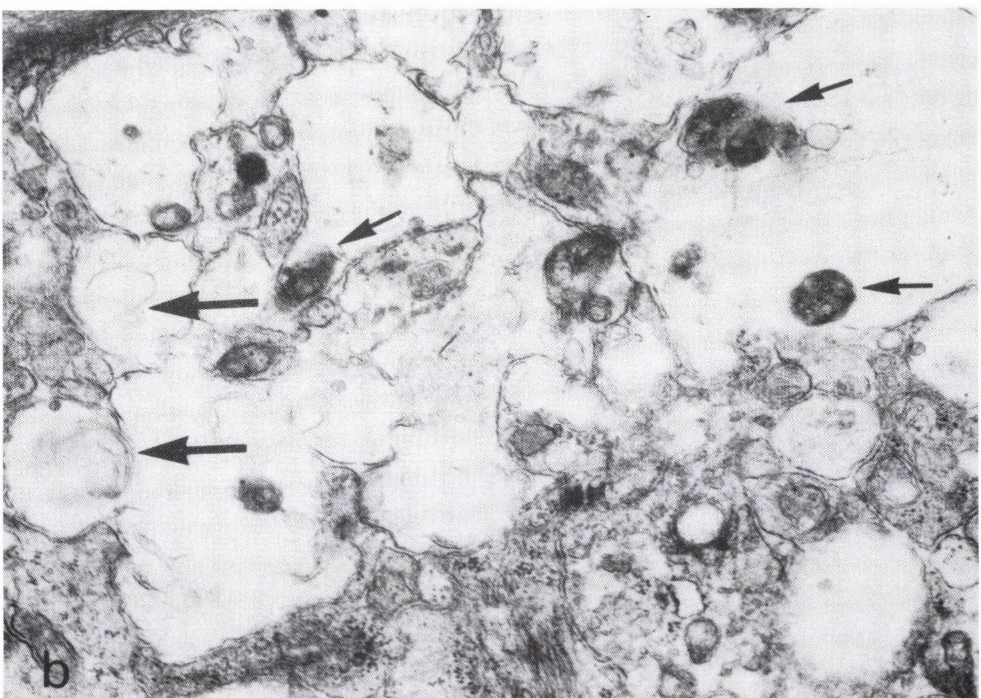


Fig. 6. At the base of the taste buds the synaptic regions show fine structural alterations. After 24 h exposition to mercury, swollen mitochondria (arrowhead) and lamellar bodies (large arrow) can be observed in the innervating nerve fibres, but their axoplasm keeps normal appearance (Fig. 6a). After three days of exposition (Fig. 6b) the axoplasm and the cytoplasm of the receptor cells at the synaptic regions become swollen and transparent. Numerous dens (small arrow) and lamellar bodies (large arrow) can be detected in the cytoplasm (arrow). a x 30.000; b x 24.000

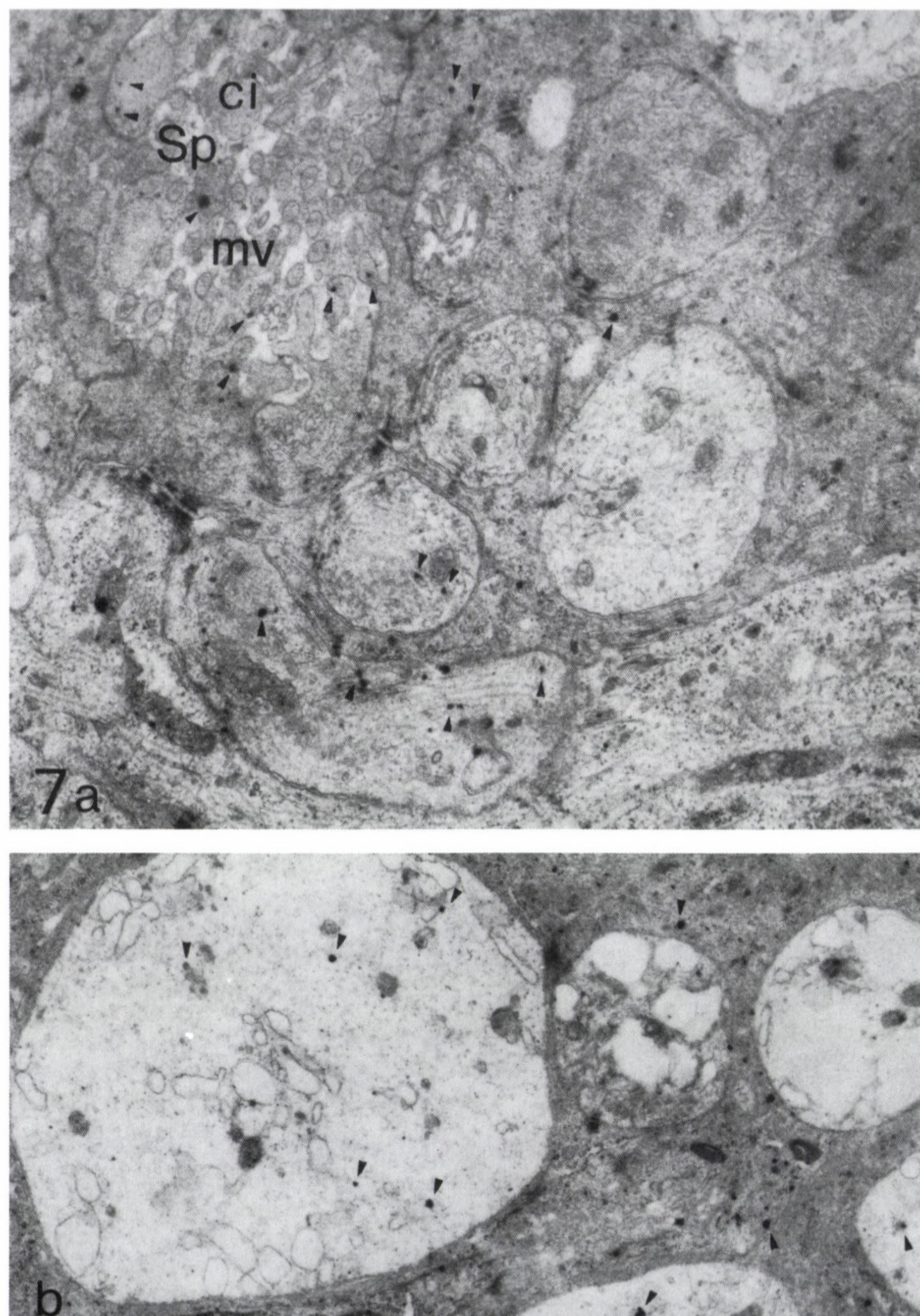


Fig. 7. The first detectable fine structural alterations after 24 h long exposition to cadmium is the appearance of small dense precipitates (arrowhead) in the sensory microvilli (mv) and sensory cilia (ci) at the sensory pits (Sp) of the taste buds, and on the microtubular system of the receptor cells. a x 20.000; b x 12.000

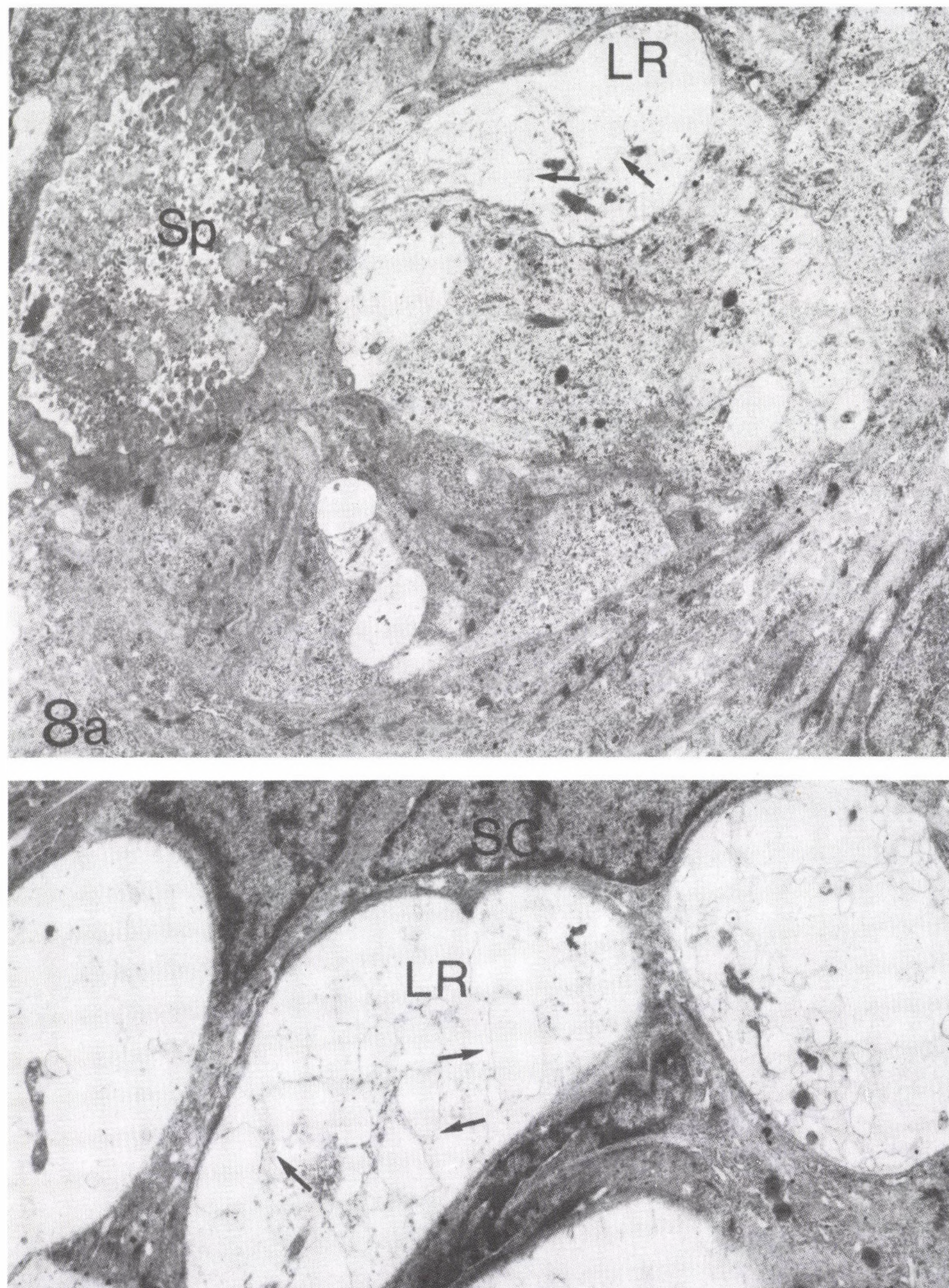


Fig. 8. After three days, one can observe besides the presence of the dense particles, the swelling and the extreme dilatation of the rER tubules (arrowhead) in a few light receptor cells (LR). Sp = sensory pit, SC = supporting cell. a, b x 8.000

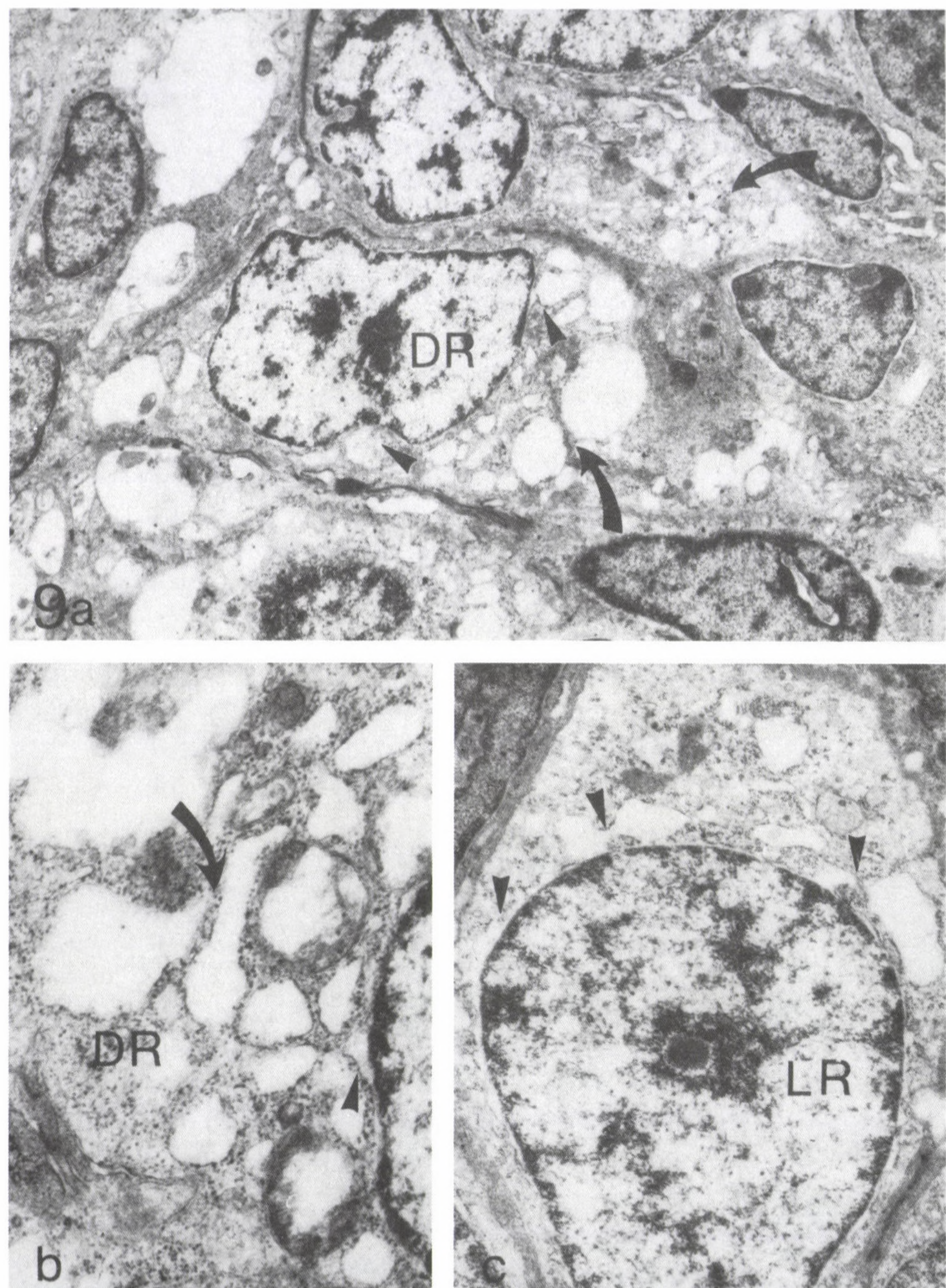


Fig. 9. After six days exposition to cadmium, significant fine structural alterations can be observed in the perinuclear regions of the receptor cells (Fig. 9a). The rER tubules are extremely dilated (arrow), and the nuclear membranes show large swelling (arrowhead) both in the light (Fig. 9c) and dark (Fig. 9b) receptor cells. a x 8.000; b x 24.000; c x 12.000

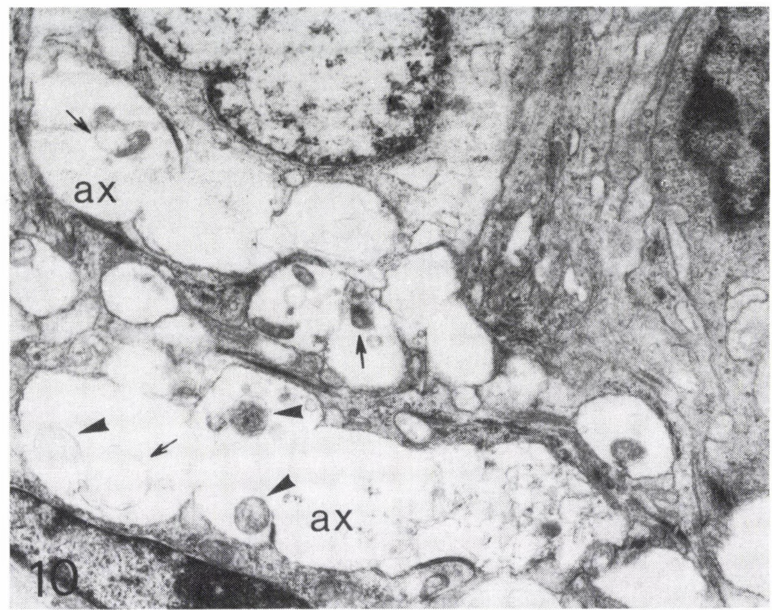
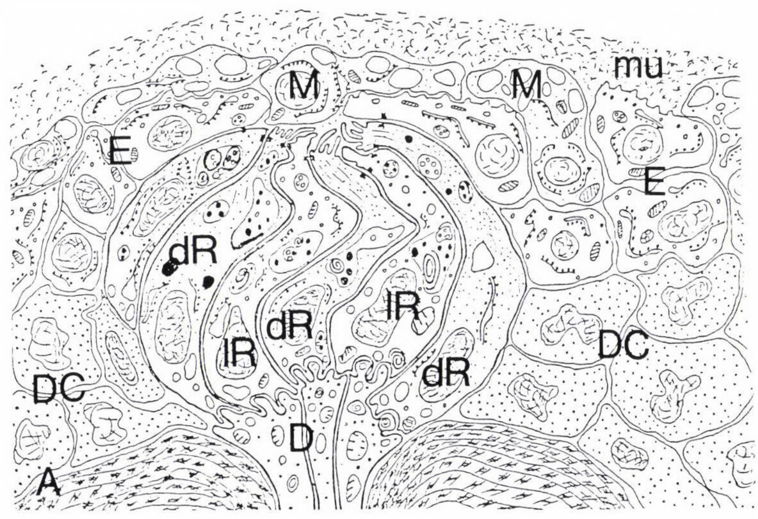
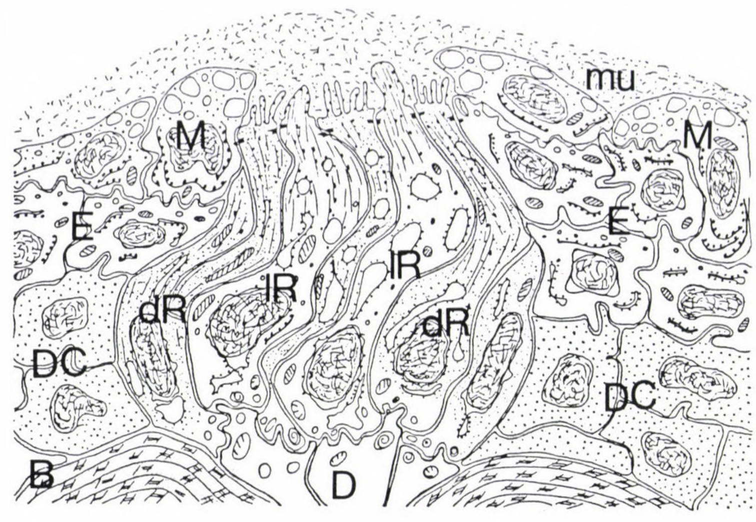


Fig. 10. At the synaptic region of the taste buds, swollen transparent segments of the nerve fibres (ax) can be seen by the 6th day of Cd exposition. Multi vsicular bodies (arrowhead) as well as lamellar bodies (arrow) can be detected in the transparent segments of the innervating axons. x 16.000





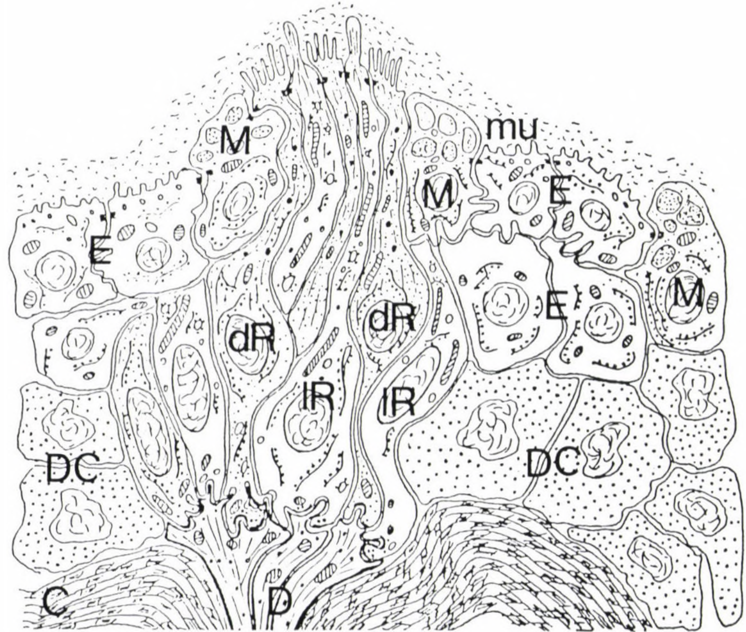


Fig. 11. The schematic drawings demonstrate the appearance of the taste bud after six days exposition to mercury (A) and cadmium (B) as well as the normal appearance (C). M = mucosal cell, mu: mucus, E = epithelial cell, D = innervating nerve fibres, lR = light receptor cell, dR = dark receptor cell, DC = developing cell

Effect of cadmium on the ultrastructure of the taste buds

Alterations induced by 1 µM $CdCl_2$ develop more slowly and slightly compared to those induced by 0.5 µM mercury. In 0.1 µM concentration cadmium ions do not evoke structural alterations in taste buds.

The degree of alteration was different in the various TB cell types. Moreover considerable differences can be observed between taste buds. Both light and dark sensory cells show structural alterations. After one day exposition dense precipitate could be observed in the sensory cilia and microvilli as well as on the neurotubules (Fig. 7a, b).

During the first three days, the earliest and most outstanding ultrastructural alterations can be observed in the rER tubules (Fig. 8a, b) and the nuclear membranes (Fig. 9), of the receptor cells. In the perinuclear region both the rER tubules and the nuclear membranes show different degrees of swelling in both the light and dark receptor cells (Fig. 9a, b, c). Sometimes enormous swelling of the ER tubules can be observed in the apical part of the light sensory cells.

The undifferentiated epithelial cells show structural alterations sporadically. Usually their cytoplasmic density increases, and sometimes the ER tubules are slightly swollen. In some cases the extracellular spaces between the epithelial cells are dilated. At the basal region of the TB sensory cell, the innervating nerve fibres show slight swelling, and they have light, sometimes transparent segments at the base of the sensory cells. In the transparent segments swollen membranous structures and dense mitochondria are present. Normal typical synaptic structures cannot be observed (Fig. 10). By the 14th day of exposition all the investigated taste buds showed a normal structural appearance.

Discussion

Heavy metals affect the sensory system of fish very rapidly, since the animals show immediate avoidance or preference behaviour in test water polluted with different heavy metals /5, 6, 9, 11/. Whitefish slightly avoided Cd^{++} at a low concentration (10 µM) but did not avoid it at higher or lethal concentrations /2/. The rainbow-trout, however, avoided Cd^{++} with a threshold of 0.5 µM /1/, thus demonstrating significant species differences in this respect. In contrast to their reaction to Cd^{++}, whitefish did

not show avoidance to Hg^{++} until the concentration reached 100 µM /2, 9/. Whitefish exposed to 0.18 µM $HgCl_2$ showed greatly reduced time dependent behavioural responses to food extract /2, 9/, thus demonstrating that the chemosensory organs were affected during the exposition.

Electrical recordings from the taste system of the carp and Atlantic salmon showed that heavy metals, including Hg^{++} and Cd^{++}, were effective blocking agents of gustatory responses /7, 12/. High concentration of mercury (100 µM $HgCl_2$) destroyed the structure of taste buds in the palatal organ of goldfish within 1 h /14/.

The effective concentrations used in our experiments were far lower (1 µM $CdCl_2$ and 0.5 µM $HgCl_2$) than that used in the case of goldfish (100 µM $HgCl_2$); therefore, the evoked structural effects were less expressed and required longer exposition time. In goldfish the 100 µM $HgCl_2$ evoked a general necrotic destruction in all TB cell types. However, at 0.5 µM $HgCl_2$ exposition bleak TB cell types showed only fine structural alterations without necrotic degeneration. The light sensory cells proved to be the most sensitive cell type: they reacted with an increased number of rER vacuolae and lysosomes as well as with the appearance of dense lamellar bodies in their cytoplasm.

TBs exposed to 100 µg Cd^{++} (1 µM $CdCl_2$) showed structural alterations in the sensory cells, such as the swollen nuclear membranes and rER tubules throughout the cytoplasm. Parallel with the observed ultrastructural changes in the sensory cells, fine structural alterations could be observed in the regions of the synaptic contacts between the sensory cells and the innervating axons after either Cd^{++} or Hg^{++} exposition. This alteration, together with that described in the receptor cell cytoplasm, may contribute to the detected behavioural changes. This is in good agreement with the electrophysiological results obtained on the gustatory system of carp and Atlantic salmon, which show that Hg^{++} and Cd^{++} are effective blocking agents of gustatory responses /7, 13/.

If we compare the effects induced by single doses of Cd^{++} and Hg^{++}, we observe that during the same exposition time mercury evokes more prominent and longer lasting structural effects than does cadmium. Although cadmium evokes avoidance — behaviour at a very low concentration /2/, it causes only mild structural alterations. Mercury, however, evokes avoidance — behaviour only at high concentrations /2/, despite of its penetration through membranes /3/, but it provokes much stronger structural altera-

tions. Thus, it appears that the two heavy metals have different abilities and use different pathways for the penetration of receptor membranes.

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THE ANTIALLATAL EFFECTS ON LOCUSTS AND LETHAL EFFECTS ON NEMATODES OF SYNTHETIC PRECOCENE-1 DERIVATIVES DIFFERING AT THE CARBON 7 POSITION

A. FODOR[1], P. DEÁK[1], MÁRIA BERÉNYI[1], T. TIMÁR[2], S. HOSZTAFI[2], I. KISS[1]

[1]Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary; [2]Alkaloida Chemical Company, Tiszavasvári, Hungary

Fourteen precocene-1 (P1) derivatives differing at C-7 were synthetized and tested for their antiallatal activities on Locusta migratoria (in vitro and in vivo) and nematocidal effects on Caenorhaditis elegans.

An outstanding antiallatal effect was produced by 7-propargyloxy-P1 in vitro. It caused an elevated rate of mortality when applied in vivo to locusts or nematodes. The antiallatal effect of 7-cyclopentyloxy-P1 was not accompanied by toxicity. Aralkyloxy substitution at C-7 eliminated the precocene activity.

Keywords: Precocenes — derivatives — mortality — locusts — nematodes

Introduction

Procócenes are 2H-1-benzopyrans synthetized by several plant species in the genus Ageratum /36/ and are therefore called ageratochromenes /1/. They were first discovered as anti-juvenile hormones /3/ or antiallatotropins /4-8/. In fact, they may be "suicide substrates" /10/ or more exactly proallatocidins /13, 29/, acting selectively and directly on the corpora allata (CA) and leading to their inactivation and destruction /6, 29, 32/ and consequently to cessation of juvenile hormone (JH) production /29/.

There are large variations in the susceptibilities of different insect species to the in vivo detectable effects of precocene treatment.

The effect of precocenes can be characterized by ED_{50}, the effective dose, the concentration causing destruction of 50% of the treated corpora

Send offprint requests to: Dr. A. Fodor, Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, P.O.Box 521, H–6701, Hungary

Akadémiai Kiadó, Budapest

allata which can be determined either in vitro or in vivo and by LD50, the half lethal dose, the concentration leading to 50% mortality of the tested animals.

The most sensitive species, such as L. migratoria can be characterized by low ED50 values and high LD50/ED50 ratios throughout their life, although these values alter in the various developmental stages /28/.

There are also moderately sensitive species, such as Diploptera puntata and Drosophila melanogaster /23, 37/, and insensitive ones, such as Periplaneta americana /30/ and Tenebrio molitor /9/. These in vivo detectable differences between insect species seem to be based upon the species-specific adsorption and/or non-specific peripheral (protective) metabolism of precocenes, rather than differences in the tissue-specific precocene-sensitivity of their CA /8, 29/.

The results of precocene treatment of nematodes is less clear, but it has been found that growth of the nematode C. elegans is markedly retarded by precocene treatment, and that this is partly reversible following treatment with the JH analogue "methoprene" /17/.

The precocenes are called proallatocidins /13/ because they become activated to "suicide compounds" by tissue-specific bioactivation mechanisms in the CA. Several hypotheses exist as to the nature of this bioactivation. Most of the circumstantial evidence undoubtedly support the "epoxidation theory" /8/, but no unambiguous experimental evidence is available about either the in situ appearance of the assumed active compound (precocene-epoxide) or the biochemistry of the hypothetical enzyme (epoxidase).

The discovery of the two major metabolites of precocene-2 (P2) led Bergot et al. /2/ to the conclusion that oxidative dealkylation is the most important metabolic pathway of P2 degradation. This conclusion, however, came from data on non-specific peripheral destruction processes of precocenes.

Precocenes might also be rearranged by the CA tissue into reactive alkylating agents called "quinone-methide", but again no unambiguous experimental evidence supports this hypothesis /6-8/.

Recently, Dinya et al. /15/ suggested (on the basis of quantitative structure-activity relationship (QSAR analysis) that oxidative dealkylation might play an important role in the antiallatal activity of precocenes. The exact mode of action of precocenes is not yet known and additional research is required to elucidate it.

Whether precocenes will eventually become "fourth generation in-

secticides", as earlier predicted /4/, or will "just" remain fine chemicals and extremely useful tools for studying physiological functions related to JH /31/, depends upon the availability of analogues (i) acting also as pro-allatocidins, (ii) more effective in their tissue specific action than the original precocenes, and (iii) resistant to peripheral detoxification. Although numerous precocene analogues have been synthetized so far /7, 13/ relatively few real structure optimization studies have been published, except those describing the basic structural criteria for the biological activity /5, 26/. It is known that the specific action of precocenes is expressed in the CA, where only about 1% of exogenous precocene is incorporated /6/. Our aim is to utilize the qualitative and quantitative structure-activity relationships to design precocene derivatives which are more effective than the original ones.

Our working hypothesis is to try to establish the roles of the different substituents on the basic molecules in the tissue-specific activity. When searching for precocenes of higher activity in the target organ, we used the _in vitro_ CA (_L. migratoria_) test system developed by Schooneveld /33/. In some cases, morphogenetic effects were also tested _in vivo_. Non-specific toxic effects of precocenes were tested on first instar larvae of the nematode _C. elegans_ /17/. As a first step we describe herein our biological data concerning P1 derivatives containing different substituents at the C-7 positions.

Materials and Methods

Chemicals

Precocene derivatives were synthetized as described elsewhere /34/. Precocene 2 (P2) (Calbiochem, La Jolla, CA., USA) and precocene-3 (P3) /35/ were used as references. The compounds were diluted in glass-distilled acetone for topical application and for nematocide tests, and in ethanol for mixing with the tissue culture medium, as described by Schooneveld /32, 33/.

Animals

Locusta migratoria migratorioides were kept under crowded conditions in a regime of 12 h light and 12 h darkness at 32 °C during the photophase and 28 °C during the scotophase. The relative humidity was kept at about 70%. Eggs were collected daily and the population was partially synchronized by keeping animals hatched within 2 days in the same cages. Animals were fed fresh wheat and maize seedlings, and bran supplemented with milk powder.

Caenorhabditis elegans CB 678 nematode strain (for the nomenclature of the C. elegans strains, see /21/ was kept on conventional NGM agar plates seeded with E. coli OP 50 bacteria, as described by Brenner /11/. Populations were synchronized for experiments in two subsequent steps: (1) dauerlarvae were collected and selected in 1% sodium dodecyl sulphate (SDS) solution and allowed to moult into L4 and subsequently to adults, as described by Cassada and Russel /14/. Twenty-four h after SDS selection, young gravid adult hermaphrodites were dissolved in alkaline NaOCl solution, as described by Emmons et al. /16/. Eggs were allowed to hatch in M9 buffer /11/ for 16 h at 18 °C.

Tests for morphogenetic activity

Precocene derivatives were applied topically to the dorsum of the abdomen of CO_2-narcotized, freshly (within 12 h) moulted L4 instar L. migratoria nymphs. Controls received the corresponding volume of acetone. Treatment was carried out in the late afternoon. Animals were examined 10 days after the last (on the case of adultiforms, the next) moult. Four hundred 100, 50 and 25 µg precocene analog per nymph doses were used. Four hundred µg of precocene analogue per nymph was used, if this does induced prothetely, doses of 200, 50 and 25 µg of the same analogue were also tested.

Tests of antiallatal effect

The method developed and applied by Schooneveld /32, 33/ to locusts was followed with a few modifications. Briefly, young L5 instar nymphs, collected in a "locusta Ringer" and transferred within 4 h to Grace's medium (Gibco, Europe, No. 159) containing the desired amount of the respective precocene derivatives. The glands were incubated for 24 h. Randomized batches of 4-6 CA were incubated in 0.4 ml medium in Linbro tissue culture multiwell plates (with cover, Linbro Sci. Inc., Hamden, Conn. 06517, USA) at 25 °C in a water vapour-saturated atmosphere. Precocene derivatives were added in ethanol the final concentration of ethanol was not more than 1%, which showed no adverse effects on CA). All experiments were run together with both positive (100 µg/ml P3) and negative (1% ethanol) controls. After 24 h of incubation the glands were stained with 0.1% acridine orange and studied under an Amplival fluorescence microscope (Carl-Zeiss, Jena, GDR). The glands which took up the stain were considered to be affected. EC50 values were calculated after log (dose)/logit (percentage of surviving glands) transformation of the inactivation curve by linear regression analysis.

Tests for nematocidal effect

The respective amount of the P-analogue under test was dissolved in aceton and brought to the (0.5 cm^2) surface of an NGM agar plate in a hole of a microtiter plate. The agar was allowed to slidify and the acetone was evaporated off. Well-synchronized C. elegans L1 larvae and an E. coli OP 50 suspension (as food supply) were pipetted in a 5 µl volume onto the surface (area per well about 0.5 cm^2). The plates were incubated at 25 °C for 42-48 h, then the healthy (egg-laying) adult hermaphrodites were removed by aspiration and counted. In short experiments 0, 5, 50, 55 and 5000 µg/ml doses were used with four repeats, with at least 15-25 animals in each. Ef-

fective compounds were retested in the same system in doses of 0, 25, 50, 75, 100, 150, 200, 250, 300, 400, 500, and 1000 μg/ml. The average number of survivors was expressed as percentage of the number of adults found at 0 μg/ml concentration. The percentages of survivors were then plotted against the doses semilogarithmically and LC50 values were determined by linear regression analysis. The precocenes did not really adsorb into the agar, they seem to cover its surface uniformly after the acetone evaporated. The nematodes cannot migrate out of the plate. Whenever a worm left the agar it died immediately because of drying out. If the agar is properly made, and if there are not too many worms on its surface, the animals usually did not dig themselves into the agar during the test period. They never dig themselves into the agar, if the compound was really toxic for them. Whether _E. coli_ did or did not metabolize the precocene compounds we do not know, but we had earlier tested several analogues including P1, P2 and 6,7-methylenedioxy-3,3-dimethylchromene killing the nematodes within 24 h (they could easily survive 24 h without food) both in the presence and in the absence of _E. coli_.

Results

In vitro and in vivo data concerning antiallatal activity in Locusta migratoria

Biological data on P1 derivatives are presented in Table 1. Three compounds (P1, 7-propargyloxy-P1 and 7-cyclopentyloxi-P1) proved effective against _L. migratoria_ both _in vitro_ and _in vivo_. 7-propargyloxy-P1 (compound 8) proved the best of all tested precocenes both _in vivo_ (Fig. 1) and in vitro (Fig. 2). Prothetelic adultiforms could be found at extremely low doses (25 μg/animal) of compound 8 (although their frequency was low). Further, a 100 μg/animal amount of compound 8, at which P2 exerts a 100% morphogenetic effect /27/, proved severely toxic for locusts. The other antiallatalally very active derivative was compound 11, which is more active than P1. 7-prenyloxy-P1 and 7-allyloxy-P1 proved moderately active _in vitro_.

Elongation of the saturated carbon chain in the C-7 alkoxy substituent did not increase the antiallatal activity. However, the negative correlation between the length of the carbon chain and the antiallatal activity is not complete, because 7-nBuO-P1 was more active than 7-nPrO-P1 (compare data on compounds 3 and 5 in Table 1). 7-iPrO-P1 proved definitely more active than 7-nPrO-P1 (compare data on compounds 3 and 4 in Table 1). Neither 7-iBuO-P1 nor 7-secBuO-P1 was more active than 7-nBuO-P1 (compare data on compounds 5, 6 and 7 in Table 1). The data on compounds 12, 13 and 14 suggest that aralkyloxy substitution at C-7 is not the way to improve

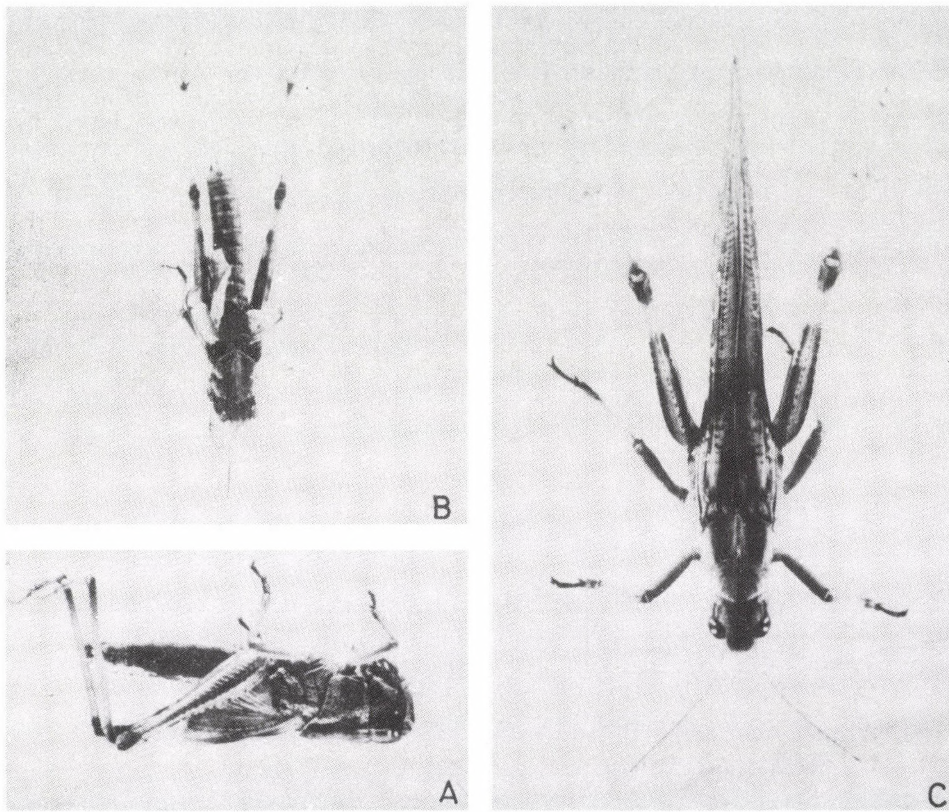
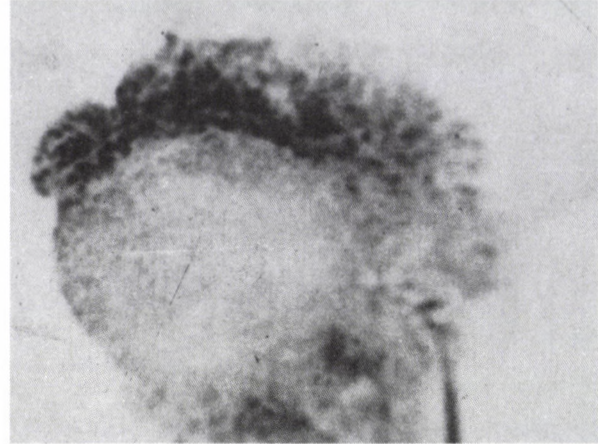


Fig. 1. Effect of compound 8 on L. migratoria in vivo. Young (moulted within 12 h) L4 instar L. migratoria nymphs were treated topically with an acetone solution of the respective dose. The controls were treated with the corresponding volume (max 10 μl) of acetone. The development of the animals was monitored daily and they were photographed 5-10 days after the following moult. Dose: 25 μg compound 8 per animal. A - adultiform lateral view; B - adultiform dorsal view, C - control



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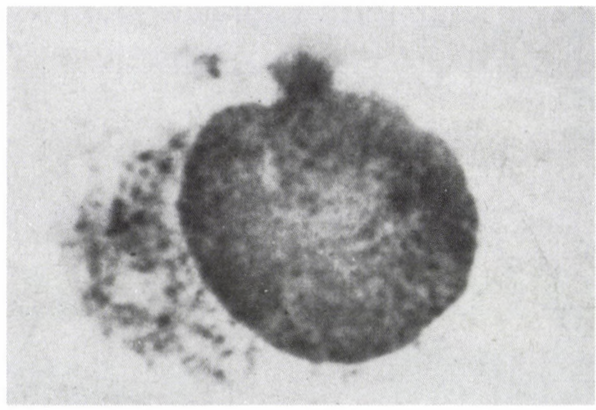

b

Fig. 2. Fluorescence microscopic picture of CA after *in vitro* incubation. CA of young L5 instar *L. migratoria* nymphs after 24 h continuous incubation in Grace medium with and without coumpound 8. The glands were stained with 0.1% acridine orange for a few minutes and then examined under a fluorescence microscope (Amplival, Carl-Zeiss, Jena). **a**. Photograph of CA treated with a 50 µg/ml dose of compound 8. **b**. Photograph of CA treated with the corresponding volume of acetone. Note that the cells of the precocene-treated glands (a) fluorescent because their membrane is premeable for the vital stain acridine orange. The cells of acetone-treated glands (b) are not permeable to it. x450

Table 1

Antiallatal and nematocidal activities of P1 derivatives containing different alkoxy substituents at C-7

Compound	R_7	Antiallatal activity[1] In vitro EC50[2] (µM)	In vivo	C[3]	Nematotoxic activity[4] LC50[2] (mM)
1	Me	43	+	-0.97	0.58
2	Et	155	+	-0.96	3.56
3	nPr	307	+	-0.96	1.39
4	iPr	174	+	-0.96	9.70
5	nBu	73	+	-0.88	1.50
6	iBu	886	+	-0.87	1.49
7	secBu	530	+	-0.91	9.67
8	propargyl	21	+	-0.80	0.81
9	prenyl	50	+	-0.95	NT
10	allyl	94	+	-0.92	13.87
11	cyclopentyl	26	+	-0.96	11.51
12	benzyl	1265	-	-0.87	1.11
13	oCl-benzyl	no effect	-	-	16.59
14	pCl-benzyl	no effect	-	-	NT
P2	reference	58	+	-0.87	0.79
P3	reference	27	+	-0.94	1.04

1 -- for details of the in vitro test, see Materials and Methods;
2 -- EC50 and LC50 were calculated as described in Materials and Methods;
3 — coefficient of correlation calculated from the log-logit line;
4 — for details of the test for nematocidal activity, see Materials and Methods;
NT — not tested

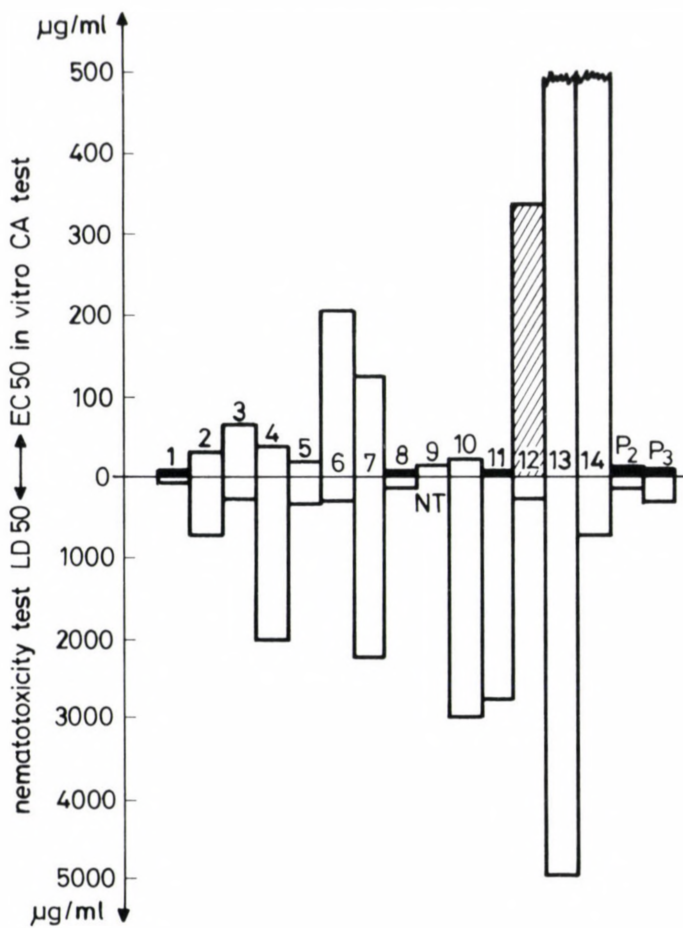

Fig. 3. Antiallatal and nematocidal activities of P1 derivatives containing different alkoxy substituents at C-7. Upper columns: EC50 values (µg/ml); precocene concentrations at which 50% of the CA tested were inactivated after 24 h continuous incubation at 25 °C in Grace medium. For details, see the Materials and Methods. Lower columns: LC50 values (µg/ml) relating to nematocidal effect determined on C. elegans. For details, see the Materials and Methods

the efficiency of precocenes. Propargyloxy and cycloalkoxy substitution at C-7 is preferable.

Data from tests for nematocidal activity

Only two compounds (P1 and 7-propargyloxy-P1) exerted considerable nematocidal activity characterized by an LC50 lower than 1mM (compounds 1 and 8). 7-Cyclopentyloxy-P1 was not a very effective nematocidal agent. On the other hand, 7-benzyloxy-P1 (compound 12), which did not exert anti-allatal activity, proved moderately nematocidal. Figure 3 demonstrates that for compounds 1, 5 and 8 the antiallatal and nematocidal activities seem to correlate, while in some cases (compound 11) the higher antiallatal activity is not accompanied by nematocidal activity, and in other cases (compound 12) a considerable nematocidal activity is not accompanied by antiallatal activity.

Discussion

Precocenes are proallatocidins requiring bioactivation in the target organ, the CA /8, 29/. If there is more than one mechanism capable of metabolizing precocenes, these compounds should be substrates for (at least) two enzyme systems. Precocene 3 is an ideal precocene, probably because of not being a good substrate for destructive enzymes, but sensitive to bioactivation in the CA. P3 can be characterized by a low effective (EC50) and a high toxic (LC50) concentrations. The dual test system we used was aimed at finding molecules with elevated antiallatal and moderate toxic activities. The antiallatal activity can be reliably tested in vitro on the isolated CA. The in vitro assay /33/ has several advantages over in vivo morphogenetic test methods, because one can determine and compare the potencies of precocene derivatives and evaluate changes in the intrinsic sensitivity of the CA to precocenes. Our in vitro test system was a less exact, less sophisticated version of that developed by Schooneveld /33/, but still sensitive enough to detect differences in the antiallatal activities of different precocenes. The duration of exposure of the CA to precocenes in vitro is important. We used continuous incubation and older (L5) instar nymphs, and found the EC50 for P1, P2 and P3 to be 43 μM (8 μg/ml), 58 μM (13 μg/ml) and 27 μM (6.4 μg/ml), respectively, which means about one order of magnitude difference from the data published by Schooneveld /33/.

This difference could be explained either by the age difference, or by different conditions of incubation, or both. In fact, all three standard precocenes (P1, P2 and P3) exerted strong and reproductible antiallatal effect in our in vitro test system, and some compounds (8 and 11) did at least as well as these.

Pratt and Bowers /30/ utilized a radiochemical assay to measure the JH biosynthesis of Periplaneta americana CA exposed to P2 for 3.5 h (without postincubation). Inactivation was achieved with 10^{-2} to 10^{-4} M. Muller et al. /25/ reported that CA from the sensitive species Oncopeltus fasciatus, incubated in vitro in a medium containing $10^{-5.35}$ M P2 for more than 24 h, lose their ability to secrete JH when implanted into last instar larvae. Bowers and Feldlaufer (1982) used a morphogenetic assay for CA activity identical to that employed by Muller et al. /25/ and reported that the CA of Tenebrio molitor, incubated in vitro in the presence of precocenes and subsequently assayed for JH activity, were inactivated in a time-dependent, dose-dependent manner, indicating that the sensitivity of an insect species to precocenes in vivo is not solely dependent on the intrinsic sensitivity of the CA of that insect. In fact, they found that the EC50 of P3 was about 10^{-5} M when CA was incubated continuously for 24 h in the presence of P3. Consequently, we think that our comparative data are reliable.

The real question is whether the antiallatal activity can be increased by chemical modification of the precocene structure. Bowers /5/ reported that 7-propargyloxy-P1 (compound 8) was inactive morphogenetically, but had a sterilant effect on Oncopeltus in vivo (jar deposit test). Brooks et al. /12/ found this compound moderately active in the same test, and in Locusta it proved morphogenetically to be a less active, but a much more toxic derivative than P1 or P2 and had a low agonist activity in vivo. Mihailovski /24/ synthetized 7-propargyloxy-P1 and qualified it as an insecticide rather than an anti-JH when tested on Oncopeltus. These results fit in well with our findings in in vivo tests. In vitro, however, this derivative was extremely effective.

We observed much higher morphogenetic activites of P2 and compound 8 and we did not find 7-iPrO-P1 as effective as reported by Brooks et al. /12/. The agreements and disagreements between the various data on the same species indicate that deviations between populations kept under different conditions could interfere with the results, while the in vitro conditions

can easily be standardized. 7-EtO-P1 (compound 2 in our work) was more active than P1 in Oncopeltus in vivo /5/, but in our test system it was less active in vitro. Ohta /26/ studied the structure-activity relationships of precocenes, and suggested that some steric requirements concerning C-7 are needed for antiallatal activity. He also proposed that the electronic distribution of biologically active and inactive precocene derivatives should be studied. The high antiallatal activity of 7-cyclopentyloxy-P1 (about which nothing has been reported so far) is the first experimental evidence supporting this hypothesis, because the cyclopentyloxy group has special steric features. Our compound 8 has a special electronic distribution and was the most active in our in vitro test system.

Although the growth retardation effect of P2 on nematodes was partly reversible following treatment with the JH analogue "methoprene", reported by Fodor et al. /17/, we considered the toxic effects of the precocene derivatives on C. elegans first stage (L1) larvae to be a rather general toxicity not necessarily coupled to anti-JH activity. In many cases, the two biological activities were parallel. The data relating to 7-cyclopentyloxy-P1 indicate that it might be possible to select precocenes of high antiallatal and low biotoxic activity by using our dual test system.

The data obtained allow us to forecast some means (and limitations) of improving the antiallatal activities of P1 derivatives through C-7 substitution. Propargyloxy and cycloalkoxy substitution at C-7 definitely improved the antiallatal activity. Our data allow exclusion of the following procedures for the improvement of antiallatal activity: (i) increase of the length of the saturated carbon chain of the alkoxy group; (ii) the use of different isomers of the saturated alkoxy groups; (iii) substitution at C-7. We have tested several other 7-araloxy precocene derivatives have also been substituted at other positions but none of them showed any antiallatal activities at all.

Our data show that the antiallatal activity can be influenced by chemical modification of P1 derivatives through C-7. We think that these findings are important and might form a basis for the development of more effective proallatocidins from 7-propargyloxy-P1 and 7-cyclopentyloxy-P1 by further structural modification.

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EFFECT OF LARD AND CORN OIL INTAKE ON SERUM LIPIDS IN YOUNG MEN

M. IMAKI, T. MIYOSHI, S. TANADA, T. NAKAMURA, Y. YAMADA, R. YAMASAKI and H. TERADA

Department of Public Health, School of Medicine The University of Tokushima, Tokushima, Japan

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An experimental diet with lard (30 g/day for 7 days) and corn oil (30 g/day for 7 days) on high carbohydrate (basal diet) was given to four healthy Japanese young men and the effect of diets containing different fat on serum lipids was examined.

Serum total cholesterol was increased significantly from a basal diet of 106±23 to 141±26 mg/dl on lard diet, and then decreased significantly ($p < 0.05$) to 111± ±22 mg/dl on corn oil diet. Serum triglycerides increased significantly ($p < 0.01$) from 66±38 to 173±32 mg/dl on basal diet. Serum HDL-cholesterol was decreased significantly ($p < 0.01$) from 41.9±1.6 to 31.2±3.8 mg/dl on lard diet and increased significantly ($p < 0.05$) to 41.9±4.6 mg/dl on corn oil diet. Serum HDL-cholesterol fraction was decreased significantly ($p < 0.01$) from 41.6±4.9 to 28.1±3.2% on basal diets, but increased significantly ($p < 0.05$) to 44.3±3.1% on lard diet, and then decreased to 36.3±2.5% on corn oil diet. Serum HDL phospholipid fraction decreased significantly ($p < 0.05$) from 62.5±6.7 to 50.7±1.8% on basal diet and increased significantly ($p < 0.05$) to 60.4±1.0% on lard and corn oil diet. Serum phospholipids did not change by experimental diets. It is concluded that lard and corn oil have different and specific roles in lipid metabolism.

Keywords: Lard — corn oil — serum lipids — young men total cholesterol — phospholipids

Introduction

It is known that dietary fats produce a hypercholesterolemic effect in man /1, 4/, increasing the hazards developing atherosclerosis and coronary heart disease. Many reports were published on the influences of high fat and high carbohydrate on serum cholesterol in animals. However,

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Send offprint requests to: Dr. M. Imaki, Department of Public Health, School of Medicine, The University of Tokushima, 3-Kuramoto-cho, Tokushima, Japan

Akadémiai Kiadó, Budapest

less work has been carried out on the relationship between the nature of dietary fat on high carbohydrate diet and serum lipids. In this work, an experimental diet with lard and corn oil on high carbohydrate diet was given to healthy Japanese men and the effect of diets containing different fat on the serum total cholesterol, serum triglycerides, serum phospholipids, serum high-density lipoprotein (HDL)-cholesterol, serum cholesterol fraction, serum lipoprotein fraction and serum phospholipid fraction was studied.

Materials and Methods

Subjects

The subjects studies were four healthy male university students aged 21 to 24 years. Table 1 shows data on their physical characteristics immediately before the experiment.

Table 1

Physical characteristics of subjects

Subject	Age(year)	Height(cm)	Weight(kg)	Broca index
K.S	21	161.4	55.0	89.6
R.Y	22	165.8	58.0	88.1
Y.S	21	172.9	69.0	94.6
S.Y	21	176.2	69.0	90.5
Mean±S.D.	21.3±0.5	169.1±6.7	62.2±7.4	90.7±2.8

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

They were all healthy as judged by their detailed histories, physical examinations, urine analyses and haematological and serum biochemical analyses. They were no drinkers and smokers.

They had average normal values for their fasting plasma lipids during the study as evaluated by the age- and sex-specific values observed in the Japanese population groups of the prevalence study of the lipid research clinics.

They received an oral and written explanation of the purpose of the study and the procedures to be followed and all gave a written consent prior to the study. They lived in the metabolic ward of our laboratory throughout the study and continued their normal daily routine.

Diet

The subjects received their usual diets for 7 days to establish their base-line fat intake. Their usual diets contained 70-80 g/day of fat, 30-35 g/day of saturated fat and 40-45 g/day unsaturated fat. Their intake of energy, protein and carbohydrate was 11087-12970 kJ/day, 80-90 g/day and 410-480 g/day, respectively. Intakes of other nutrients immediately before the onset of experimental diet exceeded daily nutritional requirements.

The experimental diets consisted of three major sections according to dietary regiment; the first involved basal diet regiment for 7 days and the second a lard regiment for 7 days and the third a corn oil regiment for 7 days. In addition to basal dietary fat, they received successively 30 g/day of lard and corn oil in three divided doses for each 7 days. Table 2 shows the composition of basal diet. The basal diet contained 10 g/day of fat, 4 g/day of saturated fat, 6 g/day of unsaturated fat and 40 mg/day of cholesterol. These levels were considered as low in terms of a possible intake in daily life. The carbohydrate intake, inversely, was high in terms of a possible intake in daily life.

Table 2

Composition of the basal diet

Subject	K.S.	R.Y	Y.S	S.Y
Energy (KJ)	10355	10920	12991	12991
Carbohydrate (g)	480	510	640	640
Protein (g)	60	60	60	60
Fat (g)	10	10	10	10
Saturated fat (g)	4	4	4	4
Unsaturated fat (g)	6	6	6	6
Cholesterol (mg)	40	40	40	40
Vitamin A (I.U)	2000	2000	2000	2000
Vitamin B1 (mg)	2.5	2.5	2.5	2.5
Vitamin B2 (mg)	3.0	3.0	3.0	3.0
Vitamin C (mg)	80	80	80	80
Fe (mg)	20	20	20	20
Ca (g)	1.0	1.0	1.0	1.0

The composition of foods were determined from standard Tables of Food Composition in Japan (4th revision). The amount of all foods eaten by the subjects was recorded. The total energy intake was settled at 188.5 kJ per kg body weight to maintain a constant body weight during the experimental periods. Deficiencies of vitamins and minerals in the diet were overcome by giving vitamin and mineral preparations in sufficient doses to meet the nutritional requirements of adult Japanese men. Tea and water were freely available. Heavy exercise was avoided, but in other respects, daily life was normal. The times of meals were as follows: breakfast 7:30 to 8:30 a.m.; lunch, 12:30 p.m., supper, 17:30 p.m.

Blood samples

Venous blood samples were taken every three or four days from subjects after overnight fasting for at least 12 h. The serum was separated and stored at 4 ^{0}C for analysis. Three blood samples were obtained from each subject for determining the basal serum lipids. These respective values were taken as control values for each individual. Serum total cholesterol, serum triglycerides and serum phospholipids were measured using an enzymatic kit method (Wako Pure Chemical Industries, Ltd.). HDL-cholesterol was measured by a heparin-manganese precipitation procedure of Albers et al. /2/. The fraction of serum cholesterol, serum lipoprotein and serum phospholipid were separated by electrophoresis on agarose film. Serum cholesterol, serum phospholipid were separated in two fractions. The fast moving band was HDL fraction, and second moving band was very low-density lipoprotein (VLDL)+low-density lipoprotein (LDL) fraction. Serum lipoproteins were separated into three fractions. The fastest moving band was HDL fraction, and middle moving band was VLDL fraction and the slowest moving band was LDL fraction. Serum Lipids were measured three times for each sample and the mean values for each sample were calculated. Pooled serum prepared in our Department was used as control serum.

Analyses

Data were analyzed by standard statistical techniques, including Student's *t*-test for paired samples. Data were analyzed in a PC-9801 Personal Computer.

Results

All subjects had normal lipid values during the periods of usual diets.

Table 3 shows changes of serum total cholesterol level during the period of experimental diets. Serum total cholesterol was not changed in basal diets, but increased significantly ($p < 0.05$) from a basal diet of 106±23 to 141±26 mg/dl on lard diet, and then decreased significantly ($p < 0.05$) to 111±22 mg/dl on corn oil diet.

Table 4 shows changes of serum triglycerides level during the period on experimental diets. Serum triglycerides increased significantly ($p < 0.01$) from 66±38 to 173±32 mg/dl on basal diets, 164±31 mg/dl on lard diet, and 169±40 mg/dl on corn oil diets.

Table 5 shows changes of serum phospholipid levels during the period of experimental diet. Serum phospholipids were not changed by experimental diet.

Table 3

The effect of experimental diet on fasting levels of serum total cholesterol (mg/dl) of the male subjects

Subject	Test diet							
	Basal		Lard		Corn oil		Usual	
	0	4	7	10	14	17	21	25(days)
Y.T.	100+12	81	98	120	138	124	110	115
R.Y.	108+14	78	77	94	106	98	80	94
S.T.	135+18	126	131	161	164	143	123	150
S.R.	155+20	138	120	142	158	140	131	140
Mean±S.D.	124±25	105±30	106±23	129±28	141±26	126±20	111±22	124±25

Table 4

The effect of experimental diet on fasting levels of serum triglycerides (mg/dl) of the male subjects

Subject	Test diet							
	Basal		Lard		Corn oil			Usual
	0	4	7	10	14	17	21	25(days)
Y.T.	42+20	176	208	194	186	188	190	64
R.Y.	35+25	150	173	172	164	158	162	46
S.T.	120+60	181	182	180	188	200	208	140
S.R.	68+17	136	130	128	120	124	115	84
Mean±S.D.	66±38	160±21	173±32	169±28	164±31	168±33	169±40	83±40

Table 6 shows changes of serum HDL-cholesterol during the period of experimental diet. Serum HDL-cholesterol was decreased significantly ($p < 0.01$) from 41.9±1.6 to 31.2±3.8 mg/dl on basal and lard diet, but increased significantly ($p < 0.05$) to 41.9±4.6 mg/dl on corn oil diet.

Table 5

The effect of experimental diet on fasting levels of serum phospholipids (mg/dl) of the male subjects

Subject	Test diet: Basal			Lard		Corn oil	Usual	
	0	4	7	10	14	17	21	25
	(days)							
Y.T.	156±21	137	163	158	144	148	152	154
R.Y.	147±26	128	141	142	138	144	150	146
S.T.	185±24	144	191	175	180	183	175	160
S.R.	175±17	169	169	164	168	172	165	165
Mean±S.D.	165±17	144±17	166±21	159±13	157±19	161±18	161±12	156±8

Table 6

The effect of experimental diet on fasting levels of serum HDL-cholesterol (mg/dl) of the male subjects

Subject	Test diet: Basal			Lard		Corn oil	Usual	
	0	4	7	10	14	17	21	25
	(days)							
Y.T.	42.9±3.8	29.6	32.3	33.4	32.5	42.8	47.5	44.9
R.Y.	42.4±4.3	28.9	29.6	27.4	25.5	31.6	36.3	35.3
S.T.	39.6±3.7	36.5	37.0	35.0	34.3	41.2	42.5	40.3
S.R.	42.9±2.8	40.1	35.5	30.5	32.4	38.3	41.3	45.7
Mean±S.D.	41.9±1.6	33.8±5.4	33.6±3.2	31.6±3.3	31.2±3.8	38.5±4.9	41.9±4.6	41.6±4.7

Table 7 shows changes of serum cholesterol fraction during the periods of experimental diet. Serum HDL-cholesterol fraction was decreased significantly ($p < 0.01$) from 36.4±5.9 to 28.7±2.3 % on basal and lard diet, but increased significantly ($p < 0.05$) to 36.8±2.8 % on corn oil diet. Serum VLDL+LDL cholesterol fraction was increased significantly ($p < 0.01$) from 63.6±5.9 to 71.3±2.3 % on basal and lard diet, but decreased significantly ($p < 0.01$) to 63.2±2.8 % on corn oil diet.

Table 8 shows changes of serum lipoprotein fraction during the periods of experimental diets. Serum HDL fraction was not changed in basal

Table 7

The effect of experimental diet on fasting levels of serum cholesterol fraction (%) of the male subjects

Subject		Basal 0	Basal 4	Basal 7 / Lard	Lard 10	Lard 14 / Corn oil	Corn oil 17	Corn oil 21 / Usual	Usual 25
		Test diet (days)							
Y.T.	HDL	42.9±2.5	36.6	29.9	27.8	27.4	38.3	38.9	36.1
	VLDL+LDL	57.1±2.5	63.4	70.1	72.7	62.6	61.7	61.1	63.9
R.Y.	HDL	39.3±3.2	37.0	34.6	32.8	32.0	37.0	39.5	37.5
	VLDL+LDL	60.7±3.2	63.0	65.4	67.2	68.0	63.0	60.5	62.5
S.T.	HDL	29.3±2.6	29.0	26.0	26.5	27.0	30.0	34.0	32.5
	VLDL+LDL	70.7±2.6	71.0	74.0	73.5	73.0	70.0	66.0	67.5
S.R.	HDL	34.0±2.7	29.1	27.1	28.8	28.5	34.7	34.8	32.7
	VLDL+LDL	65.9±2.7	70.9	72.9	71.2	71.5	65.3	65.2	67.3
Mean	HDL	36.4±5.9	32.9±4.5	29.4±3.8	29.0±2.7	28.7±2.3	35.0±3.7	36.8±2.8	34.7±2.5
	VLDL+LDL	63.6±5.9	67.1±4.5	70.6±3.8	71.0±2.7	71.3±2.3	65.0±3.7	63.2±2.8	65.3±2.5

Table 8

The effect of experimental diet on fasting levels of serum lipoprotein fraction (%) of the male subjects

Subject		Test diet: |← Basal		→|← Lard		→|← Corn oil		→|← Usual	→|
		0	4	7	10	14 (days)	17	21	25
Y.T.	HDL	39.4±3.3	41.4	42.3	41.4	35.4	39.8	41.7	42.8
	VLDL	15.6±2.5	29.8	29.1	16.1	18.2	18.2	19.2	15.9
R.Y.	HDL	45.5±2.8	46.0	47.1	37.4	39.1	39.5	40.9	41.7
	VLDL	8.9±2.9	27.0	26.7	15.9	16.7	16.6	21.8	10.2
	LDL	45.5±3.2	27.0	26.2	46.7	44.3	43.9	37.3	48.1
S.T.	HDL	34.1±2.7	30.6	29.3	28.1	26.0	37.8	41.6	37.5
	VLDL	25.1±3.2	35.1	37.8	26.4	27.5	24.5	23.4	24.5
	LDL	40.8±2.7	34.3	32.3	45.5	46.5	37.7	35.0	38.0
S.R.	HDL	40.9±3.1	41.4	42.9	36.2	34.7	40.6	41.6	38.6
	VLDL	24.2±2.9	29.6	31.8	27.3	25.3	23.7	24.9	24.0
	LDL	34.9±2.8	29.1	25.2	36.4	40.0	35.7	33.5	37.4
Mean	HDL	40.0±4.7	39.9±6.5	40.4±7.7	35.7±5.5	33.8±5.5	39.4±1.2	41.5±0.4	40.2±2.5
	VLDL	18.5±7.7	30.4±3.4	31.4±4.8	21.4±6.3	21.9±5.3	20.8±3.9	22.3±2.4	18.7±6.9
	LDL	41.6±4.9	29.8±3.1	28.1±3.2	42.8±4.6	44.3±3.1	39.8±3.8	36.3±2.5	41.2±4.9

Table 9

The effect of experimental diet on fasting levels of serum phsopholipid fraction (%) of the male subjects

Subject		Test diet: |← Basal →| (0–7 days)			← Lard →| (7–14 days)		← Corn oil →| (14–21 days)		← Usual →| (21–25 days)
		0	4	7	10	14 (days)	17	21	25
Y.T.	HDL	68.9±3.2	54.7	51.7	53.8	57.9	58.3	59.6	61.3
	VLDL+LDL	31.1±3.2	46.3	48.3	46.2	42.1	41.7	40.4	38.7
R.Y.	HDL	67.6±2.8	54.8	52.0	57.1	58.2	58.7	61.8	67.0
	VLDL+LDL	32.4±2.8	45.2	48.0	42.9	41.8	41.3	38.2	33.0
S.T.	HDL	56.1±2.7	47.7	46.4	54.9	56.5	58.7	60.3	57.2
	VLDL+LDL	43.9±2.7	52.3	53.6	45.1	43.5	41.3	39.7	42.8
S.R.	HDL	57.2±3.5	54.1	52.7	52.8	54.0	57.1	59.7	58.1
	VLDL+LDL	42.8±3.5	45.9	47.3	47.2	46.0	42.9	40.3	41.9
Mean	HDL	62.5±6.7	52.8±3.4	50.7±2.9	54.7±1.8	56.7±1.9	58.2±0.8	60.4±1.0	60.9±4.4
	VLDL+LDL	37.5±6.7	47.2±3.3	49.3±2.9	45.3±1.8	43.3±1.9	41.8±0.8	39.6±1.0	39.1±4.4

diet, but decreased from a basal level of 40.4±7.7 to 33.7±5.7 % on lard diet, and then increased significantly ($p < 0.05$) to 41.5% on corn oil diet. Serum LDL fraction was decreased significantly ($p < 0.01$) from 41.6±4.9 to 28.1±3.2 % on basal diet, but increased significantly ($p < 0.05$) to 44.3±3.1 % on lard, and then decreased to 36.3±2.5 % corn oil diet. Serum VLDL fraction was increased significantly ($p < 0.01$) from 18.5±4.1 to 31.4±4.8 % on basal diet, but did not change on lard and corn oil diets.

Table 9 shows changes of serum phospholipid fraction during the periods of experimental. Serum HDL phospholipid fraction decreased significantly ($p < 0.05$) from 62.5±6.7 to 50.7±1.8 % on basal diet and increased significantly ($p < 0.05$) to 60.4±1.0 % on lard and corn oil diet.

Discussion

Elevated levels of total blood cholesterol are considered to be a risk factor in the ethiology of heart diseases. An inverse relationship of coronary heart disease to plasma HDL cholesterol concentration has been reported. Fraser /7/ reported that consumption of cholesterol and saturated, unsaturated fat play a major role in blood lipid levels. With respect to the relation of the serum lipids and high carbohydrate diet, some researchers have found that diets of identical fat content but of different fatty acid composition produced different serum triglycerides. Beveridge et al. /3/ reported that a high carbohydrate and fat-free diet significantly increased serum triglyceride but found no change when the diet included 45% Cal from butterfat. A diet with 45% of calories from corn oil produced a significant decrease in serum triglycerides in man. Nestel et al. /9/ reported a rise in serum triglyceride in men who ate a diet high in carbohydrate or in saturated fat. Grande et al. /8/ studying effects of several diets, reported that the highest carbohydrate, lowest fat diet resulted in highest serum triglycerides. Shorain, et al. /10/ reported that the increased plasma triglyceride levels consequent to carbohydrate feeding appeared to be mainly due to an increase in the pre-β-lipoprotein (VLDL) fraction, a carrier for endogenous triglyceride in plasma, which is typical for carbohydrate-induced hypertriglycedemia. Consistent with their report, in the present study, we found that a high carbohydrate intake incrased serum triglycerides and pre-β-lipoprotein fraction significantly.

Recent studies indicated that unsaturated fats caused a redistribution of cholesterol between plasma and tissue pools. Franz and Carey /6/ reported that the content of cholesterol in liver decreased in patients fed an unsaturated diet indicating that other tissue pools must be involved. Grande et al. /8/ have suggested that feeding of unsaturated dietary fat in humans reduced plasma cholesterol by causing a redistribution of cholesterol between plasma and adipose tissues. Franz and Carey /6/ showed a decrease in the liver unsaturated fats that may increase the tissue cholesterol pool, probably in the adipose tissue. Our results also suggest that serum total cholesterol, HDL-cholesterol was significantly increased by lard addition (saturated fat was about two-thirds of the total fat), but decreased significantly by corn oil addition (unsaturated fat to six-seventh of the total fat). The mechanism by which diets rich in unsaturated fat cause reduction of the serum cholesterol in men is not clear. It has been proposed that the reduction is due to increased excretion of the total products of cholesterol catabolism, increased excretion of bile acids, or decreased synthesis of cholesterol. Another possibility made plausible by observation in animals, is that cholesterol is shifted from the serum to the tissues. Avigan and Steinberg found, for example, that rats fed on corn oil showed a large rise in liver cholesterol, while rats fed on coconut oil (mostly saturated fats) showed no significant change. In our study, the cholesterol-lowering activity produced by unsaturated fat (corn oil) was primarily due to the increase in HDL-cholesterol fraction rather than to any significant change in LDL+VLDL cholesterol. Increased HDL-cholesterol after corn oil feeding was consistent with the finding reported by Flynn et al. /5/. We also found that HDL fraction was decreased on lard, but increased on corn oil. On the contrary, LDL fraction was increased by lard, but decreased by corn oil. These results are in agreement with other findings reported in the literature. Levels of HDL cholesterol have been reported to be inversely related to the incidence of coronary heart disease.

HDL may foster the removal of cholesterol form peripheral tissue to the liver for catabolism and excretion. Moreover, high levels of HDL may compete with LDL receptor sites on arterial smooth muscle cells and thus partially inhibit uptake and degradation of LDL. It is therefore important to evaluate the results with emphasis on the alterations in cholesterol distribution among the serum lipoprotein fractions.

Grande et al. /8/ repprted that when men were eating butter diet serum phospholipid levels were significantly higher than when they were eating other diets, Ahrens et al. /1/ reported that serum phospholipid was decreased by corn oil in men. We found that serum phospholipids did not change on the experimental diets. We thought that this was bacause our experimental time was short and nutritional state was high carbohydrate and low fat. Then serum HDL-phospholipid fraction was increased on basal diets, and then decreased on lard and corn oil diets. Although the mechanism of this effect is still unknown, it is suggested that serum HDL-phospholipid fraction is influenced by the high carbohydrate diet.

Thus, the difference in the fraction of saturated fat and unsaturated fat may reflect this difference in carbon atoms numbers and it may be concluded on the basis of the evidence presented in this study that saturated fat and unsaturated fat have different and specific roles in lipid metabolism.

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EFFECTS OF VITAMIN C LONG-TERM LOADING ON THE SERUM γ-GLUTAMYLTRANSPEPTIDASE ACTIVITY IN HUMAN SUBJECTS

M. IMAKI, T. MIYOSHI, M. FUJII, S. TANADA, M. SEI, and R. YAMASAKI

Department of Public Health, School of Medicine, The University of Tokushima, Tokushima, Japan

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In an attempt to assess dietary effects on the serum enzyme activity of long-term vitamin C supplementation, the serum γ-GTP(1) fraction was studied in five healthy men. In this study, they were maintained on ordinary meals for the initial one year and the last 7 months of the 43 month experimental period, and on ordinary meals with 1 g/day of vitamin C powder for the intermediate 2 years. The serum γ-GTP(1) fraction during the first year (ordinary meals alone) was higher than that for the second or third year (ordinary meals with addition of vitamin C). The decrease after vitamin C supplementation was statistically significant. The serum vitamin C concentration tended to rise in winter. Vitamin C supplementation at 1 g/day raised the concentrations up to 1.6 mg/dl on average, where the serum was practically saturated with vitamin C.

Keywords: Serum γ-GTP activity — vitamin C — isoenzyme — long-term loading — nutritional evaluation

Introduction

The serum γ-glutamyltranspeptidase (γ-GTP) activity has become a sensitive index for hepatitis, liver cirrhosis, and hepatic carcinoma since Orlowski et al. /5/ established the assay. This enzyme differs from other serum enzyme activities in its sensitivity to alcohol intake: As more alcohol is ingested, the enzyme activity increases /6/. Therefore, it has been used as an index of the amount of alcohol intake. The authors have been seeking possible association between serum enzyme levels within normal

Send offprint requests to: Dr. M. Imaki, Department of Public Health, School of Medicine, The University of Tokushima, 3-Kuramoto-cho, Tokushima, Japan

Akadémiai Kiadó, Budapest

limits and dietary factors, both epidemiologically and experimentally, in an attempt to extend the use of laboratory data obtained from mass medical examination to the field of preventive medicine /7-8/. A previous epidemiological survey showed a statistically significant association of the serum γ-GTP(1) fraction with the amount of vitamin C intake and serum vitamin C levels. This paper describes changes in the serum γ-GTP(1) fraction in normal subjects receiving vitamin C supplementation in a study for 43 months conducted to confirm the previous epidemiological observation.

Materials and methods

Five volunteers were maintained on ordinary meals with and without vitamin C supplementation of 1 g/day for 43 months from September in 1983 to March in 1987. The serum γ-GTP(1) fraction was monitored during the period.

Subjects

All the five subjects were members of the teaching staff of a governmental institution. They were without chronic disease such as heart disease and diabetes mellitus or history of hepatobiliary disease. Informed consent was obtained from all the volunteer subjects. Table 1 showed their ages and physical condition.

1. Health conditions of the subjects during the study period.

a) Generally, all the subjects were in good condition.

b) None of them had disease which required hospital treatment.

Table 1

Sex, age, height and weight of each subject

Subject	Sex	Age (Years)	Height (cm)	Weight (kg)
No. 1	male	55	164.0	69.0
No. 2	male	43	175.0	60.0
No. 3	male	33	173.0	80.0
No. 4	male	29	171.0	89.5
No. 5	male	28	175.0	70.0
Mean		37.6	170.8	73.6
S.D.		11.3	4.7	11.1

c) None of them were on the sick list for three consecutive days or longer.
d) None of them had disease of the hepatobiliary system.
e) None of them had the onset of chronic disease such as heart disease or diabetes mellitus.
f) None of them smoked.
g) None of them drank habitually.
h) Occupationally, all continued similar service without relocation.

Study periods and dosages of vitamin C

As shown in Fig 1, the subjects were maintained on ordinary meals throughout the 43-month study period. Because of the accident in No. 5 subject, the experiment was stopped in March 1987. They received 0.5 g each of vitamin C powder after breakfast and supper daily during the second and third years.

Measurement of the serum enzyme and vitamin C

The serum γ-GTP isozyme pattern was determined by colorimetry depending on enzyme reaction after electrophoretic separation on a pol-E-film. Serum vitamin C concentrations were determined by the method of Ayekaw /4/ at least in duplicate. The mean was taken as observed value for individuals. Blood samples were withdrawn once in the middle of every month at 8 o'clock in the morning before breakfast. Routine medical examination including blood pressure was conducted simultaneously. The isozyme fraction and vitamin C were determined within the day.

1) Experimental periods: '83/9 - '87/3
First Year ('83/9 - '84/8) : Usual diets
Second Year ('84/9 - '85/8) : Usual diets + Vitamin C
Third Year ('85/9 - '86/8) : Usual diets + Vitamin C

*Fourth Year ('86/9 - '87/3) : Usual diets

2) Number of subjects : Five Japanese males

3) Serum enzyme activity was measured once a month

*; Because of the accident in No. 5 subject, the experiment was stopped in March, 1987.

Fig. 1. Experimental design

Results

Figure 2 shows serum total γ-GTP activity, mean of five subjects, during the study period. The activity showed no significant changes.

Figure 3 shows serum γ-GTP(1) fractions. The levels during the second and third study years (periods of vitamin C supplementation) were lower than those during the first year (periods of ordinary meals alone), and the difference was statistically significant ($p < 0.05$). The tendency was consistent among the subjects. When the vitamin C supplementation was withdrawn after the third year, the isozyme fraction increased again.

Figure 4 showed serum vitamin C concentrations, means of five subjects, during the study period. During the first year when subjects were maintained on ordinary meals alone, the mean serum vitamin C concentartion tended to incrase in winter. The reason of the surprising high vitamin C content in November was that Japanese used to eat a lot of oranges containing much vitamin C in winter. It rose, and then remained practically unchanged at about 1.6 mg/dl on average while the subjects were receiving vitamin C supplementation of 1 g daily, suggesting its saturation in the serum. The vitamin C content of the normal food was 125±52 mg/day.

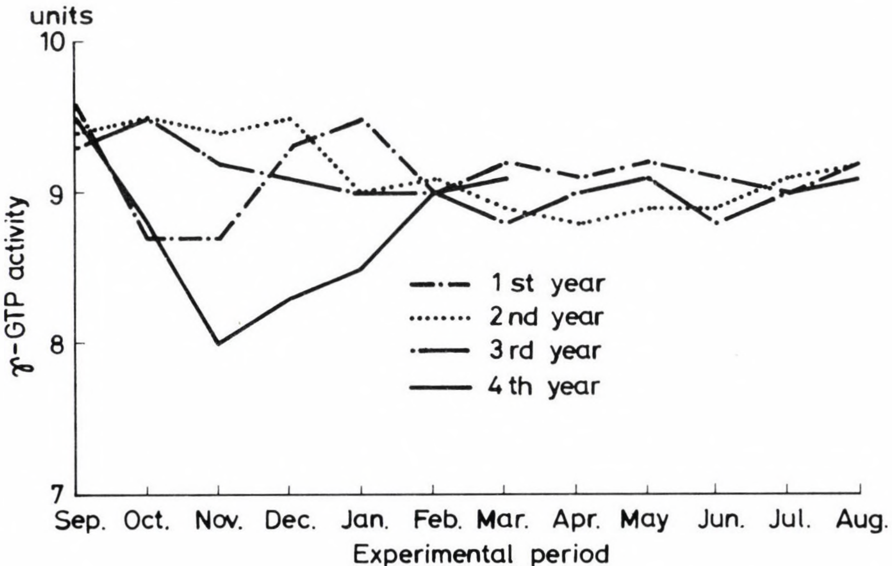

Fig. 2. Variation of serum γ-GTP activity after vitamin C administration

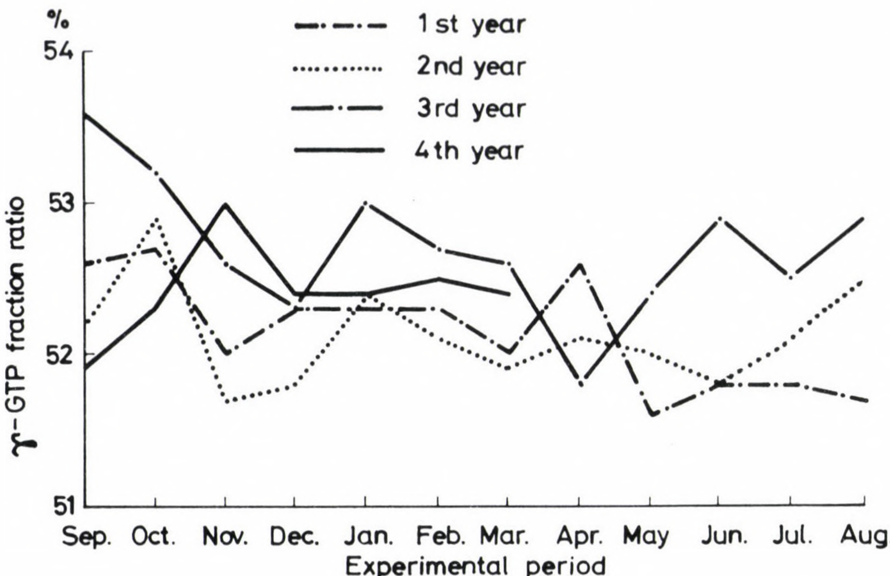

Fig. 3. Variation of the percentage of the γ-GTP(1) fraction after vitamin C administration

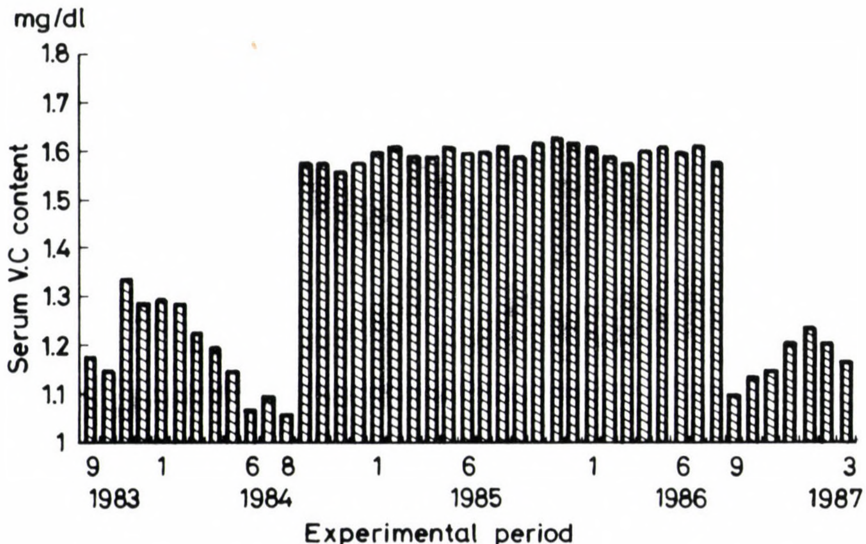

Fig. 4. Variation of serum vitamin C level after vitamin C administration

Discussion

Routine laboratory data include the activity and isozyme pattern of serum γ-GTP, which are diagnostic for disease of the liver and heart. In attempt to use these data as indices of nutritional status for normal individuals, a series of experiments and epidemiological studies have been conducted seeking possible association of the enzyme activity or isozyme pattern with nutritional factors.

An epidemiological survey on nutritional intake and serum γ-GTP in 217 healthy students disclosed a statistically significant negative correlation between the serum γ-GTP(1) fraction and amount of vitamin C intake /7/. There was also a statistically significant negative correlation serum γ-GTP(1) fractions and serum vitamin C concentrations /8/. These findings suggest that the isozyme fraction may be decreased in a population ingesting much vitamin C or having higher serum vitamin C concentrations, but do not necessarily mean that vitamin C has activity to lower the serum γ-GTP(1) fraction. To prove the activity, further study is necessary to demonstrate the decrease in serum vitamin C concentration in subjects receiving vitamin C supplementation.

The association of the serum γ-GTP activity with nutritional status has been studied only in connection with extreme malnutrition such as seen in patient with Kwashiorkor and chronic alcoholism /1-3/. In our previous experiments /4/, six healthy subjects receiving 1 g/day oral vitamin C on ten consecutive days, and then 10 g daily for the subsequent 10 days. In these subjects, the serum γ-GTP(1) fraction decreased, and the decrease was statistically significant. Actually, this experimental period was too short because physiological factors such as seasonal variation were neglected. Therefore, in our present study, the serum γ-GTP(1) fraction was monitored over an as long as 43 month period in the same subjects. Serum γ-GTP(1) fractions during the first year were significantly higher than those during the second or third year when the subjects were receiving vitamin C powder. Thus, the vitamin C supplementation led to a decrease in serum γ-GTP fraction even in the prolonged experiment. The decreasing tendency was observed in all the individual subjects, althought they varied widely in age. The decrease may be attributed to reduction of stress by vitamin C, but no experimental study has clarified this mechanism. It remained yet to be elucidated. However, our present observations suggest that the serum γ-GTP(1) fraction can reflect vitamin C intake. Therefore,

the routine laboratory data may be of value as a nutritional indicator in the field of preventive medicine.

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BOOK REVIEWS

PHYSICS IN LIVING MATTER

Lecture Notes in Physics Vol. 284.

Eds D. Baeriswyl, M. Droz, A. Malaspinas, P. Martinoli

Proceedings of the Tenth Gwatt Workshop held in Gwatt, Switzerland, October 16-18 (1986). Springer-Verlag, Berlin, Heidelberg, New York, Paris, Tokyo, pp. 1-180.

This Volume includes 14 papers in the field of interest to both biologists and physicists covering the following topics: Part I deals with the structural and functional building blocks, the biomolecules, and their roles in evolution process. - Part II is devoted to symmetry and structure. - Part III is concerned with thermodynamics and transport properties of living matter in particular of biomembranes. Both mathematical modelling and describing the electrical currents through the living membranes provide new insight into the relation between structure and biological function. - Part IV contains papers on neural networks. Models for the processes of learning, storage and retrieval of information in the central nervous system are described.

The volume can be recommended to a wide range of readers including scientific workers, university teachers, students or even non-specialists interested in new ways of thinking in science.

Katalin S.-Rózsa (Tihany)

LOW MOLECULAR WEIGHT MICROBIAL AUTOREGULATORS

A.S. Khokhlov

Moscow, "Nauka", 1988 (In Russian), pp. 271

This is a very special and pioneering work. Something really new, and not a compilation of chapters from books of other authors.

Professor Khokhlov is himself one of the first scientists who started in the sixties to look for endogenous regulatory substances in microorganisms. He and his co-workers have found A-factor a relatively small molecule which can be looked upon as a model molecule of regulatory function. If it is added to certain Streptomyces strains which do not sporulate and do not produce e.g. streptomycin, this small butyrolaction (Mr: 242 Da) — induces in minute amounts the synthesis a whole series of enzymes, proteins and secondary metabolites in large quantities. It is a pleiotropic regulatory molecule.

The main merit of this book is that the author starting out from his discovery, collected the facts (publications) from the wealth of publications considering their connection to the phenomenon of autoregulation of life processes by small molecules in microorganisms.

The result is most stimulating. It turns out that there are many different biological phenomena, like spore production in prokaryotes, sexual differentiation in yeast, antibiotic production in prokaryotes and eukaryotes, morphogenesis, etc. and they are all regu-

lated by autoregulatory molecules which are chemically as diverse as are the biological phenomena they bring about.

The 16 chapters are the following: Mating type regulators of Strptococcus faecalis; Autoinducer from Vibrio fischeri; Cosynthetic factor I; Factor C; A-factor and analogous lactone type autobioregulators from Actinomycetes; B-factor; Pamamycin and other autoregulators of Streptomyces alboniger; Anteridiol and dehydro-oogonioles; Sirenines; Trisporic acid and related regulators of (fungi) Mucorales; Sex pheromones (hormones) of yeasts (Saccharomycetes); Lipopeptide sexual hormones (pheromones); Sclerosporines and other sporulation regulators from Sclerotinia fructicole; Autoregulators of cellular slime moulds (acrasimycetae); Effect of antibiotics upon the organisms which produce the same; Microbial autoregulators not yet investigated in detail.

The difficulty to cover the vast area of biological phenomena related to autoregulators — has been successfully overcome. The book is easy to read, clearly and succiently written which is highly appreciated.

There are not only facts described but the contradictory results are also critically discussed. The author's vantage point is of a chemist: he is mainly critical on the purity of the natural products and structure of the new compounds, less profound on the genetics and metabolic connections of the problems described in the book.

The generality of the biological role of an autoregulator is often questioned throughout of the book because a marker e.g. spore formation may ensure in some mutants even in the absence of the autoregulator that is normally necessary to spore production in the wild type strain.

I think that autoregulators are signal molecules triggering specifically evolutinary fixed metabolic pathways. In the absence of specific signals non-specific factors may trigger (perhaps less efficiently) the same pathway. This, however, does not question the significance of autoregulators in natural regulatory processes.

The reader will certainly get in his/her hands a book of basic significance which will be followed soon by a series of eventually new books of more pages about similar topics but by other authors.

I hope that the successors are not going to forget the pioneer(s) too soon.

It is a pity that it took such a long time to publish this monograph because the latest references are from 1985 and the book became available only this year. (I am sure that the author agrees on this subject with me.) The monographs should be published in English as soon as possible. There is a large number of microbiologists, geneticists, biochemists, developmental biologists, etc. in the world for whom the book should be made available.

G. Szabó (Debrecen)

THE STATE OF WATER IN THE CELL

Compiled by W. Negendank and L. Edelmann

Scanning Microscopy International. 1988. pp. 114

BIOTECHNOLOGY AND BIOAPPLICATION OF COLLOIDAL GOLD

Compiled by R.M. Albrecht and G.M. Hodges

Scanning Microscopy International. 1988. pp. 312

CELL STRUCTURE AND CELL FUNCTION

Ed. G.M. Roomans and B. Forslind

Scanning Microscopy International. 1988. pp. 138

The articles included in these books were reprinted from volumes of Journal Scanning Microscopy and from the proceedings of Fourth Pfefferkorn Conference. They were selected and arranged according to the topics indicated in the titles of the books.

The first of the series focuses on facts and questions about the state of water in biosystem. Ten reviews are included which cover the problems of "bulk" and "bound" water in biopolymers, the results of electron microscopic and physical studies on the state of water and ions in different kinds of cells and the views on the organization of cytoplasm.

The second of the series provides an extensive coverage of the principles of application of colloidal gold as a marker in biological systems and of the results achieved by this method. Articles are devoted to the methods of labelling of plant and animal cells by gold-labelled reagents and to the light-, fluorescent-, transmission- and scanning electron microscopic detection of the label. Other sections of the book contain chapters on the results of investigation of cellular components (surfaces, extracellular matrix, cytoskeleton, chromosomes) based on gold-labeling technics.

The third book of the series contains papers presented at the Symposium on " Cell Structure and Cell Function" held in honor of Björn Afzelius (1985), Stockholm. They cover a broad spectrum of topics in the field of cell biology. Common in these contributions is the broad scale of ultrastructural methods used for the solution of the examined problems.

The major asset of these books is the wealth of up-to-date information on selected areas of cell biology research. The layout of the books is excellent, most of the illustrations are of highest quality. Each paper contains a discussion with at least three reviewers. Their questions and comments and the responses of the author(s) greatly help the reader to understand the background of the discussed problems and the latest achievements and/or controversies of research as well.

Overall, the effort of the Scanning Microscopy International to present volumes on hot problems in the field of cell biology seems successful and is worth to continue.

I recommend these books to researchers interested in the ultrastructural aspects of cell biology and to all who wish to be informed about the latest developments in the methodology of ultrastructural research.

J. Kovács (Budapest)

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NEW AVENUES IN THE BIOLOGICAL INDICATION OF ENVIRONMENTAL POLLUTION

J. SALÁNKI

Balaton Limnological Research Institute of the Hungarian Academy of Sciences, Tihany, Hungary

(Accepted 1989-12-14)

The history of the impact of human activity on the environment dates back to the beginning of the history of mankind itself. This impact has been multifold, it has been influencing and changing - for the worse or for the better - the environment ever since Man lives on Earth. In the past the eternal revival of nature could counterbalance the effects of human activity. Over the past hundred and even more so, over the past fifty years however, technical development, industrialization and extensive use of chemicals have reached every region of our Earth. Due to this, uncontrolled pollution of the environment has become universal by now and can justly be regarded as a global threat. It was not just by chance that the first report published by the Roman Club which deals with the problems of the limitations of economical and social development of mankind also regarded environmental pollution as one of the major limiting factors. This "high priority" classification of the problems of environmental pollution is understandable when we consider that by that time the anthropogenic loading of the environment with pollutants had created serious centers of crisis in the industrially developed countries. The quality of air has by now become intolerable in some big cities and vast regions, a large number of natural water bodies have become unsuitable for human consumption, wildlife has become extinct in scores of rivers and lakes. Acid rains were pouring down even on countries distant from the source of pollution, the contamination

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Send offprint requests to: Prof. J. Salánki, Balaton Limnological Research Institute of the Hungarian Academy of Sciences, P.O.Box 35, H-8237 Tihany, Hungary

Akadémiai Kiadó, Budapest

by chemicals of the oceans, the soil and the subsurface waters began and this process appeared to be completely out of control. It has become apparent by now that nature cannot counterbalance by self-regulatory processes the adverse effects of the ever-increasing environmental pollution. In order to ensure its own conditions of existence, it is Man, the human society itself that must put a stop to further destruction of the environment. The realization of this has lead to the emergence of a new trend with the objectives of restoration of the damaged environment, prevention or restriction to the minimum of further destruction, harmonization of economical and ecological interests and promotion of environment-friendly economical solutions. In the emergence of this new trend different branches of science, state and social institutions have all played important roles.

The biologists were the first who had to face the consequences of the universal environmental pollution at the scientific level. They registered the extinction of certain plant and animal species, the unfavorable alteration and degradation of ecosystems, the direct or indirect damage caused to the biotopes by human activity. These observations, registrations and measurements recorded the results of the environmental pollution which were justly regarded then as indicators of the pollution.

We do not want to raise any objections against that type of approach in the biological indication of environmental pollution which bases its judgement solely on the actual perishing of living organisms. This approach formed the starting point for establishing the toxicological network of environmental protection whose object is to elucidate whether different chemicals released into the environment cause death of plants or animals and if yes, what is the limit of the tolerance of the living organisms for these harmful agents.

Recently, new criteria in the judgement of the degree of the environmental pollution have also come to the fore. According to these, sublethal effects appear to be much more important than it was thought earlier and beside acute toxicity, chronic influences are also taken into consideration. This requires new approaches to the biological indication of environmental pollution, too /9/.

The starting point of these new trends of bioindication is that the protection of environment must be accomplished in a preventive fashion and in order to achieve this, the whole spectrum of biological sciences has to be used. A basic principle of these new trends is that it is not the death of living organisms or the degradation of the ecosystem that should be

regarded as criteria. Instead, the important question is whether the living conditions and tolerance of the individuals and living systems are adversely affected even by possible cumulation of sublethal effects. As a result of such cumulative effects, the natural adaptability of the living organisms to changing conditions might be impaired. Decline of the populations, increased extinction of particular species within a community can be attributed to increased damage and decreased adaptability in individuals. In turn, these can be attributed to impairment of those physiological mechanisms and processes that maintain the metabolism, regulatory functions, adaptive and protective mechanisms of the organism.

The new trends therefore regard the monitoring of such latent damages as a major means for judging the nature of environmental pollution. The investigation of long-term damaging effects on the living systems, the disclosure of the bioaccumulation of dangerous chemicals released into the environment in sublethal concentrations, the elucidation of effects and mechanisms of actions of sublethal doses of these chemicals all form parts of the problems of environmental protection are increasingly based on knowl-edge accumulated in various branches of the biological sciences and that,
ledge accumulated in various branches of the biological sciences and that, on the other hand, problems of the environmental pollution are increasingly incorporated into these scientific branches themselves. Terms like "environmental biophysics", "environmental biochemistry", "environmental biotechnology", "environmental physiology" or even "environmental neurobiology" often turn up nowadays at congresses, in various publications, research programs or recommendations of international organizations.

I mention an example from Hungary: there were mass deaths of the fish-stock in Lake Balaton in 1965 and 1975. When investigating this phenomenon no one could with certainty establish whether it was an acute poisoning or infection that caused the death of the fish. It could only be hypothesized from data in the relevant literature and from the known circumstances that it was the accumulation and subsequent mobilization of pesticides that led to the poisoning which was lethal for the majority of the animals. We do not know, however, that the accumulation took place in the sediment of the lake or in the animals themselves, whether the cumulative effects of other agents, for example toxic heavy metals, or other anthropogenic effects played some additional roles in the breakdown of vital functions and in the death of the fish. To make a judgement on the subject was impossible because we had no chemical, biochemical, physio-

logical or toxicological background information related to the particular circumstances. It should be mentioned, that similar "unsolved" cases of fish-death had occurred in Europe and the USA as well and this contributed much to the strong development of environmental protection related toxicological research all over the world.

Some living organisms possess physiological properties that make them particularly capable of accumulating and storing substances appearing in their natural environment. These organisms are good indicators of the occurrence and propagation in the biosphere of environmental pollutants. Different organisms are differentially capable of concentrating the same substances in their body and to function as indicators of the pollution. This realization led to the initiation of research activities aiming to discover suitable indicator organisms with strong accumulative properties and then to use such organisms in the process of forming judgement on the quality of the environment. The aim of our present research activites is to investigate the heavy metal content of animals living in Lake Balaton and to find organisms among these that are particularly good accumulators of heavy metals. To find such indicator organisms is important because biogenic heavy metals present in the environment either in toxic or in physiologically ineffective concentrations are very common and very dangerous environmental pollutants.

It has become apparent in our investigations that the heavy metal concentrations can be significantly different in different animal species living in Lake Balaton and also in the different organs of the same species. For example, cadmium (Fig. 1) and lead (Fig. 2) are accumulated best by the gill of mussels, whereas the best accumulators of copper (Fig. 3) are the crustacean plankton and the liver of bream /12/. Comparing the measured values for five toxic or biogenic, respectively, heavy metals with data from the literature it has become clear that the heavy metal content of fish in Lake Balaton is on the boundary of values measured in unpolluted or slightly polluted waters.

These data also unambiguously showed that of the organisms and their various organs under investigation it is the mussels and specifically, the gill of the mussels that can be regarded as best accumulators of toxic heavy metals. Consequently, the gill of the mussel can be regarded as an indicator organ which is suitable for investigations with the aim of drawing conclusions on the degree of pollution by heavy metals.

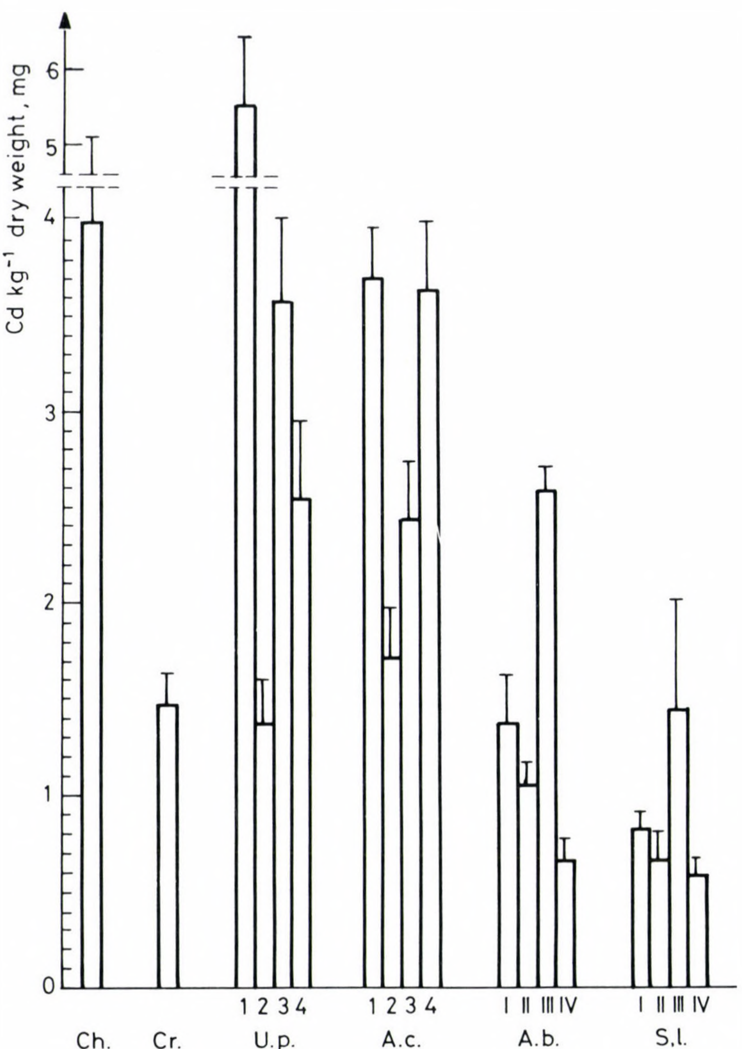


Fig. 1. Cadmium concentration of various organs of animals in Lake Balaton calculated for dry weight. Ch - Chironomidae larvae; Cr - Crustacea plankton; U.p. - Unio pictorum L.; A.c. - Anodonta cygnea L. (1 - gill; 2 - mantle; 3 - kidney; 4 - foot and viscera); A.b. - Abramis brama L. (bleak); S.p. - Stizostedion lucioperca L. (pike perch) (I - gill; II - kidney; III - liver; IV - muscle)

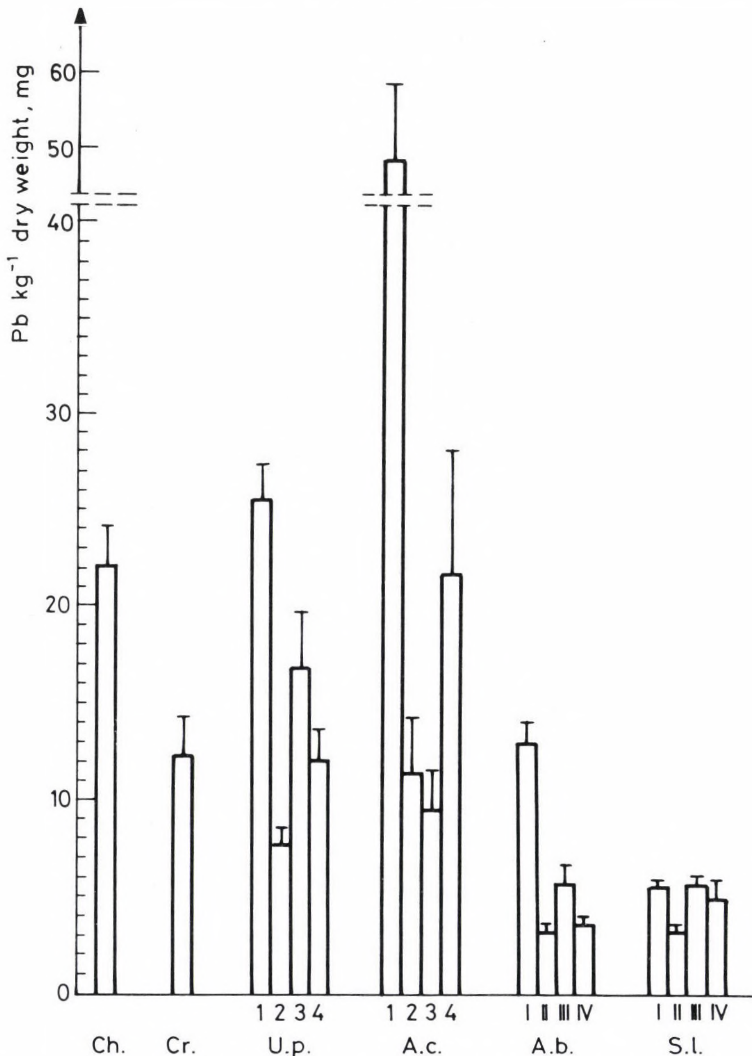


Fig. 2. Concentrations of lead (mg/kg dry weight) in various organs of animals in Lake Balaton. Symbols are the same as in Fig. 1

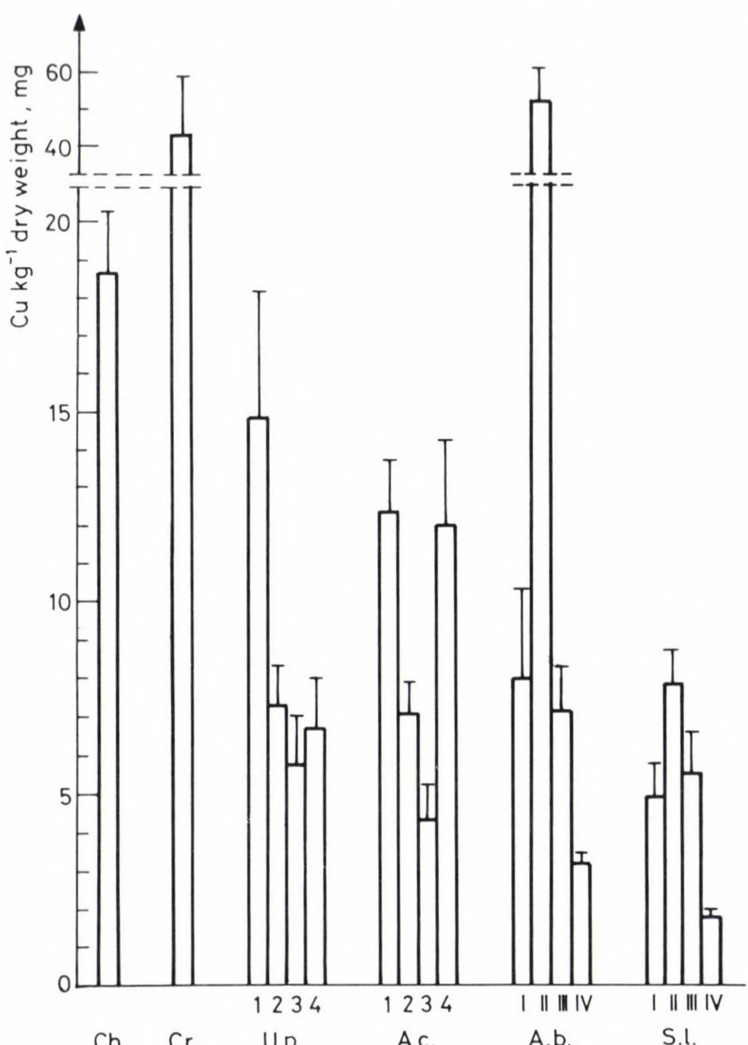


Fig. 3. Concentrations of copper (mg/kg dry weight) in various organs of animals in Lake Balaton. Symbols are the same as in Figs 1 and 2

By simultaneous sampling at different areas in the lake it is also possible to assess whether there are any differences between the pollution at different locations with particular reference to littoral zones, the vicinity of inflows, effluent discharge of sewage purification plants and sailing boat harbours.

The analysis of the accumulation capacity of crustacean plankton showed /7/ that for instance, the Hg and Cd were accumulated at a higher rate in crustaceans in the Keszthely basin than in the eastern one (Fig. 4) whereas the lead content of small crustaceans was highest in the Tapolca basin (Fig. 4). Near the inflows from sewage plants into the lake and in the vicinity of the Balatonfüred sailing boat harbour the heavy metal - especially copper, cadmium and lead - content of mussels was many times higher than the concentrations measured in animals collected from other locations on the lake. It should be noted, however, that these animals were doing well under these circumstances, no unusual mortality was noticed, but the bioaccumulation indicator clearly showed that purified sewage and protective paint on the sailing boats might be important point sources of heavy metal pollution in Lake Balaton.

Beside the analysis of the pollutant content of aquatic organisms collected in their natural habitats, there are other, more sophisticated field experimental designs to determine the degree of pollution. One such technique is to collect animals from relative less polluted habitats, transfer them to more polluted ones and to determine their heavy metal content after a shorter or longer period of time. This was the technique we used to investigate the heavy metal pollution at different locations along river Zala /20/. The biweekly sampling helped to discover that upstream from the town of Zalaegerszeg the heavy metal loading of the water is significantly smaller than downstream, after the inflow of the sewage from the town. This increased load can be detected as far downstream as the inflow of the river into Lake Balaton (Fig. 5). At present we do not know how the Kis-Balaton water quality protection system will influence this loading, our results reflect the situation as it had been before the reservoir started to function.

From our translocation experiments it has also become apparent /18/ that after a longer period of time the concentration of heavy metal under investigation showed significant increase in the experimental animals' body (Fig. 6).

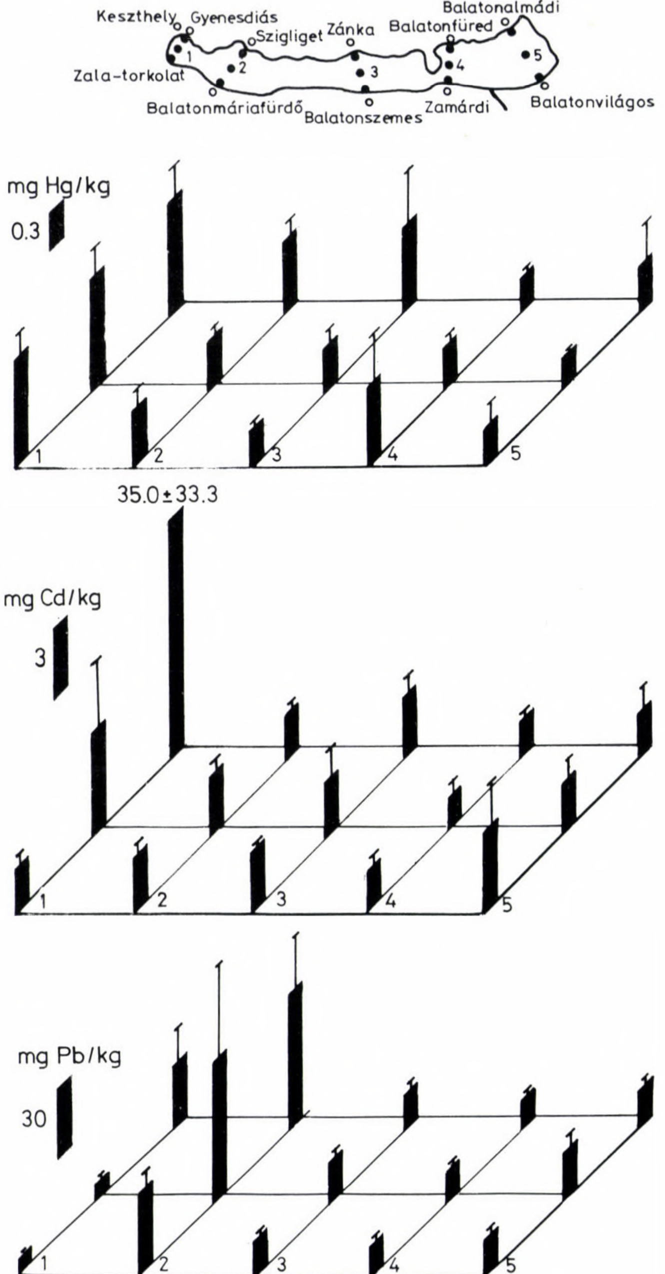


Fig. 4. Mercury, cadmium and lead concentrations in the Crustacea plankton collected at different locations on Lake Balaton. The areas indicated on the Balaton map were 200 m offshore and in the midline of the lake, respectively. Values are given in mg/kg dry weight.

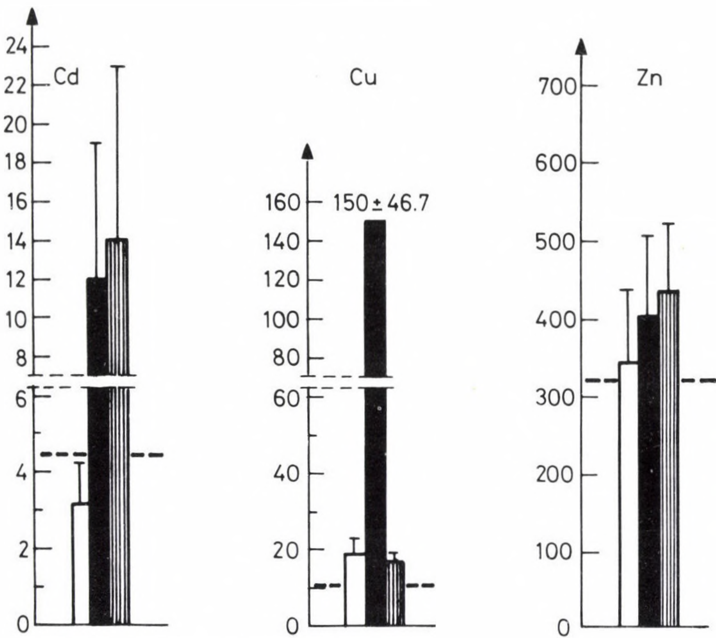


Fig. 5. The comparison of the concentration of cadmium, copper and zink in the gills of transferred mussels at the three different reaches of the Zala river. Dashed line shows the concentration at the time of the transfer. Measurements took place 2 weeks after transfer. Empty column: location above Zalaegerszeg, black column: location under Zalaegerszeg; striped column: inflow of the Zala river. Each measurement was performed with 3 animals. Y axis: mg/kg dry weight

We found 50 to 100% differences between samples taken at the same location and kept at different areas for several weeks. This raises the question whether such differences can really be explained by changes in environmental pollution. We have to ask this question whenever we talk about biological indicators and it has to be made clear, too, that to what extent the past conditions under which the experimental animal lived can influence the measured data. This is important because there are organisms which retain the accumulated pollutants throughout their life, store them in their bodies and release them into the environment only after their death. In other organisms both uptake and release take place more quickly. This is why we investigated the dynamism of the heavy metal uptake and release in mussels in long-term laboratory experiments. The animals were kept for 4 weeks in water of constant heavy metal concentration, then they were transferred into natural Balaton water. The heavy metal content of various organs of the mussels was measured at regular intervals after the transfer

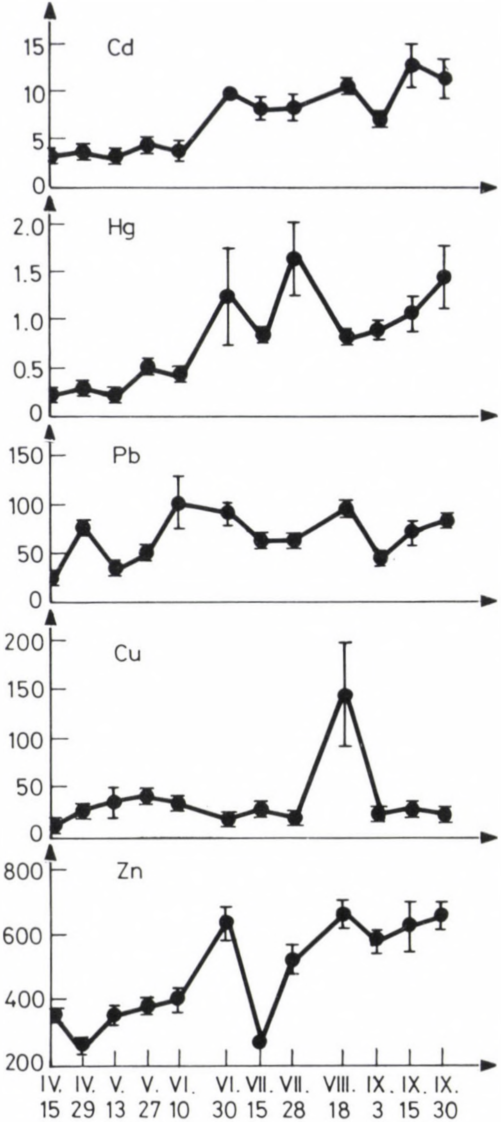


Fig. 6. The concentration changes of 5 heavy metals in gills of mussels transferred from Lake Balaton to the Zala river. The first measurement took place at the time of the transfer (15 April). Values are given as mg/kg dry weight

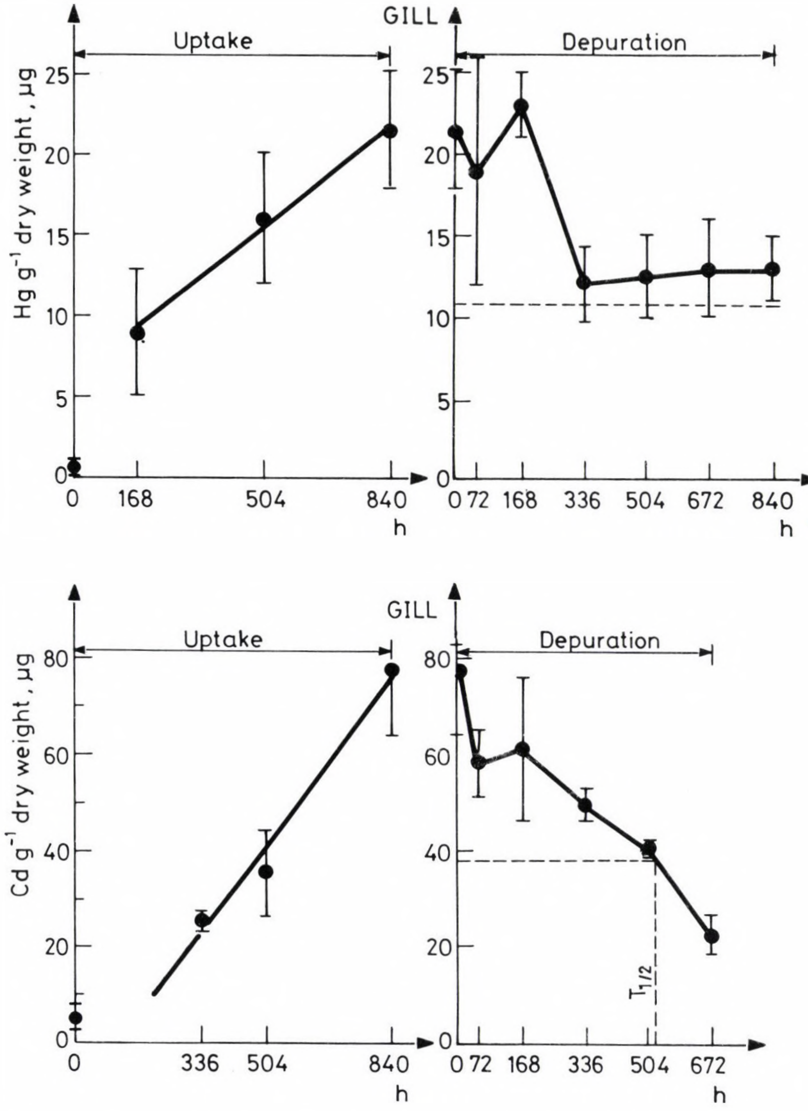


Fig. 7. The dynamics of mercury and cadmium uptake and release in the gill of mussels. The applied concentration was 12.9±2.8 and 16±1.3 µg/l, respectively, in through-flow type system. Horizontal dashed line indicates the theoretical 50% release level. $T_{1/2}$ is the time required for 50% depletion of cadmium

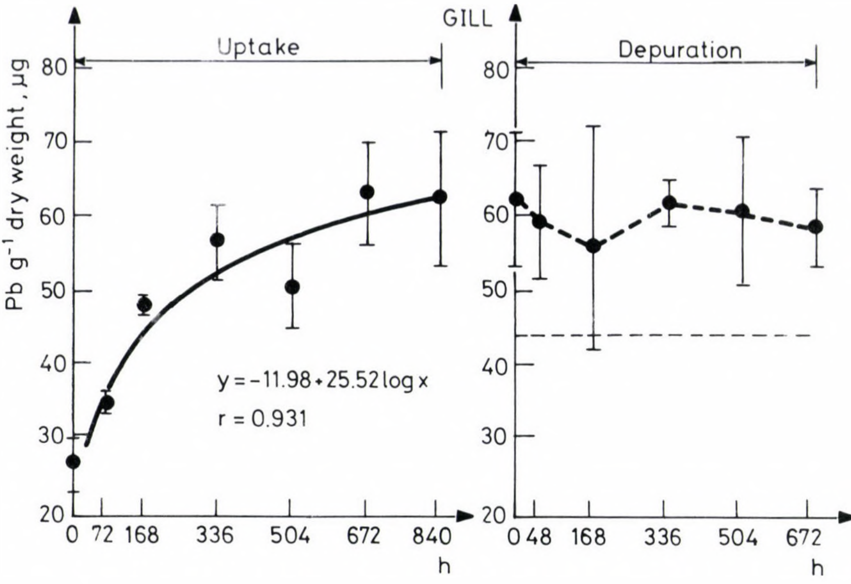


Fig. 8. The dynamics of lead uptake and release in the gill and mantle of mussels. The applied concentration was 57.6$\pm$19 µg/l in through-flow type system

into Balaton water. We found that in polluted water the heavy metal uptake was relatively slow, then the uptake rate increased and following a linear phase it went into saturation. When the animals were transferred into unpolluted Balaton water, there was a sudden drop in the heavy metal content. The concentration of the pollutants, however, never returned to the baseline value, rather, it became stable at higher levels characteristic for particular heavy metals and organs (Fig. 7). Certain tissues bound certain heavy metals very strongly with a very low release rate for weeks after treatment (Fig. 8).

The laboratory experiments have shown that heavy metals are stored in at least two different compartments within the organs and they are not or hardly released from the first type of these whereas can be washed out easily from the second one /10, 11, 19/.

The dynamism of uptake and release has great significance from the point of view of biological indication of pollution. It is characteristic of lakes and particularly of rivers that the level of pollution is not constant in them, rather, pollution occurs in waves. Besides, the sources of pollution often emit the pollutants in varying quantities, and on the

other hand, the concentration is very much dependent upon the actual rate of water flow. Transient (lasting for a few hours or days) pollution peaks can be detected in the water itself by lucky chance only, while traces of the pollutants are present in the accumulating indicator organisms for weeks or even longer.

The cause of the 1987 water poisoning on the Rhine that killed a significant proportion of the living organisms at the most polluted parts on the river was only partially disclosed by the water chemical analysis carried out a few days after the pollution. However, from the surviving animals it was possible to detect the presence of a variety of toxic substances. Only when this was presented as bioindication evidence the pharmaceutical factory emitting these substances acknowledged that they had released the toxic agents into the water. This also shows that in contrast with earlier views, not only such species are good indicators that are very sensitive and react by dying rapidly after the pollution. Those species that are more resistant and because of their great accumulating capacities are capable to accumulate and store pollutants in their bodies, can also be used as good practical indicators.

It is not difficult to realize how significant these more resistant biological indicators might be from the point of view of the control of the quality of the environment. It also hardly needs to be argued that regular control of the quality of the environment, detection of the sources of pollution and keeping the amount of emitted pollutants within strict limits are fundamental questions of economy, public health and society in our country, too. For this reason, after surveying the heavy metal pollution in organisms living in Lake Balaton we started to work on a program which aims to assess the heavy metal pollution in the entire catchment area of Lake Balaton by using a biological monitoring system /13, 21/. In the course of this work we would like to lay down the foundation of a continuously operating biological monitoring system. Similar systems have already been in existence for a long time in some industrially developed countries in order to keep track of the pollution of sea. This system in Hungary would continuously provide data for a national environmental protection network.

The biological accumulation tests and test systems serve to detect the appearance of substances emitted in low concentrations in a particular region and to direct attention in time to the danger of possible damage. Such a signalization system is also suitable for the detection of irregularly emitting sources of pollution. The early detection of pollution is an

important starting point for preventive measures and thereby can be regarded as part of the prevention system. However, there are limits to the applicability of this system. One problem with this system is that it is suitable only for the detection of substances that do not break down, or at least they are stable enough to be accumulated in biological systems. Such substances are the heavy metals, persistent chlorinated hydrocarbons and various other synthetic substances. On the other hand, there are substances with well-known toxic and pollutant effects that break down rapidly and can not be detected by accumulation tests. The new generations of pesticides also belong to this latter category.

The other problem is related to the amount of extensive analytical work required for the detection of the large number of potential pollutants in animals and plants. Besides, detectability and accumulation are not always indicative of the nature of the damaging effect. Every year hundreds of freshly licensed chemicals come into use in the industry and agriculture and also as pharmaceutical and cosmetic products. There are neither elaborated methods nor demand for the measurement of the appearance of these substances in living organisms. This is why recently a new concept has come to the fore. According to this, novel chemical substances coming into use in large quantities should undergo not only the usual toxicity and genetical tests to assess their effects on the mortality but beside these, they have to be investigated with respect to possible transient or lasting damage caused by their sublethal concentrations in biological systems.

The scientific trend that deals with the environment damaging effects of anthropogenic substances in a preventive fashion uses a large variety of approaches and biological systems. Microbes as well as plants and animals, genetical as well as biochemical, radiobiological approaches and methods all play roles in this. Neurobiological methods also appear among these and they are used to assess the toxicity and indirectly, the harmful or neutral nature of various anthropogenic substances by examining the effects of these substances on the nervous system /6/.

It should be noted that the investigation of the effects of various exogenous substances in the experimental biology was not originally prompted by environmental biological demands. In the analysis of the rules and mechanisms of biological regulation various organic and inorganic chemical substances have been used for a long time. These substances range from mutagenic and carcinogenic substances, enzyme activity influencing heavy metals and organis phosphate esters to naturally occurring poisons

and cell toxins. Also in neurophysiological studies, it is a common practice to use synthetic specific inhibitor substances for the separation of parallel occurring biological processes, for the elimination of particular components and thereby for the elucidation of the role the inhibited and uninhibited component might play within the whole regulatory process.

The environmental biological approach during the application of experimental biological methods means that our primary goal is to clarify whether the substance under investigation causes some alterations in the regulatory system. These alterations lead to impairment of life functions and although they do not cause the animal's death directly, they might adversely influence various physiological functions. In this complex set of problems our research efforts aim to investigate a special one, namely, the neurophysiological effects of persistent pollutants that occur commonly but in fluctuating concentrations in the environment. These effects include alteration of neural structures, changes in the synthesis of the chemical substances of neural regulation, influence on the excitability of neurones, on the transmission and processing of information and on the membrane processes underlying these. These structures and processes play a decisive role in the regulation of vital functions in animals, in their ability to adapt to changing conditions and in the behavioural reactions of the individuals.

In connection with our studies on the accumulation of heavy metals in organisms of Lake Balaton, we have most extensively been studying over the past few years the neurophysiological actions of the same heavy metals under laboratory conditions. It is true, however, that some aspects of this work date back to over 30 years /3/ when in studies unrelated to environmental biology, I found that the behaviour of mussels undergoes characteristic changes under the influence of the SH group blockers cadmium and mercury. Under normal circumstances the mussels display continuous, long-lasting filtering activity, whereas under the influence of the two heavy metals the active periods become shorter and the alternation of activity with quiet periods becomes more frequent (Fig. 9). The relative shortening of the active period is accompanied by a drop in the feeding and respiration activities of the animals and at the same time, by a decrease in the filtering, water cleaning activity, which, in turn is unfavorable for the environment. The behavioural pattern shows that changes in central regulatory mechanisms are responsible for the observed alterations.

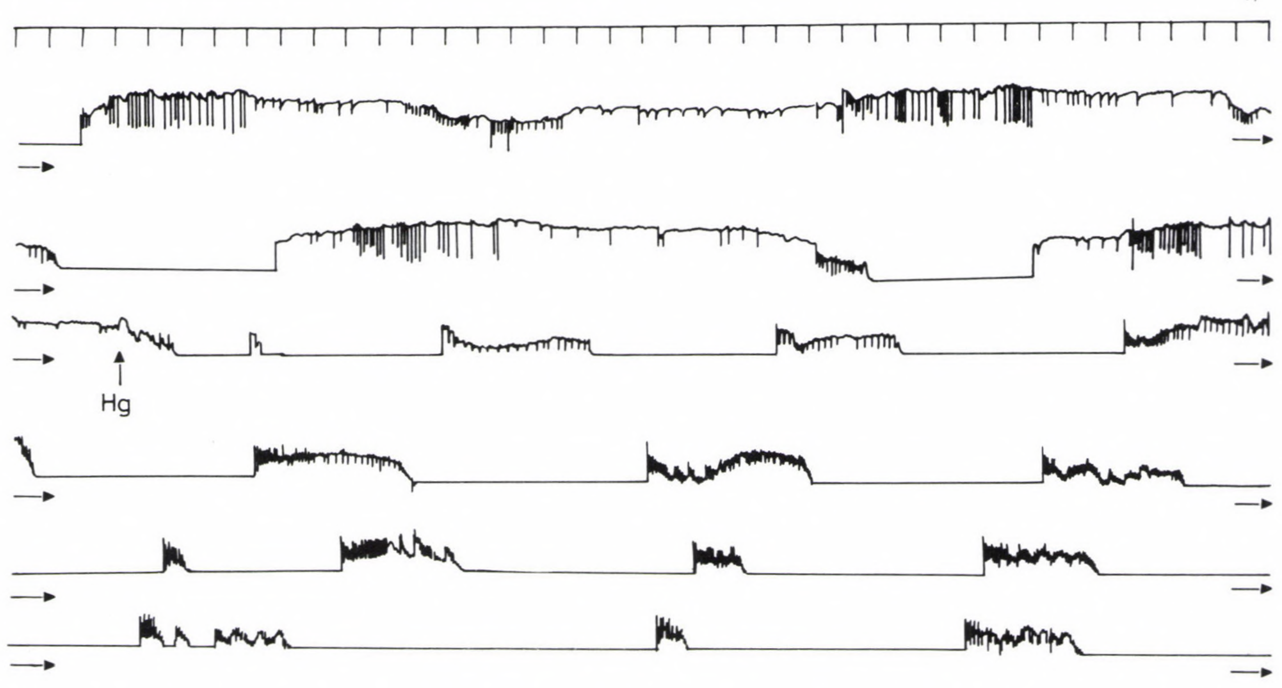
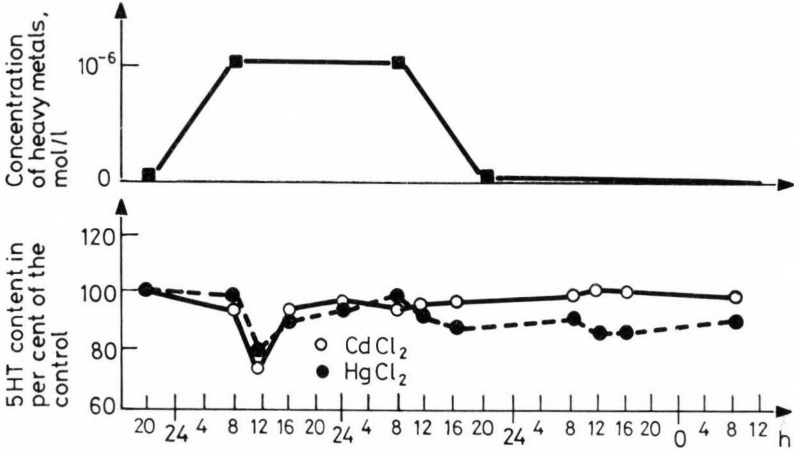


Fig. 9. Filtering activity pattern of fresh water mussels in clean Balaton water and after contamination with $HgCl_2$ (10^{-4} mol/l) The duration of active periods is 20 to 60 h in clean water and can be as short as 2 to 5 hours in polluted one



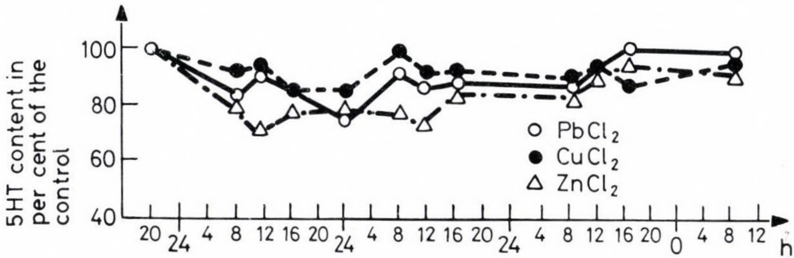


Fig. 10. The effect of 10^{-6} mol/l cadmium, lead, copper and zink (added to the water) on the serotonin content of the CNS of mussels. Samples were taken every 4 h during the treatment. The decrease of serotonin (5-HT) differs for each metal but never exceeds 30%

Over the past years we have elucidated that two endogenous monoamines in the nervous system of mussels, serotonin and dopamine, play important roles in the regulatory processes of these animals /2, 5/. Based on these observations we hypothesized that heavy metals might exert their regulatory mechanism influencing effects by altering the levels of these monoamines in the brain. We tested this hypothesis and found that Cd, Hg, Zn, Cu and Pb applied for 24 h in 10^{-6} - 10^{-8} mol/l concentration indeed resulted in a transient depletion of dopamine and serotonin content from the brain (Fig. 10).

The toxic agents might interfere with various steps in the synthesis, liberation and elimination of behavioural pattern regulating monoamines. The inhibition or stimulation of these processes influence both the con-

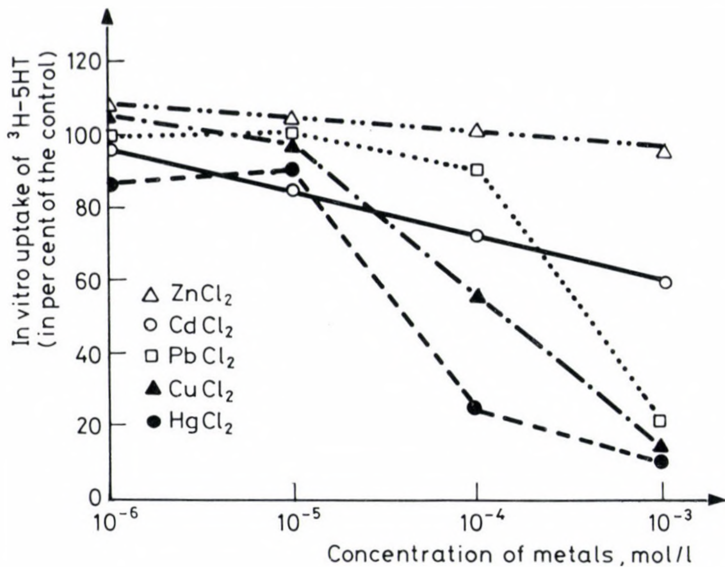
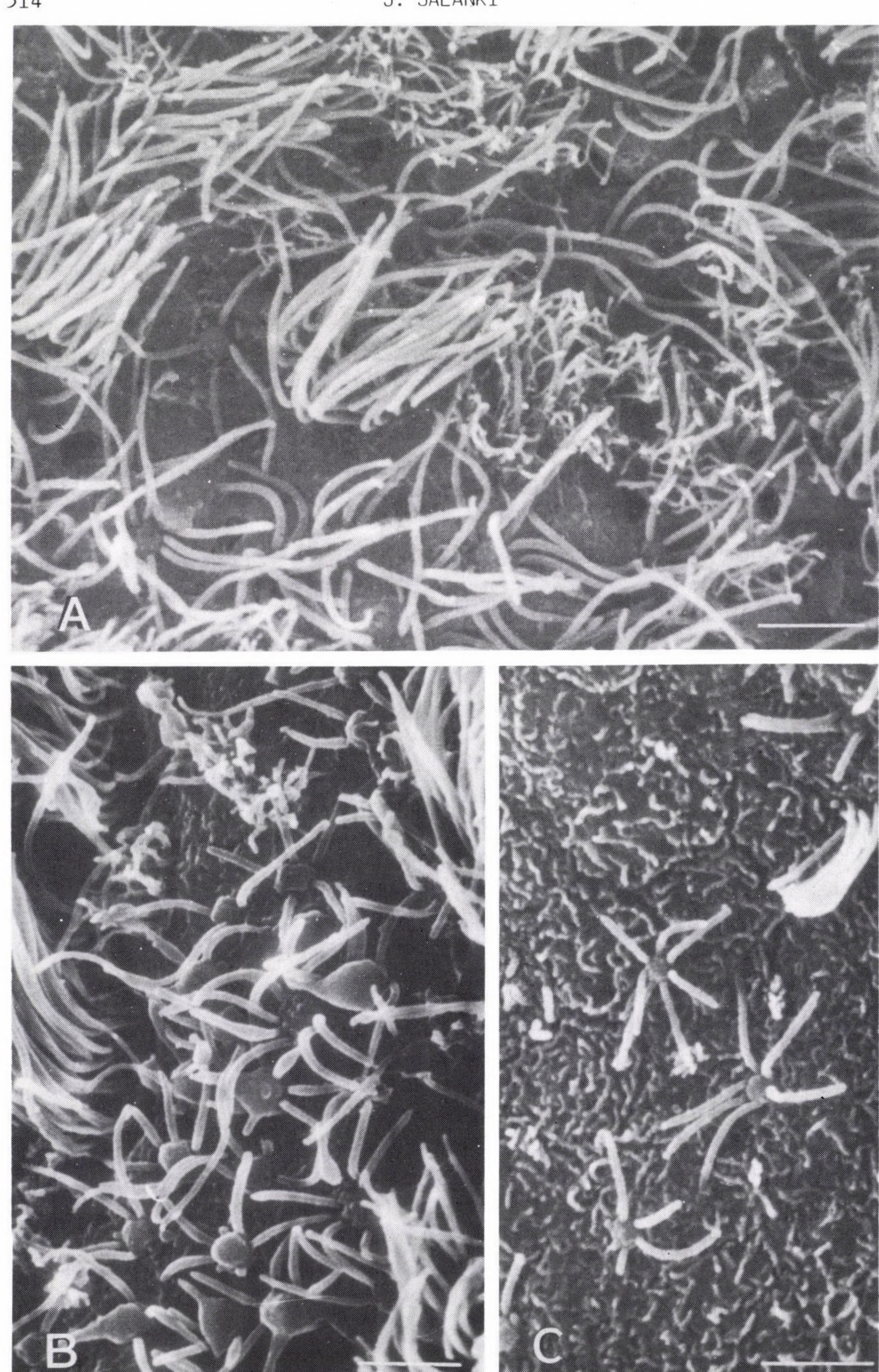


Fig. 11. The effect of heavy metals on the uptake of serotonin by nervous tissue of the mussel, in vitro. Different metals blocked the uptake to different extent, in 10^{-4} mol/l concentration mercury was the most effective blocker, in 10^{-3} mol/l concentration mercury, copper and lead had nearly the same blocking effects, while Zn was ineffective even when applied in high concentrations

centration of the active substance and the function regulated by it. From the results of the relevant research I refer here only to one finding: heavy metals influence the re-uptake of serotonin to the storage sites to different degree /8/ which is an important means of the elimination of the transmitter in the nervous system of the mussel (Fig. 11).

It is also possible that different agents act at different points in the system but the final behavioural output is the same. This is supported by our investigations on the effects of some pesticides compared with the effects of heavy metals. As it turned out, heavy metal free pesticides influence the mussels' activity pattern the same way as cadmium or mercury did. We suggest, that this activity test and the analysis of the influence on the monoamine levels, respectively, are suitable methods for the prediction of the effects of other harmful agents in the aquatic environment.

Environmental pollutants may occur in large concentrations in surface waters and here, they are in permanent contact with living organisms. Beside the respiratory organs it is the gustatory and olfactory system that is most exposed to the effects of dissolved chemical substances. The chemo-


A
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receptors play an important role in the orientation, food finding and other behaviours of the aquatic organisms. The external chemosensory epithelium might be damaged by exposure to pollutants and the animals lose their ability to obtain important information about the outer world and this, in turn, leads to behavioural defects. Our research included the investigation of heavy metal induced structural changes in the chemosensory organs of fish which might serve as a background for functional deficiencies.

We studied the olfactory organ of bleak Abramis brama L., and we found that following treatment with mercury the number of receptor cells decreased dramatically. Exposure to cadmium resulted in a different type of damage to the sensory units, namely the swelling of the apical region of the sensory cells (Fig. 12). Long-term exposure to mercury had degenerative effect also on the gustatory epithelium of the bleak's palate (Fig. 13). In contrast, during short-term exposure to Hg, the sensory cells are protected by mucus secretion and by the supporting cells and this prevents morphological degeneration /1, 4/.

Metabolic and structural changes occur after exposure to the heavy metals for several hours or days. On the other hand, the rapid, acute membrane effects of the environmental pollutants can be best assessed by using cellular electrophysiological techniques.

Elementary processes of excitation such as the generation of action potentials, membrane effects of biologically active substances, specific activation of ionic channels upon the binding of transmitters to receptors are all present in the nervous system of every animal. These phenomena are identical in animals of simple and more complex nervous organization and even in the human brain. The complex, interconnected systems of elementary processes form the background of neural functioning and regulation involved in both internal regulatory processes and adaptive responses to environmental effects.

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Fig. 12. The different effect of cadmium and mercury on the olfactory epithelium of bleak (Alburnus alburnus L.). Scanning electron microscopical picture. A: control; B: 14 days after treatment with 100 μg/l cadmium; C: 7 days after treatment with 300 μg/l mercury. Following cadmium treatment the apical region of the ciliated dendrites swells and becomes deformed, treatment with mercury causes complete degeneration of the ciliated elements. Calibration bar: 2 μm

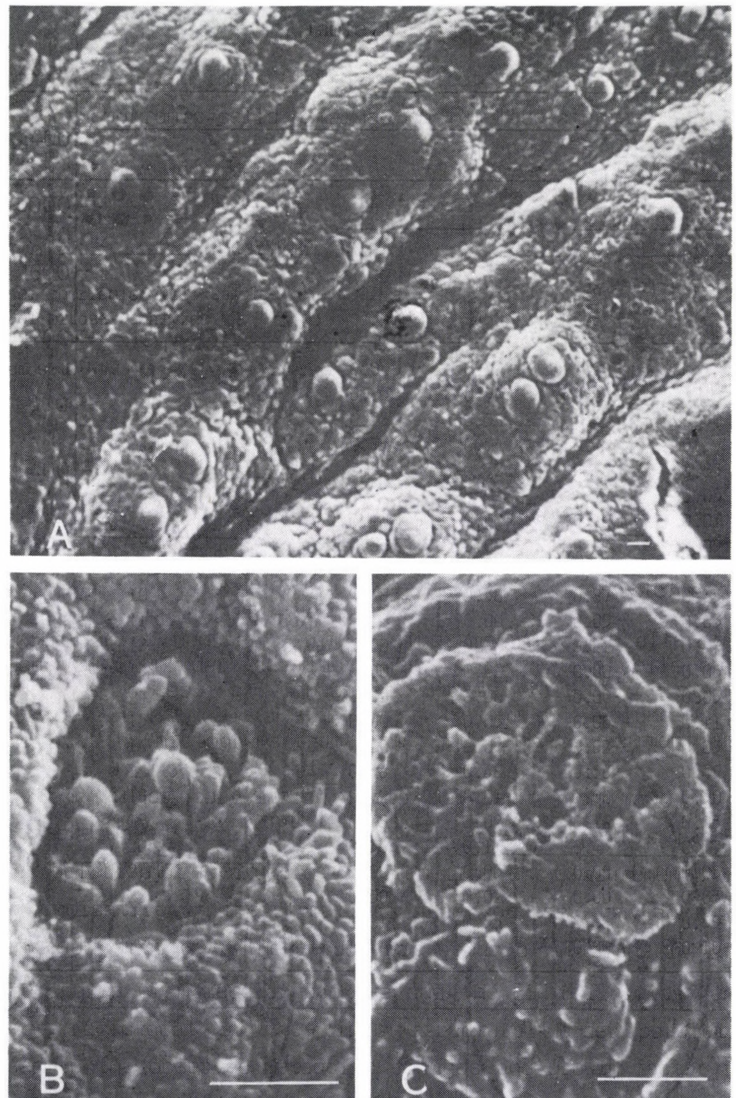


Fig. 13. The effect of mercury on the structure of the gustatory epithelium of bleak. Scanning electron microscopical picture. A: the distribution of the taste buds on the surface of the palatal organ (low magnification; B: an individual taste bud at higher magnification; C: 3 days after treatment with 300 μg/l mercury. The taste bud is damaged and covered with mucus. Calibration bar: 10 μm

For the evaluation of the neurophysiological effects of environmental pollutants we used two snail species, Helix pomatia L. and Lymnaea stagnalis L. Both species have long been used as experimental animals in neurobiology and both possess giant nerve cells in their central nervous system the majority of which can be identified by function /15/.

In this system the effect of chosen transmitter substances can be tested with sufficient accuracy. It can also be studied in these systems how various anthropogenic agents and other exogenous substances influence the functioning of the nerve cell membrane.

The starting point for these effect analyses is that in the neuronal membrane certain proteins embedded in the lipid bilayer may function as ionic channels in response to specific transmitter or modulator actions. The flow of ions through these channels generates the action potentials which can be measured as a sudden potential change between the two sides of the membrane. The binding of the released transmitters to membrane proteins activates such channels. Of the widely known chemical transmitter substances acetylcholine (ACh), serotonin (5-HT) and dopamine (DA) occur commonly in the snails nervous system but the neurones do not respond uniformly to these substances. The responses of different neurones might differ depending on the particular ionic channel activated by the binding of a particular transmitter to the receptor.

The activation of Na and Ca channels causes depolarization of the membrane which excites the cell, the activation of K channels, on the other hand leads to hyperpolarization of the membrane and this is expressed in the inhibition of the cell's activity. Activation of Cl channels might result in either excitation or inhibition. The picture is more complex because, for instance, the inactivation of K channels might also cause excitation and the entry of Ca into the cell may also increase the K permeability of the membrane. The same transmitter may activate different channels of the same membrane and in this case the resulting response is the product of more than one factor. The direction and actual value of the measured response also depends on the resting potential of the cell. It is well-known that various modulators can alter the membrane effects of transmitters and in our investigations we wished to elucidate the possible transmitter action influencing effects of various environmental pollutants. I would like to present a few examples to illustrate the nature of this modification and to characterize the effectiveness of these substances /14, 17/.

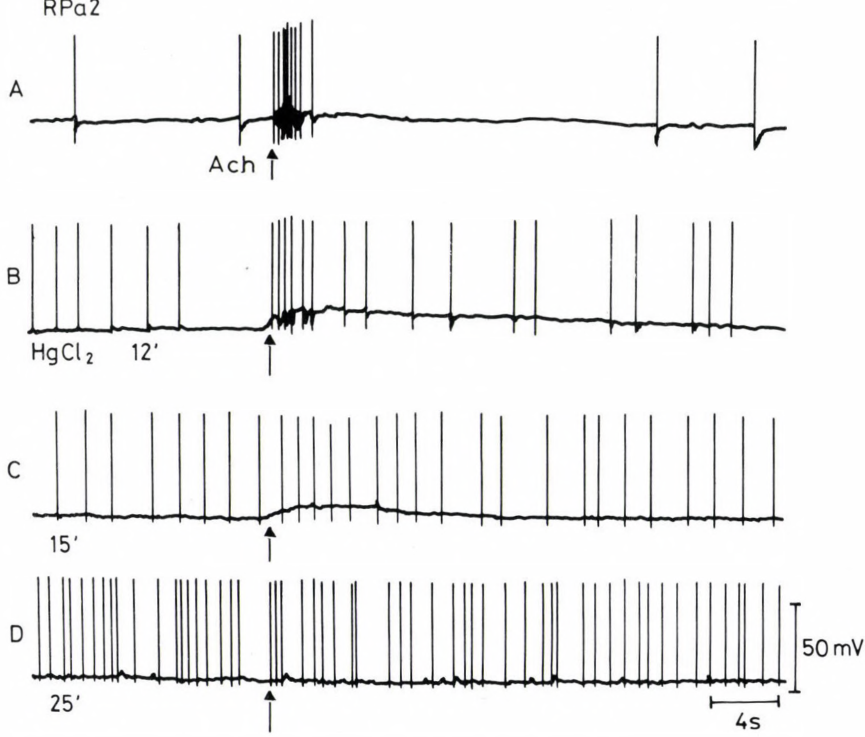


Fig. 14. The effect of mercury on the acetylcholine response in the identified RPa2 neurone of Helix pomatia L. A: ACh depolarizes and excites the neurone; B, C, D: after treatment with mercury the response becomes weaker and finally ceases

We know that a previously identified neurone gives an excitatory response to ACh. As it is shown in Fig. 14, ACh applied to the some membrane produces a characteristic depolarization and activity increase similarly to the effect of cholinergic excitatory inputs. If the preparation is exposed to $HgCl_2$ for a few minutes, the response of the neurone to ACh decreases with no apparent impairment of the general action potential generating ability of the cell. The effect of Hg therefore is the abolishment of the ACh generated depolarization of the soma membrane.

When the same neurone was exposed to Cd, the observed consequences were entirely different from those of the Hg preexposure. The cell gradually lost its ability to generate action potentials, at the same time it was still capable of producing depolarization upon the effect of ACh (Fig. 15). This clearly shows that the two different toxic heavy metals attack at dif-

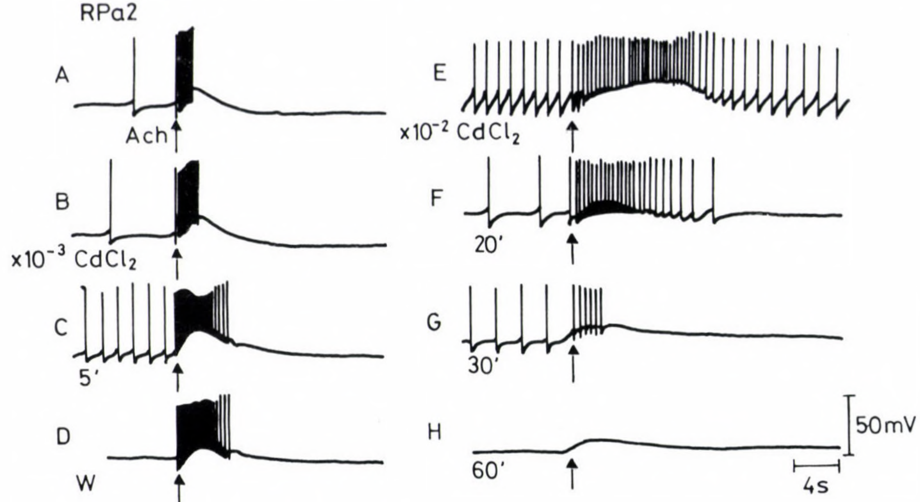
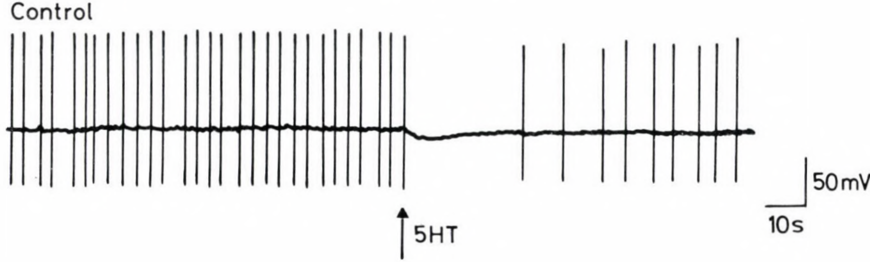
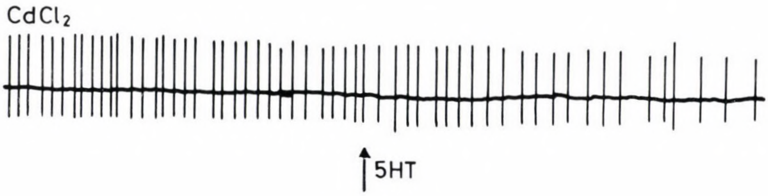


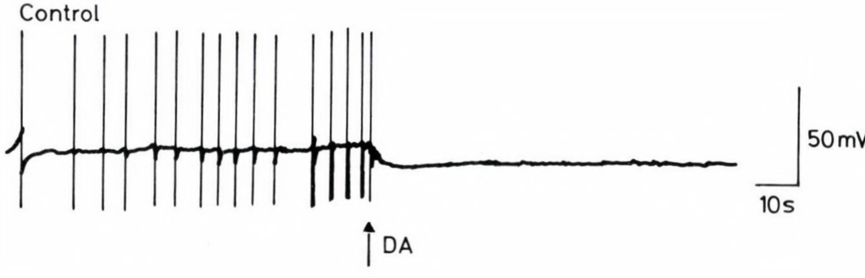
Fig. 15. The effect of cadmium on the acetylcholine response in the identified RPa2 neurone of Helix pomatia L. A: ACh depolarizes and excites the neurone; B, C: 10^{-3} mol/l $CdCl_2$ is ineffective when applied for 5 min; D: wash out; E, F, G, H: 10^{-2} mol/l $CdCl_2$ when applied for a longer period of time, causes the abolishment of the spike generating ability of the cell but does not affect the ACh induced depolarization.

ferent points on the membrane. Hg clearly inhibited the binding of ACh to the receptors and the ionic channel activated by this, whereas Cd did not affect this system, instead it blocked the channel responsible for the generation of action potentials.This also shows that different mechanisms are responsible for the spike generation and for the ACh-induced depolarization.

Cd also produced characteristic changes in the dopamine and serotonin sensitivity of the membrane. In different neurones serotonin might induce either excitatory or inhibitory responses while dopamine is mostly inhibitory. In neurones where both serotonin and dopamine were inhibitory, preexposure to Cd abolished the inhibitory effect of serotonin and reversed the inhibitory effect of dopamine into an excitatory one (Fig. 16). This latter phenomenon indicates that the transmitter was activating a variety of channels and inputs and the actual response of the cell is always a kind of summation of these effects. When one of these components is eliminated as a result of preexposure to a heavy metal, others come to the fore and the overall result may be the opposite of what we saw first. Similar cell function and chemosensitivity influencing, modulatory, inhibitory effects







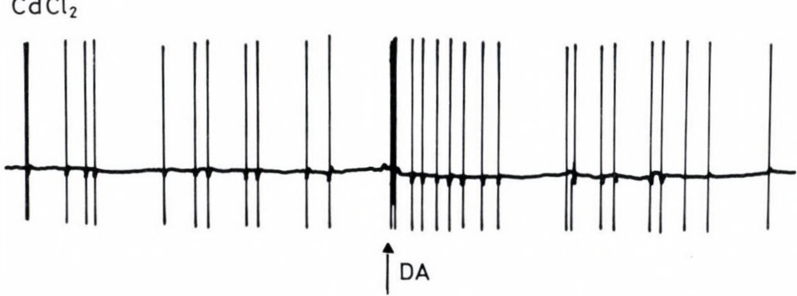


Fig. 16. The effect of cadmium on the serotonin and dopamine response of the same neurone. Both transmitters hyperpolarize the cell membrane (control). $CdCl_2$ treatment abolishes the serotonin response and reverses the dopamine response

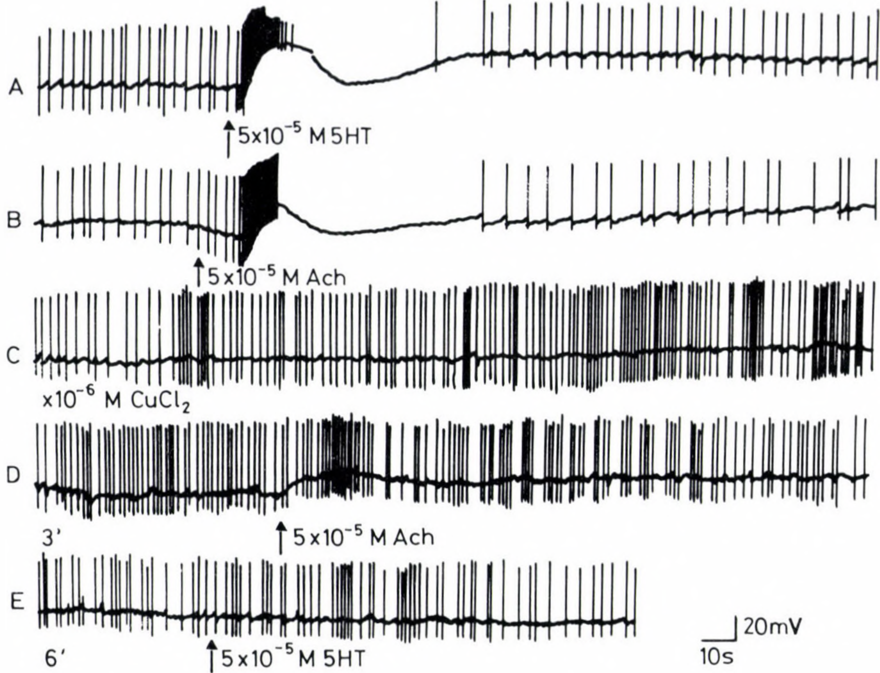


Fig. 17. The effect of copper on the serotonin (5-HT) and acetylcholin (ACh) response in a pond snail (Lymnaea stagnalis L.) neurone. A, B: controls; C: 10^{-6} mol/l $CuCl_2$ increases the spontaneous activity of the cell, D: in the 3rd min of the treatment ACH effect is strongly suppressed; E: the 5-HT effect is blocked in the 6th min of the treatment

were found following preexposure to copper or lead, too. In the experiment shown in Fig. 17 copper eliminated both ACh and serotonin induced responses. The effect of lead was different on the serotonin response of different neurones (Fig. 18) while acetylcholin responses of the same cells were blocked in most cases (Fig. 19).

The above results showed that transmitter substances can have either excitatory or inhibitory effects on the neuronal membrane and these effects can be modified as a result of previous exposure of the membrane to heavy metals. As for the activity of the neurone itself, excitation and inhibition are well defined phenomena, however, the membrane processes underlying them cannot be directly analyzed by measuring the overall potential changes in the cell. We can characterize the membrane processes only by directly measuring ionic currents flowing through the membrane. The essence of this technique is that we fix the polarity of the membrane at certain present levels (we clamp the membrane potential) and then we measure the amount of

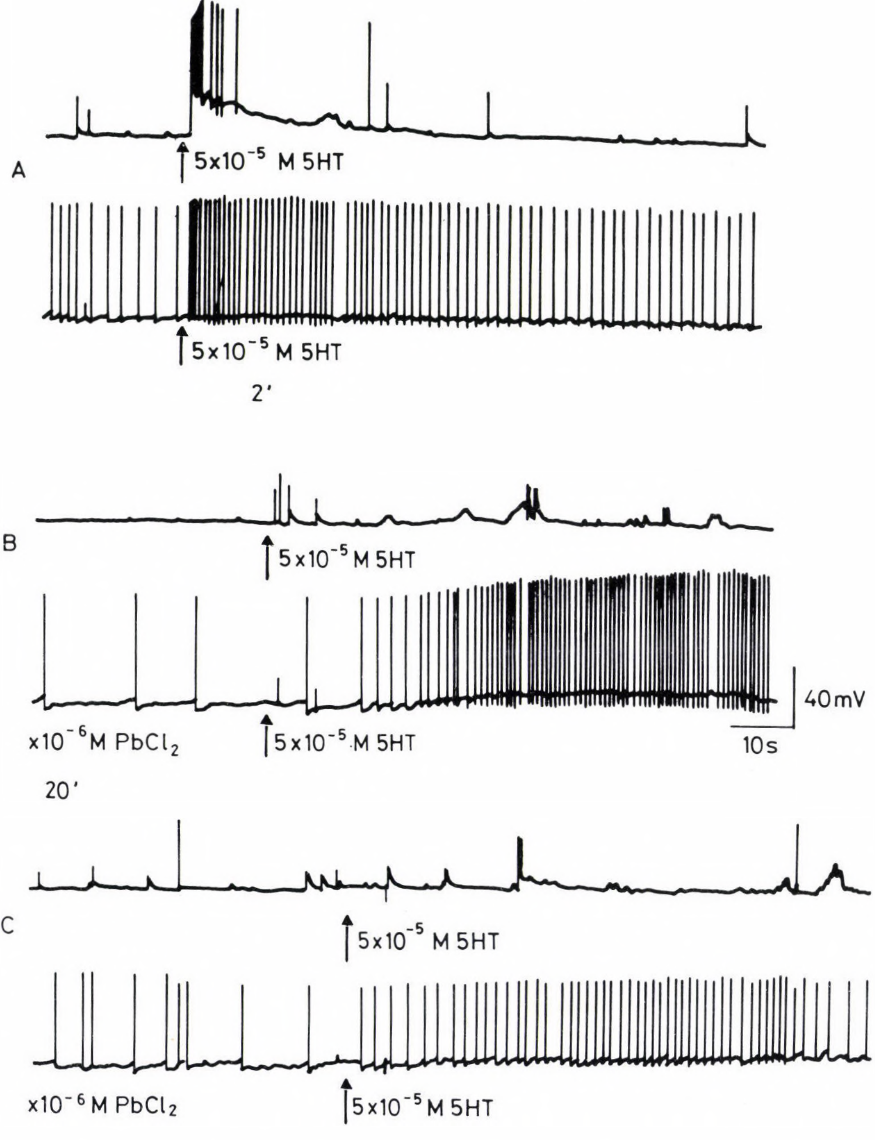


Fig. 18. The effect of lead on the serotonin (5-HT) sensitivity of two simultaneously recorded neurones. A: control responses of the neurones; B: the responses of the neurones 2 min after $PbCl_2$ treatment; C: the same after 20 min treatment. The neurone responding with brief excitation to 5-HT becomes insensitive following treatment with lead. The other neurone responding with a longer lasting excitation, loses only the fast component of the response

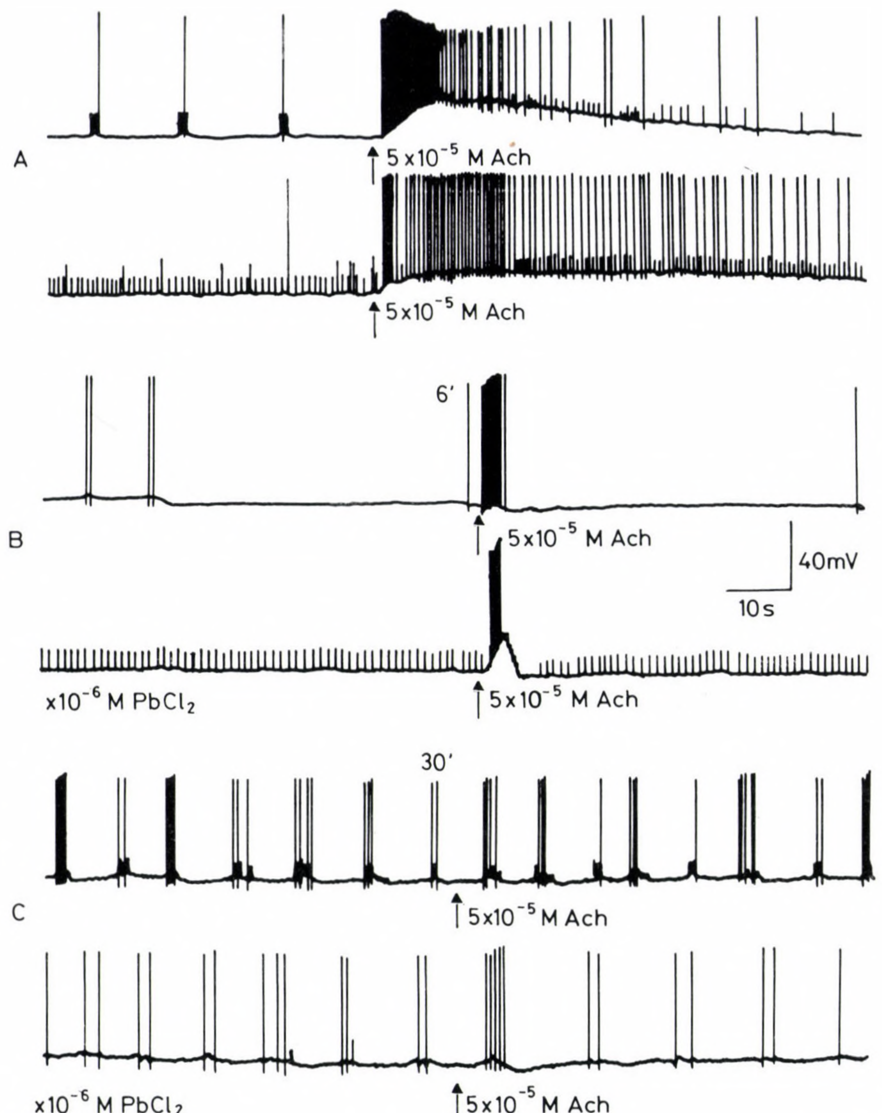
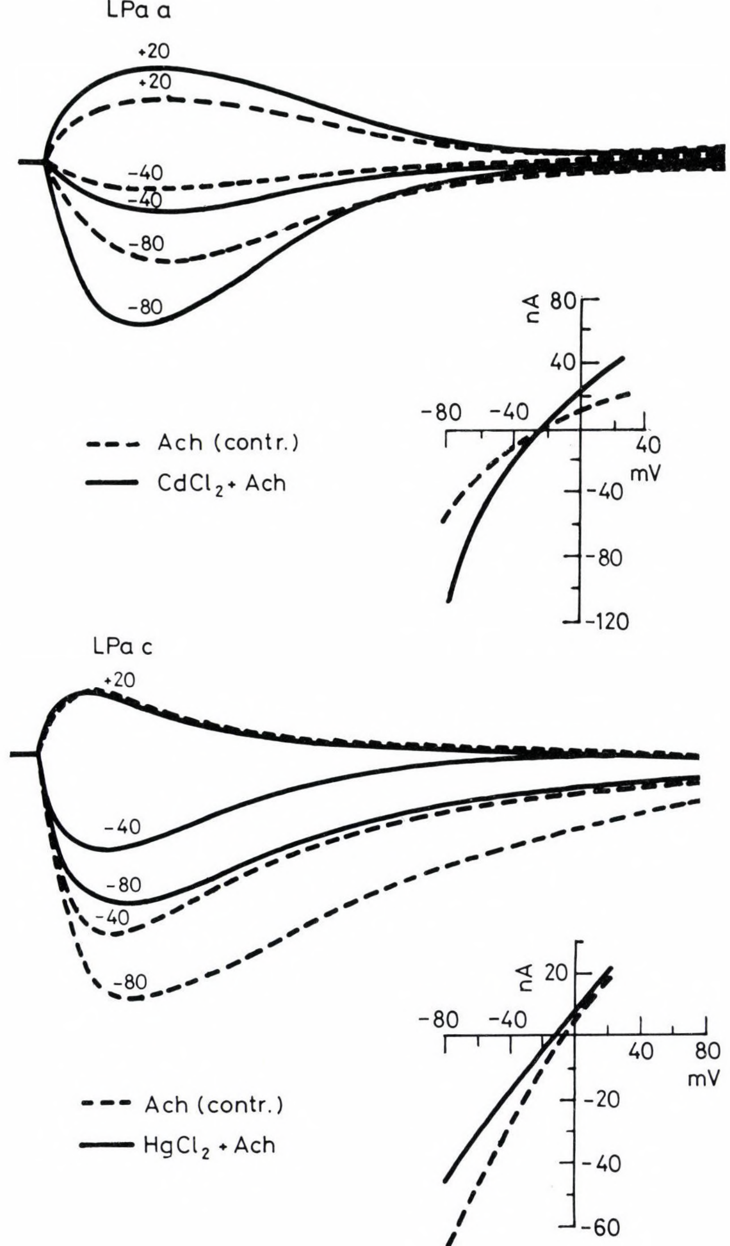


Fig. 19. The effect of lead on the acetylcholin sensitivity of two simultaneously recorded neurones. A: excitatory response of the neurones to ACh; B: 6 min after treatment the evoked response is significantly reduced; C: 30 min after treatment there is no or hardly any response to ACh



LPa a
+20
+20
-40
-40
-80
-80
nA
80
40
-80
-40
40
mV
-40
-80
-120
Ach (contr.)
CdCl2+ Ach
LPa c
+20
-40
-80
-40
-80
nA
20
-80
-40
40
80
mV
-20
-40
-60
Ach (contr.)
HgCl2 + Ach

current flowing through the membrane at different holding potential levels. Since we know the flow of various ions, like Na, Ca, K or Cl, at different holding potentials, we can form a judgment on the nature of the channels activated by the transmitters and on the type of channel that was influenced by the exogenous substance in question.

In our earlier studies we found that application of Cd to the some of an identified neurone mainly suppressed the outward K current, which in turn led to a delayed restoration of the membrane sensitivity thereby decreasing the excitability of the membrane. When we studied in detail the effect of Cd and Hg on the primary ACh effect we found that changes in the ionic current might be different from one cell type to another. Heavy metal ions may influence both inward and outward currents and the effect appears to be specific for the particular neurone rather than for the particular metal ion /16/.

In the case shown in Fig. 20 both the ACh induced outward and inward current increased following Cd treatment and this can be regarded as potentiation of the transmitter effect. The reversal potential of the current response indicates that ACh activated a Cl current which is also affected by Cd. In a different cell, adjacent to the previous one, Hg decreased the ACh induced inward current, i.e. blocked the excitatory action of ACh, but did not affect the outward current. This corresponds to the blocking of Na channels.

This method is also suitable for the measurement of the membrane effects of other toxic substances, and at the same time it is also possible to gain information on the characteristics of the processes of neural excitation in general.

The investigation of the effects of environmental pollutants on neural regulatory mechanisms and the application of the methods and results for purposes of biological indication have great significance for the protection of wildlife and human life alike. Changes in the ability of the

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Fig. 20. Upper traces: The effect of cadmium on the ACh-induced ionic currents in the LPa a neurone. Currents were induced at -40 and -80 mV holding potential levels. There is a noticable shift in the current-voltage characteristics in both inward and outward directions.
Bottom traces: The effect of mercury on the ACh-induced ionic currents in the LPa c neurone. Only the inward current is decreased, the outward current remains unaltered. This is also shwon on the I-V characteristics. The duration of the evoked currents is 50 s

nerve cells to react to inputs have profound effects on the entire process of neural regulation. Since transmitter substances play a major role in the connections between the neurones themselves and between neurones and effector organs, the entire neural regulation depends on the undisturbed functioning of these transmitter systems. Any intervention that disturbs the chemical sensitivity and reactivity of the neurones opposes this undisturbend functioning. If changes similar to those observed in our in vivo experiments occurred in the nerve cells of intact animals, their entire regulatory system would collapse and normal life functions would be impossible. This is particularly true when we consider that the effects studied in separate experiments by us might occur simultaneously in the natural environment.

Apart from cases of poisoning, such grave damage is rarely sustained in everyday life resulting from pollution. However, the initially subtle effects of pollutants entering even in trace amounts the nervous system, accumulated and suddenly released from the tissues, might manifest themselves as serious defects on the long run. Recently more and more publications suggest for example, that Alzheimer disease, the old age dementia is probably brought about by such subtle but cumulative environmental pollution.

The chronic effects damaging the central regulatory system of both animals and humans in a latent manner can be assessed only by investigating the effects on excitability and the fundamental mechanisms of neural functions. It follows from this, that for the elucidation of the possible consequences of pollutants entering the environment and the living organisms, these types of investigations are indispensable as preventive methods in the environmental protection.

It is apparent that the short-term economical/production interests of the human society can be balanced with long-term interests also including the protection of the healthy environment, only if the environmental protection steels itself with new attitudes and seeks and finds new ways for the protection of the biosphere and of the health of man living in it. I believe that the above described new approaches which we also follow in our research, enrich our fundamental knowledge on the biological systems and beside, they open new avenues for the environmental protection in the practice. These approaches will have to become integral parts of the national and international system of environmental protection.

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DEFENSIVE BEHAVIOUR IN THE POND SNAIL, LYMNAEA STAGNALIS: THE WHOLE BODY WITHDRAWAL ASSOCIATED WITH EXSANGUINATION

D.A. SAKHAROV[1] and KATALIN S.-RÓZSA[2]

[1]Institute of Developmental Biology of the USSR Academy of Sciences, Moscow, USSR, [2]Balaton Limnological Research Institute of the Hungarian Academy of Sciences, Tihany, Hungary

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The freshwater snail L. stagnalis is known to be able to respond to a strong, noxious stimulus with a full retraction of the foot and head into the shell accompanied with expelling the blood through the hemal pore. We have found that this behavioural response, besides graded local retractions, can be triggered by mild tactile stimulation provided that the stimulus is repeated several times. Only a complete exsanguination could be obtained, indicating that it is an all-or-none defensive behaviour. In an electrophysiological investigation of isolated brain, a number of similarities were found between this all-or-none behaviour and the so-called input 3 to central neurons, as described by Benjamin and Winlow /3/. These include ability to be selectively activated by high calcium solutions, and blocked by keeping the snails in a spoiled water. Injection of snails with naloxone (0.5-2.0 μg/g) or ergotamine (0.4 μg/g) blocked selectively the whole body withdrawal induced by tactile stimulation, but not that induced by injection of a high calcium saline or acetylcholine solution, indicating that enkephalinergic and/or dopaminergic mechanosensory neurons might be involved. The consideration of available data has led to a working hypothesis that the activity of input 3 might be the neurophysiological correlate of the high threshold all-or-none whole body withdrawal associated with exsanguination.

Keywords: Pond snail, defensive behaviour, exsanguination, mechanosensory neurons, acetylcholine, ergotamine

Send offprint requests to: Dr. Katalin S.-Rózsa, Balaton Limnological Research Institute of the Hungarian Academy of Sciences, P.O.Box 35, H-8237 Tihany, Hungary

Akadémiai Kiadó, Budapest

Introduction

Gastropod snails exhibit a wide range of defensive behaviours in response to tactile and noxious stimulation, from graded local contraction of the affected part of the body to withdrawal of the entire body into the shell (see /1, 2/). In the pond snail Lymnaea stagnalis, a strong noxious stimulus can elicit withdrawal associated with exsanguination. Blood is expelled through the hemal pore located near the pneumostome /6/. It has been suggested that only after eliminating blood from haemocoel (and also air from the lung) can the animal fully retract and hence avoid attacking predators /1/.

This goal-oriented avoidance behaviour is hereinafter referred to as WIEX (WIthdrawal plus EXsanguination). WIEX has proved useful in obtaining samples of the snail blood for analysis (see, e.g. /9, 12/). As far as we know, however, neurobiologists have not paid attention to this form of defensive behaviour. The present paper reports on the results of a series of experiments attempting to approach the cellular analysis of WIEX. Our experimental strategy was as follows: we attempted, first, to find the conditions under which WIEX can be reliably induced or prevented in whole animals, and second, to examine the electrical activity of the neurons of the isolated CNS exhibiting similar responses to the above conditions. It was believed that the central program for WIEX should be generated by isolated CNS both spontaneously and in response to factors capable of inducing WIEX in the intact animal.

Material and Methods

Experiments were carried out mainly on juvenile specimens of Lymnaea stagnalis, weighing from 1.5-3.3 g, reared in the laboratory. Mature animals collected from ponds were also used. Snails were kept in dechlorinated tap water at room temperature (22-24 ^{0}C) and fed on lettuce. All observations were made in the months of October-December.

Injections were made with needle inserted through the foot and directed so that the tip lay in the vicinity of the central ganglia in the cerebro-pedal hemocoel. Gentle insertion of the needle did not induce WIEX in itself. Standard volume of injected solution was 0.05 ml for juvenile snails and 0.2 ml for adults.

To estimate the body fluid loss connected with WIEX, animals were carefully dried with filter paper and weighed prior to and after exsanguination. Samples of blood expelled during WIEX were collected and centrifuged for microscopical examination of the precipitate.

In neurophysiological experiments, the isolated central nervous system with the cerebral commissure cut was pinned so as to expose the neurons of the dorsal surface of the pedal ganglia and the visceral loop ganglia. Two neurons could be recorded simultaneously using intracellular microelectrodes. A conventional microelectrophysiological set-up was used to record and display electrical activity of the neurons. Current injection could be carried out via recording electrodes using bridge circuit inserted into the recording amplifiers.

The following substances were used: acetylcholine chloride (Sigma); ergotamine tartrate (Sigma); naloxone hydrochloride (Sigma).

Results

1. WIEX evoked by summation of mild tactile stimuli

As a standard non-noxious, mild tactile stimulus, a fine paint-brush was stroked along the sole. We have found that a series of such stimuli is effective in evoking WIEX.

The procedure was as follows. A snail held in the hand was repeatedly stroken by a brush from the posterior to the anterior end of the sole with a frequency of one brushing per second. The first stimulus evoked a transient local retraction of the foot. With repeated stimulation this response, typical of graded defensive withdrawal /1/, could gradually increase or decrease in size. However, at some unpredictable moment, the animal could abruptly change the graded retractions to a qualitatively different response: it expelled a fluid and withdrew deep into the shell for several minutes. This can be regarded a typical WIEX. Snails were qualified unresponsive if they failed to produce WIEX after 100 repetitions of the standard stimulus.

Using animals with partially removed shells, it could be seen that following WIEX, heart beat was inhibited and movement of the animal was completely eliminated. In several minutes, movements of the foot, head, lips and tentacles abruptly reappeared, heart beating became strong and fast, and general relaxation developed.

Change in weight due to fluid loss at WIEX was measured in a group of 15 snails. As a result of a single WIEX, the animals were found to lose 31.7±2.12% of their initial weight. The collected fluid was cnetrifuged. The precipitate, examined under light microscope, contained an uniform population of the cells corresponding to *Lymnaea* hemocytes /9/, while the supernatant was of a light-blue colour and slightly opalescent, indicating that the expelled fluid was the blood.

The animals of this group required less than 10 standard tactile stimuli for triggering WIEX, four snails required from 10 to 15 stimuli, and in one animal the threshold was achieved after 18 trials. This range of distribution was generally characteristic of snails maintained in fresh, well aerated water. However, keeping the animals in slightly spoiled water prevented them from producing WIEX though otherwise their behaviour looked normal. As a rule, all snails kept in a particular aquarium behaved similarly: they either exhibited WIEX in response to standard stimulation or they remained unresponsive.

WIEX could also be prevented pharmacologically. Naloxone, in a dose of 0.5 μg/g injected into an animal taken from the responsive group blocked WIEX for no less than 1 h; with 2 μg/g, naloxone made the animals unresponsive for more than 5 h. Injection of ergotamine tartrate had a similar effect; the dose of 0.4 μg/g blocking WIEX for more than 24 h. Control animals of the group, injected with the snail saline, required 15 brushing stimuli or less for triggering WIEX.

2. WIEX evoked by injection

As already mentioned, the injection procedure itself did not trigger WIEX, provided that the needle was sharp enough and its insertion was gentle. Injection of a diluted (25%) physiological saline or even distilled water - which is known to induce blood venting in land pulmonate slugs /8/ - did not evoke WIEX in *Lymnaea*.

Of a number of solutions tested, two were found highly reliable in triggering WIEX in *Lymnaea*: first, the physiological saline with elevated Ca^{2+} second, the acetylcholine (ACh). The high Ca^{2+} saline was unequivocally effective at 40 mM (10x normal) while, with 20 mM, WIEX was obtained only in one part of the animals. ACh diluted in the snail saline had an effect at $4x10^{4}$ M but was ineffective at $2x10^{-4}$ M. The $2x10^{-4}$ M solution of ACh was found to be ineffective even when its volume was 5 times higher than the standard.

Naloxone and ergotamine in doses being effective for blocking WIEX response to tactile stimulation did not prevent WIEX response to injection of ACh or 40 mM Ca^{2+}. Neither ACh nor high Ca^{2+} solution could, however, evoke WIEX in snails of the unresponsive group, i.e. the group kept in spoiled water and therefore being unable to respond to tactile stimulation with WIEX.

3. Neurophysiological correlates

Our working hypothesis to be tested was based on an observation found in the literature. In a paper dealing with spontaneous synaptic activity in the isolated CNS of L. stagnalis, Benjamin and Winlow /3/ have mentioned that the so-called input 3 may be initiated "by altering the bathing medium of the preparation" (p. 302). This notion is illustrated in the paper by a figure in which activity of input 3 had been evoked by changing the normal saline, containing 4 mM Ca^{2+}, to a high Ca^{2+} (20 mM) saline.

Input 3 is described as a very powerful synaptic activity generated by an unknown neuronal source and influencing many follower neurons (see also /14/). It appeared that input 3 might be the neurophysiological correlate of WIEX. The aim of our experiments on the preparation of isolated CNS of L. stagnalis was to check whether other similarities between input 3 and WIEX might also exist.

The large neuron RPeD1 of the right pedal ganglion was selected as a reference cell. This unpaired dopaminergic interneuron has a number of benefits. The activity of input 3 can be easily identified in the RPeD1 neuron due to characteristically grouped large EPSPs originating in relay neuron(s) /3, 7, 14/. Further, firing in RPeD1 may initiate the activity of input 3 by a presumably post-inhibitory rebound mechanism /3, 7/.

Altogether 44 preparations of the isolated CNS were investigated. Of them, 30 were made of responsive snails (they were kept in fresh water and responded with WIEX to tactile stimulation) and 14 of snails belonging to the unresponsive group. The two sorts of preparations are referred to as $WIEX^{+}$ and $WIEX^{-}$, respectively.

Spontaneous activity of input 3 could be found in $WIEX^{+}$ preparations. If it was absent, we could induce it by activation of the cell RPeD1, as it was in an experiment shown in Fig. 1A. In the figure, two bursts of large EPSPs can be seen, representing input 3 in this neuron. Adding 2-3 drops of high calcium saline (40 mM Ca^{2+}) to the bath led also invariably to the activation of input 3 in $WIEX^{+}$ preparations (Fig. 1B and C). Figure 1B shows

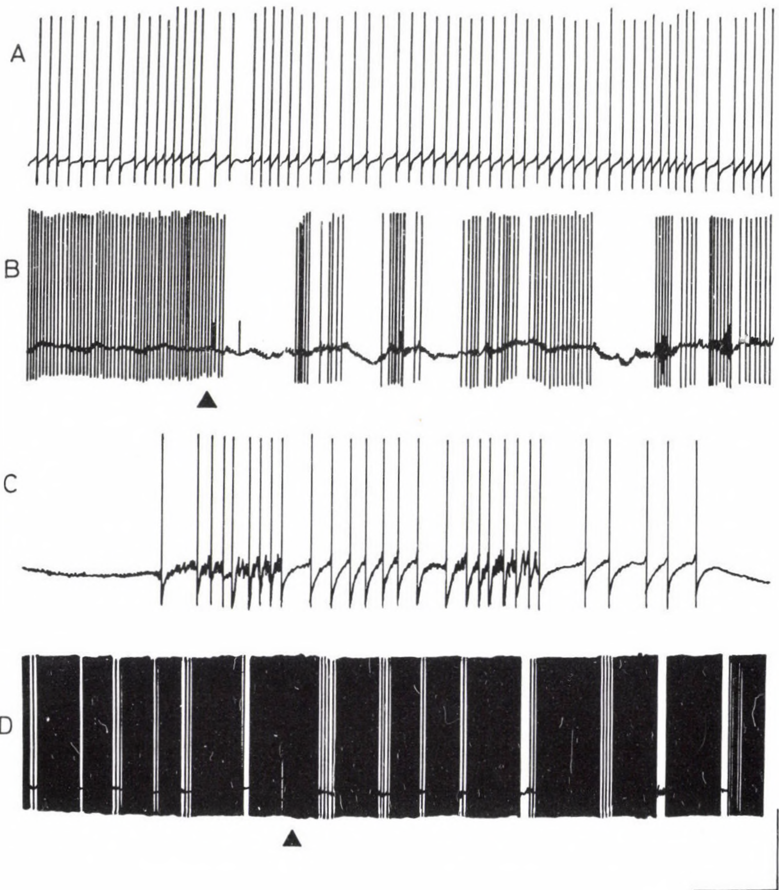

Fig. 1. Factors affecting the activity of input 3 in WIEX$^+$ (A,B,C) and WIEX$^-$ (D) preparations. All recordings from RPeD1 neruons.

A. Activity of input 3 induced by a preceding activation of RPeD1. Two discharges of input 3 are shown.

B. Induction of activity of input 3 by adding the high calcium saline (40 mM Ca^{2+}, arrow).

C. The same activity of input 3 as in B, at a higher speed. Two discharges of input 3 are shown.

D. Lack of induction of the input 3 activity by adding the high calcium saline (40 mM Ca^{2+}, arrow) in a WIEX$^-$ preparation. Note that background activity of RPeD1 did not change significantly after adding calcium.

Voltage calibration 20 mV. Time calibration (A, C) 6 s, (B, D) 24 s

that the initially tonic activity of the neuron RPeD1 turned to the bursting one due to the bursts of EPSPs evoked by the high Ca^{2+} saline. When 20 mM Ca^{2+} saline was similarly added, input 3 was either activated in some $WIEX^{+}$ preparations, or remained inactive in others. Both stimulation of RPeD1 and addition of 40 mM Ca^{2+} maintained their effectiveness on the input 3 in the presence of naloxone (0.5 μg/ml) and ergotamine (0.4 μg/ml).

$WIEX^{-}$ preparations behaved rather differently. Spontaneously active input 3 was never observed in them, though they generally had an intensive synaptic and spiking activity. Neither activation of RPeD1 nor addition of high Ca^{2+} saline could activate the input 3. Actually, only single large EPSPs appeared. These were, however, very rare and unable to affect significantly the initial background activity of the cell RPeD1, as it was in the experiment demonstrated in Fig. 1D.

Using RPeD1 as a reference cell for monitoring activity of input 3, it was not possible to study the effects of ACh on the activation of input 3, as ACh was found to exert a strong direct excitatory action on RPeD1.

4. Neurons related to input 3

Evidences presented in the foregoings indicate that input 3 behaves similarly to the behavioural WIEX. For this reason, additional studies on input 3 and its central connections were carried out.

Besides large amplitude EPSPs arriving sporadically, cell RPeD1 receives a continuous excitatory input of a smaller emplitude (Fig. 2A-C; see also /3/). We have found that the two excitatory inputs can be distinguished from each other in terms of the feed-back inhibition. Figure 2E shows that the generation of the large amplitude EPSPs is abolished for a short time after spike generation in RPeD1. This might mean that the spike inhibits the relay neuron of the input 3. There is no such inhibition in case of the continuous excitatory input (Fig. 2C).

In the experiment shown in Fig. 2E, the level of Ca^{2+} in the bath was elevated, and therefore the amplitude and duration of EPSPs (and thence the amount of released transmitter) were increased. At the same time, the cell RPeD1 was maintained at a slightly hpyerpolarized level to make its firing rare. These conditions allowed the feedback inhibition of the relay neuron to be observed in a well pronounced form. The inhibitory effect of RPeD1 on the relay neuron of input 3 was not so well seen if the firing had

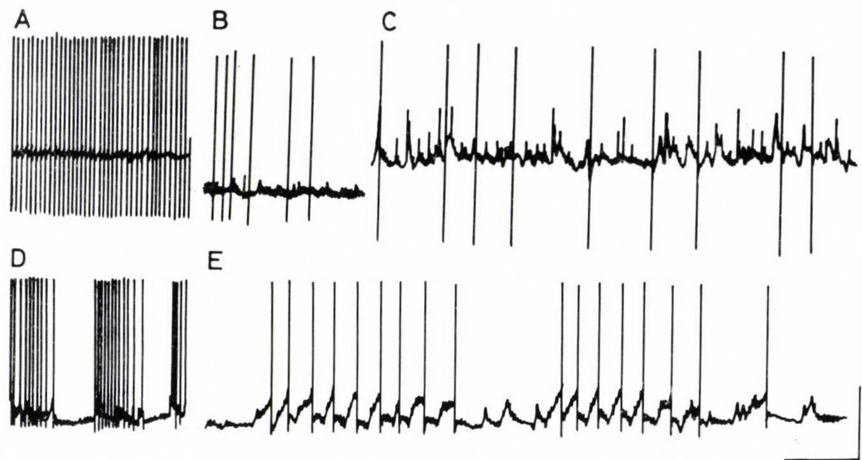

Fig. 2. Two excitatory synaptic inputs to RPeD1 distinguishable in terms of feed-back inhibition.
A. Background activity of RPeD1.
B. The same cell at a hyperpolarized level of membrane potential. Continuous excitatory bombardment is seen.
C. As in B, at a higher speed and amplification. Note that only a portion of action potentials is shown.
D. Background activity of a slightly hyperpolarized RPeD1. The activity is underlain by sporadic discharges of input 3.
E. As in D, at a higher speed. Note that generation of the high amplitude EPSPs is stopped for some time by a preceding action potential. There is no such inhibition in C.
Voltage calibration (A, B) 40 mV, (C) 10 mV, (D. E) 20 mV.
Time calibration (A, D) 24 s, (B) 48 s, (C, E) 6 s.

a higher frequency, as in Fig. 1A and C. It seems possible that a sort of desensitization may modify this inhibitory transmission.

Another central connection related to input 3 is illustrated in Fig. 3. In this experiment, activity of input 3 induced by a high Ca^{2+} saline (A, arrow) was recorded from the cell RPeD1 (upper trace) while the second recording was made from a neuron of the right pleural ganglion. Figure 3B demonstrates a regular character of the bursting activity in RPeD1 detrmined by regularly repeated activity of input 3. Intracellular stimulation of the pleural neuron via bridge scheme led to the temporary cessation of bursts in RPeD1. It appears that the pleural neuron may somehow affect the input 3 generation.

The pleural neuron shown in Fig. 3 is a member of the group of rather large cells localized close to the pleuro-parietal connective.

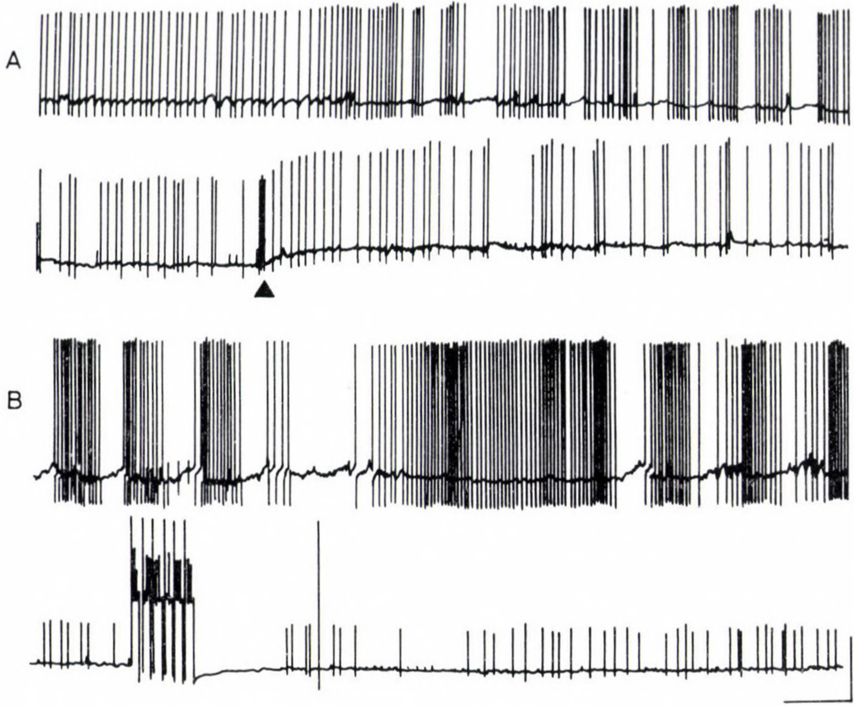

Fig. 3. The effect of activation of a pleural neuron (lower trace) on the occurrence of input 3 in RPeD1 (upper trace). A WIEX^{+} preparation.

A. Induction of the input 3 activity by adding the high calcium saline (40 mM Ca^{2+}, arrow). The input 3 activity can be seen in RPeD1, but not in the pleural neuron.

B. The same experiment, one minute later. A series of short bursts in the pleural neuron results in a transient elimination of the input 3-underlain periodic activity in RPeD1.

Voltage calibration (A upper, B lower) 20 mV, (A lower, B upper) 10 mV. Time calibration 24 s

Neurons of this cluster usually generate action potentials of varying amplitudes, similarly to another cluster of pleural neurons localized near the pleuro-pedal connective (see /5/). We have found that these pleural neurons are, on their turn, under inhibitory control of a previously unknown interneuron of the left parietal ganglion. This cell was labelled LPaIntl is a rather large cell lying between the root of the parieto-visceral connective and that of the left mantle nerve. In Fig. 4C inhibition of a pleural neuron can be seen caused by insertion of microelectrode into LPaIntl. In Fig. 4D inhibitory effects of discharge in the LPaIntl on another cell of the pleural cluster are shown. It can be seen that although

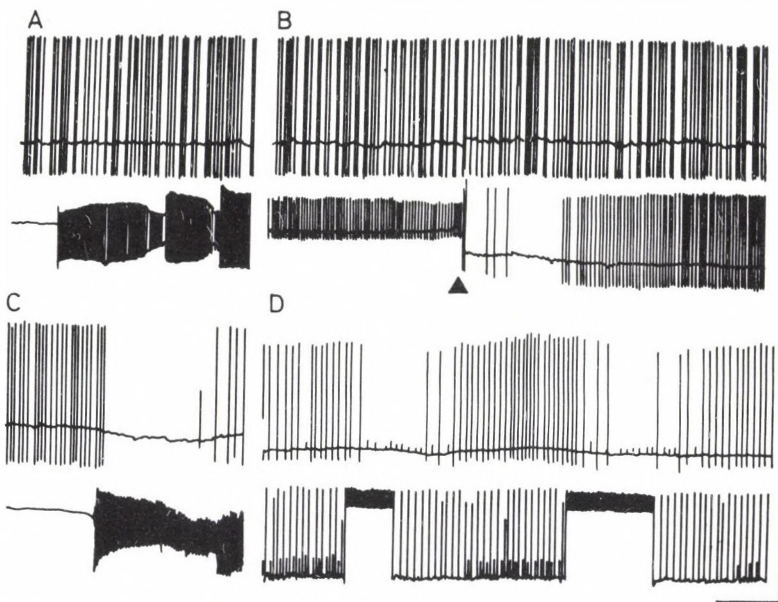

Fig. 4. The left parietal interneuron LPaInt 1.
A. Simultaneous recording from RPeD1 (upper) and LPaInt1 (lower, the moment of insertion is shown). Note a variable character of the activity in LPaInt1. A WIEX$^-$ preparation.
B. The same cells as in A, two minutes later. Lack of induction of the input 3 activity by adding the high calcium saline (20 mM CA^{2+}, arrow). Note a strong effect of Ca^{2+} on LPaInt1.
C. Simultaneous recording from a pleural neuron (upper) and LPaInt1 (lower, the moment of insertion is shwon). Discharging of LPaInt1 inhibits the activity of the pleural cell.
D. Simultaneous recording from another pleural neuron (upper) and the same LPaInt1, as in C (lower). Activation of LPaInt1 results in inhibition of the higher spike generation in the pleural cell.
Voltage calibration (A, B, D upper) 40 mV, (C upper) 20 mV, (A, B, C, D lower) 10 mV.
Time calibration 24 s

the generation of large amplitude action potentials was inhibited, a low amplitude activity of unidentified nature appeared in the postsynaptic neuron as a result of activity in the LPaInt1.

Electrical activity of the LPaInt1 is highly unstable with respect to its frequency and amplitude (Fig. 4A). A peculiar feature of this cell is that its activity changes dramatically if a high Ca^{2+} saline is added to the bath (Fig. 4B). It appears, therefore, that this neuron may represent a population of neurons responsible for triggering the CNS response to high Ca^{2+}.

DISCUSSION

Although the ability of the pond snail to expel its blood in the process of full body retraction was known for years, the neurobiology of this interesting behaviour referred to here as WIEX remained unstudied. Our experiments seem to represent the first attempt to investigate WIEX and its neurophysiological basis.

Our results allow the suggestion that WIEX is an all-or-none behaviour. This suggestion is based mainly on two observations. First, the loss of blood was maximal although withdrawal was evoked by mild stimulation. Indeed, the total weight of blood in *L. stagnalis*, as measured by two independent methods including exsanguination caused by a noxious stimulus, is about 34% of the body weight /12/. In our experiments, a nearly similar value of loss in body weight was obtained as a result of body retraction caused by a mild tactile stimulation. This implies that in *L. stagnalis* only a complete autoexsanguination may take place, and there is no graded "blood venting" similar to that described in land slugs /8/. Second, the motor component of WIEX is obviously distinct from graded withdrawal responses in that it is a full and long-lasting retraction. We may thus conclude that - along with the low threshold graded withdrawal - a qualitatively different, high threshold and all-or-none avoidance behaviour exists in the pond snail.

The two types of defensive withdrawal are distinguishable in terms of pharmacology: naloxone or ergotamine were shown to effectively prevent the WIEX response to tactile stimulation, while the graded withdrawal responses were unaffected. It would be premature to go deep into the interpretation of this finding. Both dopamine and enkephalins function as transmitters of mechanosensory neurons in other invertebrates /4, 10, 11, 13/. In this connection, it seems natural that pharmacological antagonists of these transmitters might prevent a specific behaviour induced by tactile stimulation.

The behaviour of input 3 in the preparation of isolated CNS closely paralleled WIEX in our experiments. The list of similarities includes - besides parallel responses to 20 and 40 mM Ca^{2+} - the same specific sensitivity to keeping the animals in spoiled water. It can be added that the high calcium saline effectively evoked both WIEX and activity of input 3 in the presence of naloxone or ergotamine. Actually, we did not observe any

differences between the two events as far as their responses to changing conditions are concerned.

An obvious distinction is that input 3 is a sporadically repeated event while WIEX is a single, though long-lasting motor action. The long-lasting contraction of muscular organs may, however, be maintained by sporadic bursts in motor neurons and, if so, then the distinction is without a difference.

In summary, the obtained results seem to lend further support to the hypothesis that casually connects the specific defensive behaviour, WIEX, with activity of input 3. In this connection, newly found neurons related to input 3 may be of interest. It will of course be necessary to investigate the input 3 activity during all-or-none retraction in semi-intact preparation before the relation of input 3 generator to defensive exsanguination can be conclusively established.

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CORTICAL POTENTIALS EVOKED BY MECHANICAL STIMULATION OF DIFFERENT NUMBER OF VIBRISSAE OF THE RAT

A. ANTAL, J. TOLDI

Department of Comparative Physiology, József Attila University, Szeged, Hungary

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Distribution maps of cortical potentials evoked by mechanical stimulation of different number of contralateral vibrissae were studied. It was found that stimulation of all the contralateral vibrissae led to more extensive activation than the barrel field in the somatosensory cortex. The activation was most widespread when all the vibrissae were synchronously deflected. With reduction of the number of synchronously stimulated whiskers the activated cortical area did not decrease in parallel. Deflection of only a few whiskers activated significantly smaller cortical areas.

Keywords: Vibrissa stimulation, barrel field

Introduction

The central sensory system of vibrissa projection in rat has become one of the most fascinating themes in the last decade. This is probably because of the unique anatomical feature, the postero-medial barrel subfield of the somatosensory cortex in these animals /17, 18/. Welker has proposed "one whisker-to-one barrel" scheme in her original electrophysiological study /14/. However, it has been somewhat modified by subsequent investigators, who have found some convergence from more than one whisker /8, 10/. In spite of this convergence, the discrete nature of each barrel is obvious; all neurons within a barrel respond optimally to deflections of a specific whisker /10/. Detailed studies have been published about the quantitative

Send offprint requests to: Dr. J. Toldi, Department of Comparative Physiology, József Attila University, P.O.Box 533, H-6701 Szeged, Hungary

aspects of vibrissa-driven neuronal responses of barrels /3, 5/. However, in earlier studies we observed, that electrical stimulation of the skin of the vibrissa pad and also the mechanical stimulation of the vibrissae evoked cortical responses not only within the barrel subfield of somatosensory cortex, but in more widespread cortical areas /1, 13/. We are not aware of any studies analyzing the distribution of cortical potentials evoked by deflection of different number of vibrissae. The present electrophysiological analysis was undertaken in order to clarify the following issues: (i) Does the deflection of all whiskers evoke potentials in the most widespread cortical areas? (ii) If so, is there any correlation between the number of stimulated vibrissae and the extension of the activated cortical area? (iii) Is there any difference in the latencies of evoked potentials recorded in the barrel field and beyond it? (iv) Could be observed any directional dependence of whisker deflections on cortical evoked potentials, or does it exist only at unitary level (as published in detail e.g. by Simons, /8/ and Ito, /3/).

These questions are particularly exciting taking into account that the vibrissae of rodents play an outstanding role in their orientational behaviour. Moreover, under "natural circumstances" not only a single whisker is stimulated e.g. deflected separately, as usually studied in the laboratory, but all, or most of them. Therefore, we decided to explore the effects of multiwhisker stimulation on the cortical evoked potentials.

Material and Methods

In the experiments 27 adult Sprague-Dawley (CFY) albino rats weighing 300-350 g were used. Rats were anaesthetised with i.p. injections of chloralose-urethane (770/110 mg/kg). After tracheal cannulation the head was fixed in a stereotaxic frame, the left hemisphere was exposed and the dura was removed. In order to prevent cooling and drying, the cortex was covered with warm paraffin oil saturated with physiological saline. After surgery, the animals were left at rest for 1.5-2 h. The body temperature was maintained at 37 ^{o}C. The mechanical whisker stimulation was performed on the right side with the aid of a MECHANOSTIM, developed by our engineer. The stimulator consisted of an oscillator attached to an electromagnete driven by a servo amplifier. The shaking movements to the whiskers were transmitted by a spoon-shaped wire net (Fig. 1). The movements of the

MECHANOSTIM were recorded on one beam of the oscilloscope. Various stimulus waveforms (e.g. sinusoids, trapezoids, triangles) were available from the generator. With the aid of the system mentioned above, the actual parameters of deflections: amplitude, duration, slope of onset and offset could be adjusted. The whiskers were cut to a length of 12-13 mm and all of them were contacted with the net. The distance between the body surface and the net was 7-8 mm. In a part of experiments reduced number of whiskers were stimulated (half of them; fifteen, or less and in other cases a single hair, respectively). In these cases we used trapezoidal waveform of deflection with the following parameters: amplitude: (in most cases from the resting position of the whisker to caudal direction): 1.2 mm, duration: 400-800 ms, slope: 30-50 ms. Mapping of evoked potentials was carried out in a stereotaxic frame, with the aid of a Narishige micromanipulator, under microscopic control. Ball-tipped silver-wire electrodes of 0.3 mm diameter served for recording of evoked potentials. Groups of 10 evoked responses were averaged with the aid of a MOTOROLA MC-6800 computer and drawn by an X-Y plotter. The maps were evaluated in the following manner. The amplitude of positive phase of the averaged potential recorded at the punctum maximum, was taken as 100% and the positive phases of evoked potentials recorded from other sites were expressed as percents of it. Discharges of single units were recorded extracellularly, via tungsten microelectrodes (A.M.

Fig. 1. The spoon-shaped net to stimulate the vibrissae. Vibrissae were cut to a length of 12-13 mm and were positioned into the net

System, Inc.) advanced by a Narishige hydraulic micromanipulator. Photos were taken from the screen of a Tektronix storage oscilloscope. Post-stimulus time histograms (PSTHs) were made by the same MOTOROLA computer. Other details of stimulation, recording and mapping of evoked responses have been described in detail /12/.

Results

Mechanical vibrissa stimulation evoked potentials in widespread cortical areas of the contralateral hemisphere. On-off responses with larger positive phase (peak latency: 17-18 ms) followed by small negative deflection, were recorded in the barrel field (Fig. 2A, B). In simultaneous microelectrode record the majority of reactive units showed on (-off) effects (Fig. 2C, D, E). The response latencies ranged from 10 to 17 ms. In a few cases sustained activation was also observed, while some neurons reacted with direct supression of activity. However, here we focus on the cortical maps of gross potentials evoked by deflection of different number of vibrissae. Interestingly enough, reducing the number of synchronously stimulated whiskers, the activated cortical area from which evoked potentials could be recorded, did not decrease in parallel (Fig. 3a, b, c). Only responses to deflection of a single or two whiskers, were restricted to the barrel field (Fig. 3d). In some cases larger activated cortical areas were observed when about half of the contralateral vibrissae were deflected, while the synchronous stimulation of all the contralateral vibrissae (about 35), resulted in a similar, even smaller activated cortical areas (Fig. 3a, b). However, the deflection of only five contralateral vibrissae, activated similarly extended cortical field (Fig. 3c). Generally, it can be said that synchronous deflection of different number of contralateral vibrissae evoked potentials in rather widen cortical areas, (without significant differences) involving not only the barrel field, but the whole somato-motor cortex, including even the frontal region, too (Fig. 3). It seemed interesting to examine the latencies of evoked potentials (positive peak latency) especially recorded beyond the barrel field (Fig. 4). While, within the focus of evoked activity (in the barrel field) the latencies were about 17-18 ms, at some mm apart (in SII area and motor cortex) they went up to 20-30 ms, or even exceeded 30 ms, in the frontal region (Fig. 4a-c).

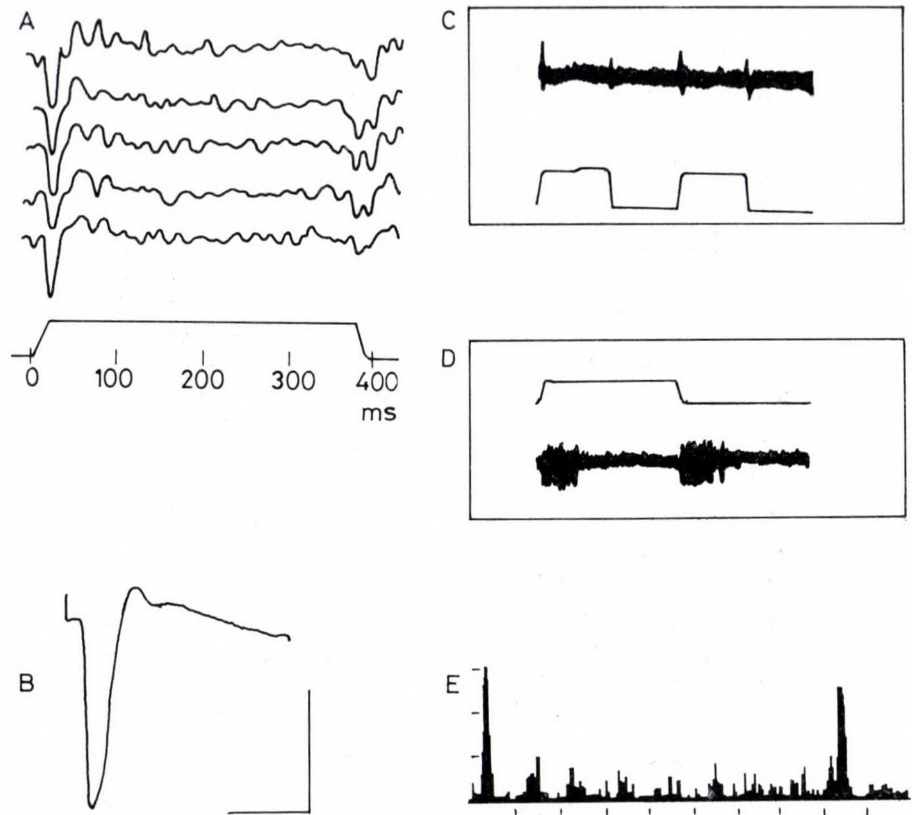

Fig. 2. Evoked activites recorded in the barrel field. A: successively averaged (on-off) potentials evoked by deflections of a single whisker. Potentials were recorded at the punctum maximum, at coordinates: -3; 5. B: averaged potential evoked by synchronous deflection of all the contralateral vibrissae. Recording was made in coordinates: -3; 5.5. Calibration: 30 ms, 200 µV. C: unit responses in the barrel field activated by deflection of a single whisker. D: burst-like responses of a neuron (population?) evoked by synchronous deflection of all the contralateral whiskers. It has to be noted that single spike responses were also found in case of synchronous deflection of all the contralateral whiskers, and in other cases burst-like phasic responses could also be evoked by single hair deflection. E: post-stimulus time histogram (PSTH) of a neuronal activity recorded in the barrel field, evoked by contralateral multiwhisker deflection. Abscissa: 100 ms/dots, ordinates: 50 spikes/division. Vibrissa deflections in all cases (A-E) happened in posterioral direction with 1.2 mm amplitudes and in the following durations: 400 ms (A), 520 ms (C,D) and 840 ms (E)

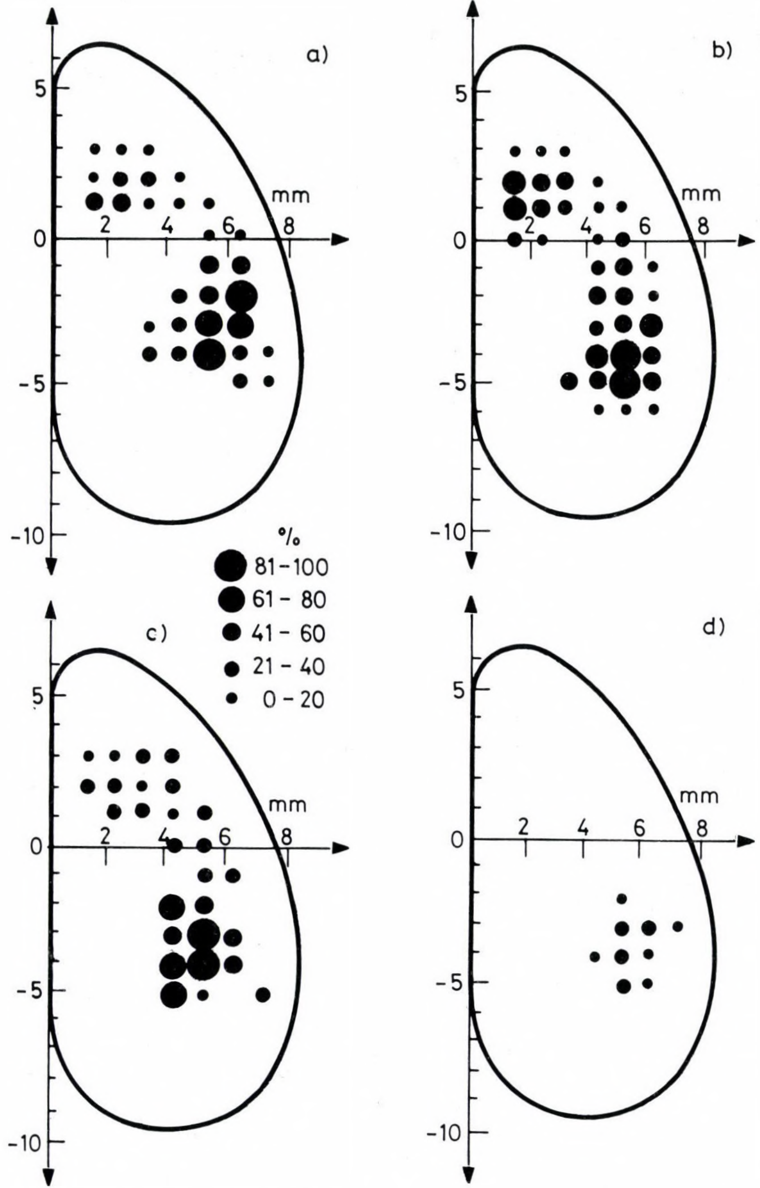

Fig. 3. Activated cortical sites by synchronous deflection of all contralateral vibrissae (a), by deflection of half of the contralateral vibrissae (b), by stimulation of five whiskers (c) and by stimulation of a single whisker (d). Amplitudes are epxressed in % of maximal response recorded in the experiments

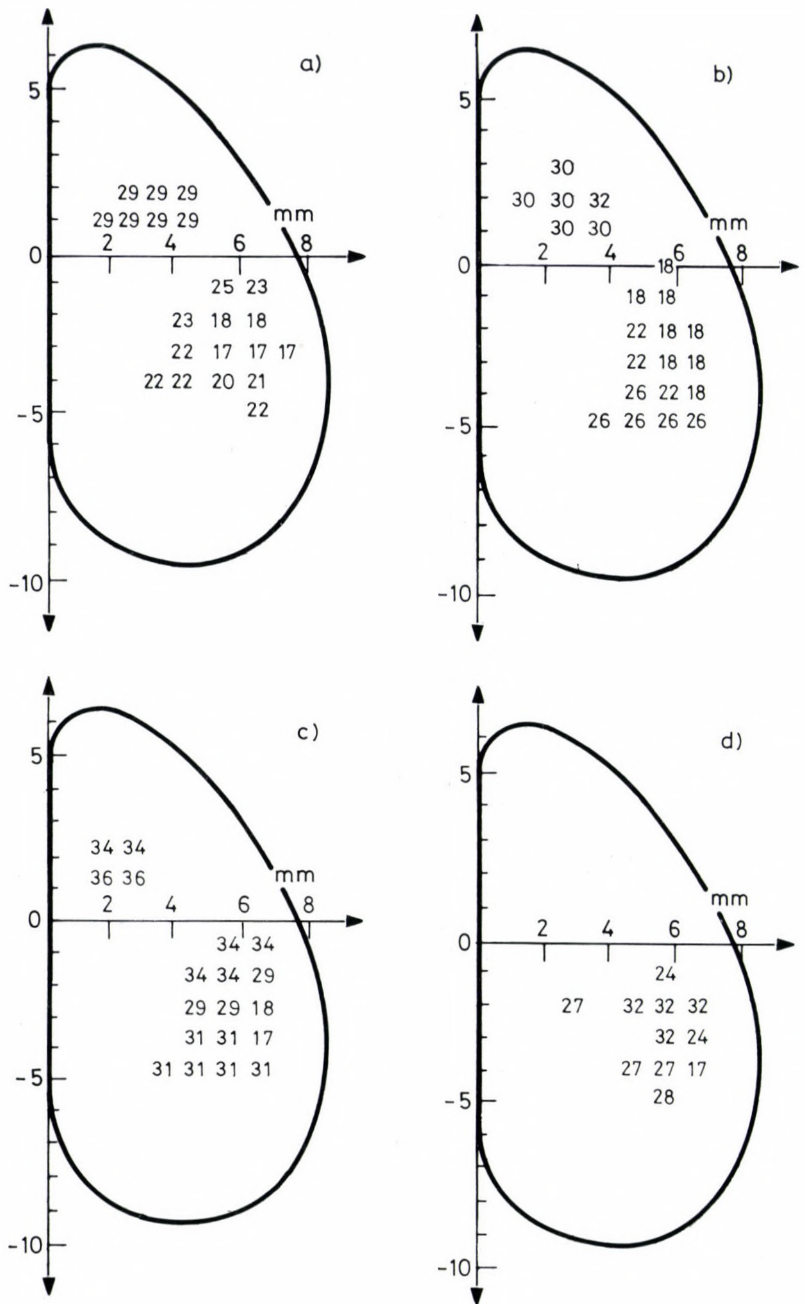

Fig. 4. Latency maps of potentials (positive peak latencies in ms) recorded throughout the hemisphere evoked by deflection of all the contralateral vibrissae (a), by deflection of half of the vibrissae (b), by stimulation of four vibrissae (c) and by stimulation of a single vibrissa (d)

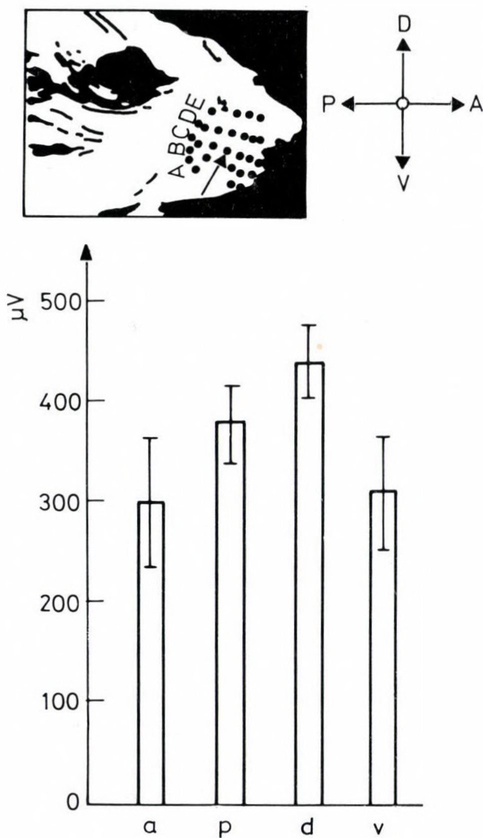

Fig. 5. Averaged amplitudes of cortical potentials (with S.D.) evoked by anterior (a), posterior (p), dorsal (d), and ventral (v) deflection of C3 whisker (arrow in the insert). Potentials were recorded in the barrel field, at coordinates -4, 5 (n=10).

The response properties of directionally sensitive units in barrels were studied in detail /3, 4, 5/. However, the question has remained, if there exists any directional sensitivity of evoked potentials, too? If so, is there any difference in this respect between potentials recorded within the barrel field and beyond it? As it was expected, the synchronous deflection of all (or most of the) contralateral vibrissae did not show any directional sensitivity neither in barrel cortex nor beyond it. The same was obtained in all cases when more than one hair was stimulated. However, in some cases, in the barrel field, significant amplitude differences were found at deflections of whiskers in different directions (Fig. 5). It was found e.g. that deflecting C3 whisker (with the same parameters) in anterior-, pos-

terior-, dorsal-, and ventral directions, potentials with different amplitudes appeared at coordinates -3; 5. Potentials evoked by dorsal deflection of C3 whisker were significantly larger than by other directions (Fig. 5). However, in most cases this kind of directional sensitivity could not be observed (at least they were not significant) neither in the barrel field nor beyond it.

Discussion

The first question to be discussed may be, why did we use this unusual way of stimulation, namely, the deflection of all or more than one whisker? In most of electrophysiological studies of this sensory system (cited above) the deflection of a single whisker was usual, while the neuronal responses were delineated with microelectrode recording. However, under "natural circumstances" a separate stimulation of a single whisker is hardly imaginable. On the other hand, this sensory system is exceptionally important in the behaviour of rodents /2, 7, 11, 15/. Taking together, it seemed to be interesting to study the effect of synchronous stimulation of more than one whisker. In earlier studies it was found that electrical stimulation of whisker pad evoked potentials not only in the barrel region of somatosensory I (SI) cortex, but nearly all over the hemisphere, contralateral to the stimulation /1/. Similarly, extensive activity was found beyond limits of SI cortex, in case of synchronous multiwhisker stimulation /13/.

First, we tested the effect of multiwhisker stimulation on the vibrissa units in the barrel cortex (Fig. 2). The latencies, time courses, and other properties of responses were similar to those published by Simons /8, 9/. Mapping of the gross potentials evoked by multiwhisker stimulation pointed to more extensive activated cortical areas than the barrel field in the SI cortex (Fig. 3). Interestingly enough, reducing the number of stimulated whiskers, the activated cortical area did not shrink significantly, with exception for cases when only one or two whiskers were deflected (Fig. 3). As compared to the "one whisker- to one barrel" scheme /14/, these results are at variance. Is there any anatomical basis of this extremely extensive cortical activity, or it reflects only some kind of passive spread of potentials? Anyway, the latency maps of evoked potentials recorded in the hemisphere oppose the passive spread. Melzer et al. have found a

more extended labelling of the cortex in a deoxyglucose study, when they stimulated with the "highest intensity" /6/. Recently, Welker et al., reported on the organization of the feed forward projections of the barrel cortex, using iontophoretic injections of Phaseolus vulgaris leucoagglutinin. They have found extended feedforward (and also feed back) projection of barrel cortex which involved e.g. the motor cortex, the SII cortex, the perirhinal cortex, etc. /16/. It seems probable that synchronous deflection of all (or more than one) contralateral whiskers, can activate throughout the "whisker-to-barrel pathway" and through a further feed forward cortico-cortical system, a more extended cortical area than the barrel cortex itself. Furthermore, our results suggest that this extensive cortical system can be activated by more than a threshold number of stimulated whiskers (Fig. 3).

Although, in a part of experiments directional sensitivity could be detected (Fig. 5), the evoked potential method does not seem the most adequate method for studying it. However, it has to be noted that, in these experiments we did not study the dependence of amplitudes of the evoked potentials on the stimulus intensity.

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THE RELATIONSHIP OF MEMBRANE FLUIDITY TO GROWTH AND NUCLEAR CYCLE IN MITOGEN STIMULATED LYMPHOCYTES

L. PAJOR, E. KÁLMÁN and T. KŐSZEGI

Department of Pathology and Clinical Chemistry, University Medical School, Pécs, Hungary

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Fluorescence polarization (P) and anisotropy (r_s) of the diphenyl-hexatriene labeled, stimulated human lymphocytes were followed in relation to cell cycle progression. Fluorescence anisotropy is proportional to the structural order parameter of the apolar lipid region, thus, indicative of the membrane fluidity. Cell cycle progression was defined by monitoring the RNA, protein and DNA synthesis as well as the expression of interleukin 2 and transferrin receptors. Cellular growth in size was also measured. A decrease in P and r_s values was seen when the RNA and protein synthesis as well as the density of growth factor receptors and cellular volume increased. At the same time no DNA synthesizing cell was detected. The low P and r_s values remained unaltered throughout the cycle, thus proving to be independent from cell proliferation (DNA synthesis) and from further progressive increase in cellular volume. The data indicate that the structural change in the membranes is an early event of cell activation and occurs in the course of G_0-G_1 transition as part of the growth cycle.

Keywords: Membrane fluidity, growth/nuclear cycle

Introduction

It has been demonstrated both in tumour and reactive proliferating cells that membrane fluidity (reciprocal of lipid structural order) is related to cell proliferation although their exact relationship remains unclear /4, 8, 22/. There are conflicting data on the issue whether increased membrane fluidity is a prerequisite of or only accompanies cellular proliferation. Determining this would be of great significance because of the

Send offprint requests to Dr. L. Pajor, Department of Pathology, University Medical School of Pécs, H-7643 Pécs, P.O.Box 99, Hungary

intimate relationship of the structural order of membrane lipids to the position and functional activity of membrane embedded and membrane related cytoskeletal proteins /5, 7, 14, 21/. Furthermore, changes in fluidity induced by artificial modification of membranes can expose or mask surface proteins of receptor functions /20, 24/. Thus, one may also speculate about the putative regulatory role of membrane fluidity changes in the cell cycle phase specific expression of activation-proliferation related surface proteins.

The aim of the present study was to relate the alterations in lipid structural order to the cell cycle phases in mitogen induced human lymphocytes. For this the steady state fluorescence polarization of the diphenyl hexatriene (DPH) labeled cells and the onset of protein, RNA and DNA synthesis as well as the expression of interleukin 2 and transferrin receptors (IL-2rs and tr-rs) were followed in stimulated cells. Furthermore, the fluorescence polarization data were correlated to cellular growth in size. The follow-up of these cellular parameters enabled us to distinguish the cell cycle phases, thus to establish that the restructuring of the membrane is not associated with cellular proliferation (entry into the S phase), but seems to be an early event of cellular growth.

Materials and Methods

1. Materials

RPMI 1640 and L-glutamine were purchased from Flow laboratories; Ficoll-Paque and percoll were from Pharmacia; α-methyl-mannoside (α-MM), bovine serum albumin (BSA), sulphorhodamine 101 (SR 101), bisbenzimide trihydrochloride (Hoechst 33342) and propidium iodide (PI) were from Sigma; 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), bisbenzimide trihydrochloride (Hoechst 33258), diphenyl hexatriene (DPH) were from Serva. (^{3}H) uridine, (^{3}H)thymidine, FITC-labeled streptavidin adn antimouse-immunglobulin biotin labeled were from Amersham; FITC labeled antitransferrin receptor monclonal antibody was from Becton-Dickinson; anti-interleukin 2 receptor monoclonal antibody OKT26a was from Ortho. Fetal calf serum (FCS) was purchased from Human; phytohemagglutinin type V (PHA) was from Difco; Triton-X-100 was from Reanal.

2. Cell culture and stimulation

Mononuclear cells obtained by Ficoll-Paque densit gradient centrifugation of blood samples from healthy donors were seeded in 10 ml plastic dishes at a cell density of $5x10^5$ cells/ml RPMI 1640 supplemented with 4.3 µm L-glutamine, 2 mg/ml sodium-bicarbonate, 10% heat inactivated (56 ^oC, 30 min) fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, at 37 ^oC, in a CO_2 thermostat (5% CO_2 in air, humified atmosphere). Stimulation experiments were carried out in the presence of 3 µg PHA/ml. The optimal concentration of the mitogen was determined by (^3H)thymidine uptake. One hour before cell harvesting α-MM (20 mM) and EDTA (5mM) was added to the cultures. Treating the cells with these compounds we eliminated cell agglutinates and a single cell suspension was obtained without any mechanical agitation. The harvested cells were layered onto 30% Percoll, centrifuged for 10 minutes with 1200 rpm, washed with saline, and picked up either in isosmotic sodium chloride or in PBS containing 2% BSA.

3. Determination of RNA and DNA synthesis

Cells cultured in 96 well, flat-bottomed microplates at a cell concentration of $5x10^5$ cells/ml in the presence or absence of mitogen were pulse labeled either with (^3H)uridine (10 µCi/mM) or (^3H)thymidine (10 µCi/ml, 25mCi/µM) for 4 h. The radioactivity of acid insoluble precipitate was measured in a Beckman LS 203 liquid scintillation spectrometer /1/.

4. Flow cytometric measurements

Measurements were carried out in a PAS II flow cytometer (Partec, Münster) using mercury arc lamp illumination.

Measurements of protein versus DNA: The cell samples were fixed in cold 70% ethanol. A given aliquot of the stored sample was washed in physiological saline. The centrifuged cells were dispersed in one part of pepsin solution (0.5% pepsin, pH 1.8) for 5 minutes at room temperature and stained with nine parts of DAPI-SR 101 solution (1.24 µg/ml DAPI, 7.5 µg/ml SP 101 in Tris buffer). Excitation was carried out by UV light (G1 filter). The emitted light was split at 560 nm. For the SR 101 derived fluorescence an OG590 long pass filter was used, whereas for the DAPI derived fluorescence an OG515 long pass filter was used.

Expression of tr-r and IL-2-r: for detection of tr-r, 10^6 cells in PBS containing 2% BSA were incubated with 20 times diluted, FITC labeled

anti tr-r monoclonal antibody. For staining the IL-2-r 10^6 cells were incubated with unlabeled IL-2-r specific monoclonal antibody OKT26a (1:100) followed by incubation with biotinated anti-mouse-immunoglobulin (1:100) finally with FITC labeled streptavidin (1:400). Each incubation step was carried out on ice for 45 min . The washed cells were then fixed in 50% methanol for at least 30 min. The fixed cells were centrifuged and stained with PI at a final concentration of 50 μg/ml TRIS buffer. Excitation was carried out by means of an EX488 interference filter. The fluorescence light was split at 560 nm, for PI derived fluorescence an OG590 long pass Schott filter was used, while for the FITC derived fluoresence an GO515 long pass Schott filter was used.

Measurement of cell volume: In this study stimulated lymphocytes and their unstimulated counterparts were investigated after 24 and 72 h. In addition to α-MM and EDTA, Hoechst 33342 (2.5 μM) was also added to these cultures for the last 1 h of the cultivation. Staining the cells with this dye at this molar concentration was shown to be informative about the cell membrane permeability, thus, e.g. activated lymphocytes could be identified as early as 12 h after initiating the culture /12/. The cultures were prepared as described above, the washed samples were kept at 4 °C and analyzed within 60 minutes. The measurements were carried out in a Coulter chamber. Simultaneous detection of Hoechst 33342 fluorescence was carried out using UV excitation (UG1 filter) and OG515 Schott barrier filter. The cell volume parameter was gated by means of Hoechst 33342 derived fluorescence.

Cell cycle analysis: For cell cycle analysis, human peripheral blood lymphocytes were cultured in 24 well, flat-bottomed plates and stimulated as above. The cells were fixed in 1% paraformaldehyde-PBS for 30 minutes. DNA staining was with one part of fixative containing 10^6 cells diluted with one part of 1 mg Hoechst 33258/ml PBS containing 0.1% Triton-X-100. After 30 min at room temperature the samples were filtered through a nylon mesh of 50 μm diameter. Measurements carried out in PAS II flow cytometer using a UG1 UV excitation filter and an OG515 Schott filter. The percentage of cells in different cell cycle phases was calculated by means of the cell cycle analysis program distributed by the manufacturer.

5. Fluorescence polarization measurements

A given volume of 2×10^{-3} M DPH in tetrahydrofuran (stock solution) was diluted 500 times in Hank's solution (without Ca^{++} and Mg^{++}) while stirring vigorously for 40 min . The cell suspension (2×10^6/ml) were

labeled with an equal volume of DPH suspension for 40 min at 25 ^{o}C. Steady state fluorescence polarization measurements were performed using a Hitachi-Perkin-Elmer fluorescence spectrophotometer 203 with a thermostatically controlled sample holder; a correction factor (0.91) was taken into account /10/. The measurements were carried out at 25 ^{o}C. The exitation light was 365 nm, the emission was measured at 430 nm. From the data obtained, the degree of fluorescence polarization (P) and the fluorescence anisotropy (r_s) was calculated according to the equations 1 and 2 /19/.

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (1) \qquad r_s = \frac{2P}{3 - P} \quad (2)$$

where $I_{\perp}$ and $I_{\parallel}$ mean the intensity of the emitted light at perpendicular and parallel positions of the analyzer to the polarizer. The viability controlled by the Trypan blue exclusion test, remained 95% during the whole procedure.

Results

Table 1 shows the fluorescence polarization (P) and anisotropy (r_s) data obtained from freshly isolated peripheral blood mononuclear cells and from those stimulated for 24 and 72 h. Stimulated cells exhibited a lower degree of fluorescence polarization and anisotropy as compared to resting lymphocytes. Althought there was some decrease in these values with increasing duration of stimulation, highly significant ($p < 0.001$) change was already seen after 24 h of stimulation. We have also investigated the possibility whether the culturing per se and not the stimulant was responsible for these changes. Despite some decrease in fluorescence polarization values, this possibility could be ruled out because cells cultured for 24 h in the absence of mitogen proved to be significantly different ($p < 0.002$) from their stimulated counterparts (not shown).

The changes in fluorescence polarization and anisotropy were related to time course of macromolecular synthesis and the expression of surface receptors in stimulated cells. Figure 1 shows the time course of RNA and DNA synthesis in function of time whereas the induction of protein synthesis and expression of IL-2 and tr receptors are demonstrated in Figs 2a-f. These

Table 1

Fluorescence polarization (P) and anisotropy (r_s) data of resting (0 h) and stimulated (24 h, 72 h) lymphocytes

	0 h	24 h	72 h
P	0.2447	0.2277	0.2301
	0.2637	0.1970	0.2205
	0.2614	0.2369	0.1880
	0.2456	0.2371	0.2067
	0.2575	0.2000	0.2052
	n.d.	0.2210	0.2234
$\bar{X} \pm$ S.D.	0.2546± 0.0080	0.2220± 0.0180	0.2123± 0.0150
r_s	0.1776	0.1643	0.1661
	0.1928	0.1406	0.1587
	0.1908	0.1715	0.1337
	0.1783	0.1716	0.1480
	0.1878	0.1429	0.1468
	n.d.	0.1591	0.1609
$\bar{X} \pm$ S.D.	0.1855± 0.0060	0.1580± 0.0125	0.1524± 0.0110

Fluorescence polarization (P) and fluorescence anisotropy (r_s) data obtained from human peripheral blood mononuclear cells at 0, 24, and 72 h after stimulation. Compared with resting cells, 24 h stimulation resulted in significantly lower P and r_s values ($p \leq 0.001$ and $p \leq 0.001$). In contrast, stimulated cells after 72 h failed to exhibit significant changes in the P and r_s values (even at $p = 0.05$) when compared with those stimulated for 24 h. The data are from six independent measurements

measurements revealed that at the time of a significant decrease of fluorescence polarization values the protein and RNA synthesis were already turned on; whereas significant increase in the surface density of IL-2 and tr receptors was also seen. In contrast, induction of DNA synthesis was seen when the change of structural order had already been completed, i.e. after 24 h of stimulation. From 24 to 72 h of stimulation the degree of fluorescence polarization slightly decreased, however, this did not prove to be significant. On the other hand, in the same interval (from 24 to 72 h) the DNA synthesis increased from the base level to the maximum (see Fig. 1). This was in correlation with data of cell cycle analysis as the proliferation fraction ($S+G_3+M$) in this period increased from 0 to 20-30%.

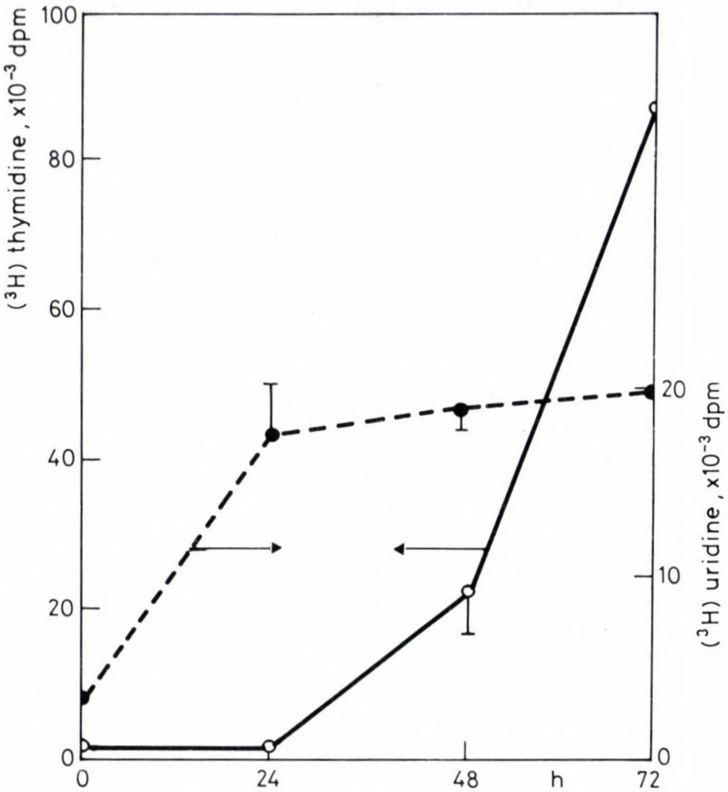
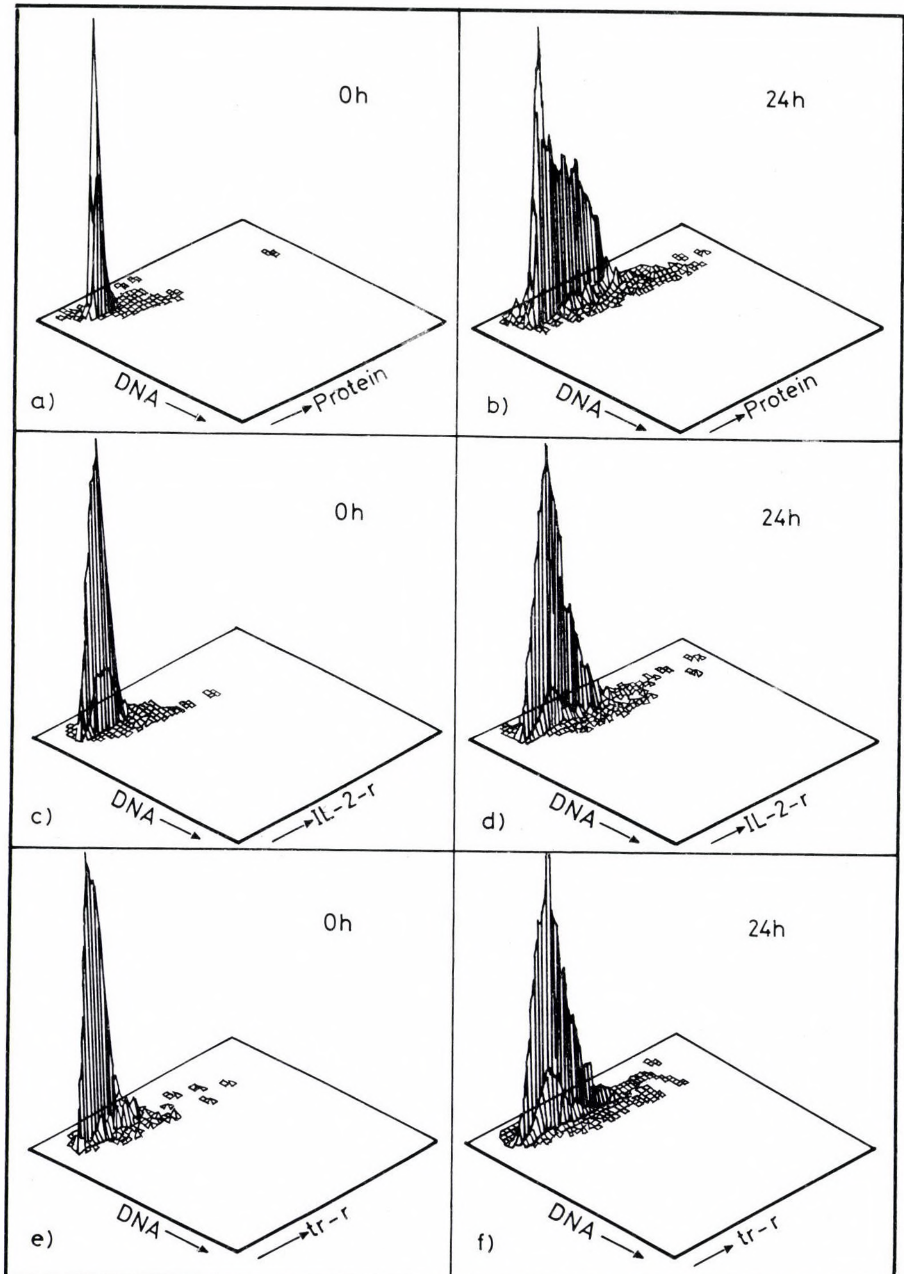


Fig. 1. Time course of RNA and DNA synthesis of PHA stimulated lymphocytes. Means $\pm$S.D. are from six independent experiments (^{3}H) thymidine o—o (^{3}H) uridine •---•

The average proliferation fraction in our experiments was 26.4%, a representative measurement is shown in Fig. 3.

The relationship of the structural order of membrane lipids to cell volume was also investigated (Fig. 4). The histograms obtained from the two parameter analyses after gatiny for the Hoechst 33342 derived fluorescence showed a definite increase in cellular volume as a function of stimulation time. Medians of Coulter volume histograms ranged from 182 (0 h) to 438 (72 h). The analysis of cell volume indicated that despite the definite volume increase as early as at 24 h of stimulation most of the cellular growth occurred after 24 h continued up to 72 h while control cells remained unaltered (not shown).


0h
a)
DNA
Protein
24h
b)
DNA
Protein
0h
c)
DNA
IL-2-r
24h
d)
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IL-2-r
0h
e)
DNA
tr-r
24h
f)
DNA
tr-r

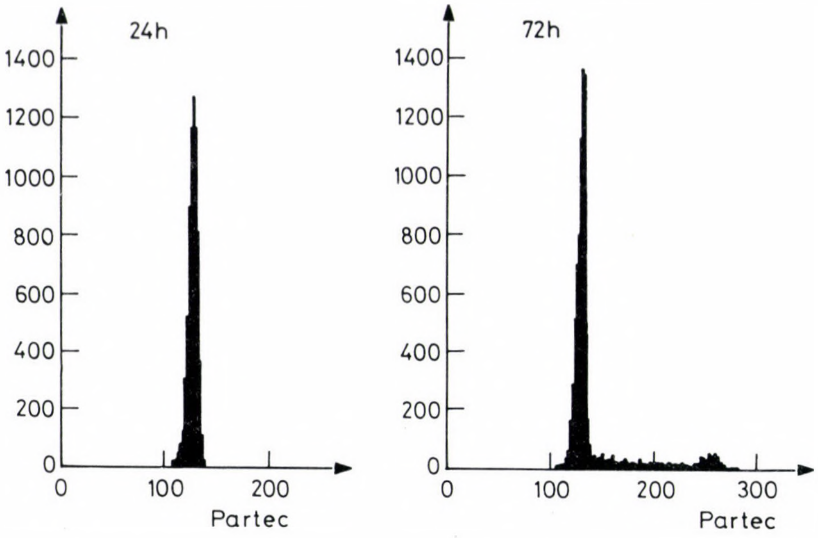

Fig. 3. Representative DNA histogram of lymphocytes stimulated for 24 and 72 h and stained for DNA by Hoechst 33258. There are no DNA synthesizing cells at 24 h, whereas the proliferation fraction was almost 30% after 72 h. The average proliferation fraction in the six experiments was 26.4%

Discussion

In this study we have followed the fluorescence polarization and anisotropy of stimulated cells related the cell cycle progression. The biological meaning of these values merits some consideration. Fluorescence anisotropy (r_s) is composed of a kinetic (r_f) and a static (r_∞) component /9/. The previous one is related to the rotational relaxation time of the fluorophore, which in turn, is proportional to the microviscosity; whereas the latter one is determined by the molecular packing in the apolar regions of the membrane and is proportional to the square of the structural order parameter (S_{DPH}) of apolar lipid core /9, 18/. If the r_s values range from

←

Fig. 2. Typical changes in protein synthesis (a-b), IL-2-r (c-d) tr-r (e-f) expression in lymphocytes stimulated for 24 h as compared with resting cells. While there is no increase in the DNA staining at 24 h, a considerable increase in protein synthesis is seen. The FITC derived green emission shown in c and d represent the unspecific signals in the range of 515 nm to 560 nm due to spillover of PI emission and autofluorescence. While there is no increase in the red fluorescence, a shift in the green fluorescence (d+f) is indicative of IL-2-r and tr-r expression

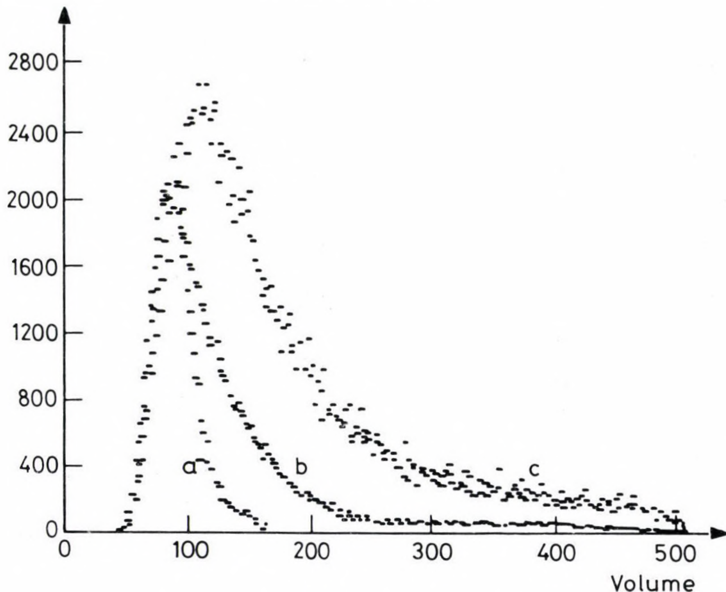

Fig. 4. Comparison of Coulter volume histograms, obtained after gating by means of Hoechst 33342 derived fluorescence, of resting cells and stimulated lymphocytes. a resting lymphocytes, median: 182; b 24 h stimulation, median: 210; c 72 h stimulation, median: 438. The cell volume is given in arbitrary units. The histograms from a representative experiment are shown

0.13 to 0.28 a linear correlation exits between r_s and r_{∞} /3/. In the measurements presented here the r_s values were within this range. Thus, fluorescence polarization values calculated from our measurements represent a parameter indicative of the structural order of the fatty acyl chains of the membranes. Furthermore, the structural order of membrane lipids is related to the degree of molecular packing and the lipid fluidity can be defined as the reciprocal of the lipid structural order parameter /3/.

Based on the observed metabolic changes and growth factor receptor expression the progression of cell cycle could be defined. One of the earliest changes after cell activation is the induction of RNA and subsequent protein synthesis. The RNA synthesis leveled off after 24 h, thus, at least the majority of PHA responding cells have already moved out of G_0 in the first 24 h of stimulation. This is further supported by the observation that a definite increase in cellular volume, reported to be an early sign of lymphocyte activation was already seen at 24 h /23/.

Additional support that the cells were in the G_0-G_1 transition at 24 h is the increased surface density of Il-2 and tr receptors, indicating

development of receptiveness to further activation signals /13/. Furthermore, based on the (^{3}H)T incorporation and cell cycle analysis data there was not any signs of DNA synthesis, thus a significant number of cells in the S phase at 24 h. On the other hand, DNA synthesis was high after 72 h of stimulation. Cell cycle analysis indicated that on average 26.4% of cells were in the S+G_2+M phases of the cycle. Assuming that G_1 constitutes about one half of the cycle time /12/, this rate of proliferation fraction indicates that more than half of all cultured cells were in cycle at this timepoint. Because the percentage of T lymphocytes in the peripheral blood mononuclear cells is 60-66%, it means that 86-94% of these cells were reactive to PHA stimulation.

The comparison of the fluorescence polarization (P) values of resting and stimulated lymphocytes revealed that those stimulated for 24 h exhibited a significantly lower degree of fluorescence polarization when compared to both 0 and 24 h control cells. This lower P level failed to exhibit any significant change during further progression of the cell cycle. The biological meaning might be that the changes in molecular packing and structural order have already been completed after 24 h of stimulation.

In summary, the decrease of the structural order, thus the remodelling of membrane core, is related to the G_0-G_1 transition and is not associated with cellular proliferation (DNA synthesis). Fluorescence polarization measurements on stimulated lymphocytes, fibroblasts and hepatocytes as well as on neuroblastoma cells indicated decreased P values in late G_1 and during G_1-S phase transition and were reported to depend on the proliferation fraction /6, 11/. Furthermore, P values were found to remain constant throughout the cell cycle in mouse leukaemia L 1210 cells /15/. Our conclusion is that the restructuring of membrane acyl chains is an early event of G_0-G_1 transition and may differ in quantity depend on the type of cell activation. This is also supported by our previous finding that significant differences exist between fluorescence polarization values of circulating acute and chronic lymphoid leukaemia cells; although no major difference in proliferation fraction was detected /16/.

The cell cycle can be divided into two closely related, but clearly separate processes that are under different molecular control /2/. One of this is the growth cycle (increase in volume and dry mass) and the other one is the nuclear cycle (DNA replication). Because, restructuring of the apolar regions in membranes clearly preceeded the onset of DNA replication, in our experiments, these structural changes are supposed to be associated

with the growth cycle. It can be ruled out that the structural changes in membranes are due to the passive stretching of the membranes by increased cellular volume because further progressive enlargement of the cells is not followed by alteration of strucutral order. Thus, the above described structural change is an inherent feature of cells in cycle and occurs in the course of the G_0-G_1 transition as part of the growth cycle, however, appears to be controlled independently from the growth cycle. The biological significance of this phenomen might be that the structural change in the cell surface has a permissive function in the interactions of proteins involved in the active membrane processes in that specific phase of cell cycle when major regulatory control of cellular proliferation occurs /17/.

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DIFFERENTIATION OF POTATO (SOLANUM TUBEROSUM L.) PLANTS FROM CULTURED LEAF PROTOPLASTS

A. FEHÉR*, JOHANNA PREISZNER*, and D. DUDITS*

Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

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Through induction of totipotent stage in cultured cells plants were regenerated from protoplast derived callus colonies of potato. Growing of the plants *in vitro* with optimal aeration and pretreatment of the leaves in dark and cold prior to protoplast isolation has improved the protoplast yield and frequency of cell division. Protoplasts of four potato genotypes have started to divide within 2-5 days after plating them into culture medium containing 2,4-D (0.2 mg/l); ZEA (0.5 mg/l); and NAA (1 mg/l) as growth regulators. Embedding of the cells into agarose proved to be favourable to avoid cell browning and to increase colony formation. The series of hormon treatments based on complex action of NAA and BAP promoting colony growth and greening, ZEA and IAA inducing shoot redifferentiation, and GA_3 plus NAA supporting shoot elongation and rooting, finally resulted in high frequency of plant regeneration from microcolonies.

Keywords: Protoplast isolation, plant regeneration, potato, *Solanum tuberosum*

Introduction

In contrast to all the higher eukaryotic cells the somatic plant cells exhibit a flexible differentiation program. Under *in vitro* conditions a fully differentiated cell can become totipotent during cell divisions in-

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*Present address: Institute of Plant Physiology, Biological Research Center, Hungarian Academy of Sciences, P.O.Box 521, H-6701 Szeged, Hungary

Abbreviations: 2,4-D,2,4-dichlorophenoxyacetic acid; KIN, kinetin; ZEA, zeatin; NAA, naphthaleneacetic acid; BAP, 6-benzilamino purin; IAA, indoleacetic acid; GA_3, gibberellic acid; MES, 2-(N-morpholino)-ethane sulfonic acid

Send offprint requests to: Dr. Dudits to the above address

Akadémiai Kiadó, Budapest

duced by plant hormones. From the two major redifferentiation pathways the organogenesis is characteristic for several Solanaceous species /5/.

Initiation of shoot and root meristems in the dedifferentiated callus mass is strongly influenced by several factors, mainly by the hormone component of the culture medium /5/. Since the in vitro response is highly species and genotype dependent the establishment of regeneration systems requires optimalization for each species and variants. Various genotypes of Solanum tuberosum have already been extensively used in tissue culture experiments and successful plant regeneration from cultured protoplasts have been reported /2, 6, 7, 19, 22, 23/. Though these studies outlined the significance of some of the key factors there is still a lack of a generally applicable approach.

In the present paper we describe the plant regeneration from cultured potato protoplasts. These studies show the effect of preconditioning of plants used as protoplast source on culture efficiency. Furthermore the frequency of cell division and plant regeneration will be analyzed by using different culture techniques and potato cultivars.

Materials and Methods

Plant material

Solanum tuberosum cv. Gracia, Boro, Desirée, Gülbaba and Somogygyöngye were aseptically propagated by cuttings on MS-P medium /9/ supplemented with 0.7% agar (Difco Bacto) and 1% sucrose, at 24 ^{o}C under 16 h fotoperiod of fluorescent light.

Protoplast isolation and culture

Prior to protoplast isolation donor plants were put into darkness for two days. Than leaves were cut, weighted and floated on top of liquid conditioning medium for additional one day at 4 ^{o}C in dark /8/. Leaves were cut into small strips and placed in enzyme solution containing 1% cellulase (Onozuka R-10) and 0.5% Macerozym (Onozuka) dissolved in V-KM culture medium /2/ with elevated amount of $CaCl_2 x 2H_2O$ (10 mM) and 0.42 M sucrose as osmotic stabiliser. Digestion took place in dark at 24 ^{o}C for 16-20 h. Protoplasts were filtered through a 44 µm steel mesh and one half volume of 0.6 M sucrose solution was added. Then they were floated by centrifugation (1000 rpm, 3 min.) into an overlay of 0.19 M mannitol; 100 mM $CaCl_2 x 2H_2O$;

0.5% MES; pH 5.6 (Fig. 1/b). Protoplasts were collected from the interface and washed two times with the overlay solution.

Protoplasts were cultured in V-KM medium /2/ containing 0.45 M glucose and 3 mM MES, at a density of $5\text{-}10 \times 10^4$ protoplasts/ml. Protoplast yield was determined by haemocytometer counts. At fourth day of culture cells were embedded in 0.6% Seaplaque (FMC Marine Colloids) or Sigma Type VII agarose supplemented culture medium, and they were further cultured in "agarose-bead" type culture system /20/. To reduce the osmotic pressure and change the glucose to sucrose stepwise half of the reservoir liquid medium were weekly changed for 0.28 M sucrose containing V-KM medium.

The viability data and the division frequency were determined 7-10 days after isolation via microscopic investigation. A protoplast was considered to be viable if it retained its turgidity and organelles.

Plant regeneration

After three or four weeks of culture the series of media "C", "D", and "E" according to Shepard /17/ was used for plant regeneration from the microcolonies.

Results and Disucssion

Condition of the donor plants

Leaf tissues from in vitro grown plants are frequently used as starting material for isolation of protoplasts. The actual metabolic stage of leaf mesophyll cells at the time of culturing can significantly influence the in vitro response. In addition to some previous results /7, 19/ we have analyzed the effect of growing conditions on quality of explants in vitro. For this reason we compared several media which have been used for potato shoot culture /8, 11, 15/. Finally we found, however, that simple MS /11/ and MS-P medium (a modified form of MS; 10) supplemented with 1% sucrose and 0.7% agar resulted in the fastest growth to our plants. In the later experiments MS-P medium were used.

It is well known that in vitro potato shoots produce ethylene during culture /4, 9/, this is why shoot sgrowing in carefully closed jars or boxes form thick shortened stems and small leaves. It can be hampered providing sufficient ventillation for the cultures /4, 9/ or using ethylene inhibitors such as silver thiosulfate in the medium /14/. We used cotton-

wool caped Erlenmeyerflasks (500 ml in size; three shoots per flask) in which shoots developed large leaves (Fig. 1/a) suitable for mesophyll protoplast isolation.

Treatment in dark of potato plants before protoplast isolation has been reported to be beneficial in increasing protoplast yield /8, 16/. For example Haberlach et al. /8/ put cut leaves on top of "flotation medium" (simple NH_4NO_3, $CaCl_2$ solution) for two days in dark followed by a 24 h incubation in sugar free liquid "conditioning medium" at 4 ^oC in dark, and reported high amount of viable protoplasts isolated. We found, however, that the flotation step makes the leaves too soft for subsequent handling (e.g. cutting) and finally resulted in reduced yield of healthy protoplasts. Putting the whole plants into darkness for two days at 24 ^oC suggested by Shahin /16/ proved to be superior resulting in about two times more protoplasts as compared to the untreated plants. It can be further augmented using the conditioning medium of Haberlach et al. /8/ (Table 1). Similar results were obtained by others in the case of tomato as well /21/. The reason of the beneficial effect of these pretreatments is not yet fully understood. The mobilization of starch into osmotically active surgars in dark /13/ and the adaptation of cells to stress conditions during cold treatment /21/ can play role in this phenomenon.

Pretreatments of Gracia plants and leaves definitely enhanced division frequency determined at 7th day of culture, while percentage of viable cells were not influenced (Table 1), but of course the total number of viable protoplasts was higher in the case of treatments. The earlier division of cells probably due to the one day incubation of the leaves in hormone containing liquid medium prior to isolation.

Protoplast isolation and culture

Protoplasts were isolated from healthy leaves of 3-6 weeks old *in vitro* explants as described in Materials and Methods (Fig. 1/a, b, c).

In agreement with previous observations /6, 8, 15/ protoplast yield considerably depends on genotypes used /5, 8, 15/. In our experiments the number of isolated protoplasts ranged between 2-5x10^6 per gram leaf tissue of preconditioned plants (Table 2).

For protoplast culture V8PCL, VKCL /6/ and V-KM /2/ medium were tested. V-KM medium was slightly modified by decreasing the osmotic pressure to about 575 mOsm. This was achieved by reducing of glucose concen-

Table 1

Effect of pretreatment of Solanum tuberosum (cv. Gracia) plants/leaves on the yield, survival and division frequency of protoplasts*

Treatment[a]	Yield[b]	Survival[c]	Division[c]
untreated	2.0±1.7	50.7±8.0	8.9±3.9
treated	4.3±1.2	55.2±8.6	14.8±3.8

*Data from four independent experiments

[a]Two plants with the same age were used putting one into darkness and leaving the other in light, respectively. Two days later leaves were cut, weighted and the leaves of the dark treated plant were floated on top of conditioning medium (8) for another one day at 4 °C in dark, while the leaves of the other plant were used directly for protoplast isolation.

[b]Numbers of protoplasts were determined based on four independent haemocytometer counts, and referred to one gram of leaf tissue.

[c]Percentage of viable (intact) and dividing cells were determined based on light microscopic investigations at seventh day of culture. One sample consisted of around one thousand protoplasts

tration to 0.45 M. Furthermore 3 mM MES was added to the medium to avoid changing of the pH during cell divisions. In this way the three media differed only in the organic addenda and the hormones. We found that the mixture of 0.2 mg/l 2,4-D; 1 mg/l NAA; and 0.5 mg/l ZEA as growth regulators essential to maintain sustained cell divisions. In later experiments this medium was used.

At 2-5 days of culture cell wall synthesis and the first cell divisions could be observed (Fig. 1/d). In one week old cultures usually more than 10% of plated protoplasts went through at least one cell division, with the exception of cv. Somogygyöngye. Protoplasts from this cultivar died some days following isolation.

At this stage sometimes browning of the cells occurred which finally resulted in cell death. The harmful effects of poliphenolic compounds could be reduced by using of agarose embedding and a high volume of liquid

Table 2

Effect of genotype on protoplast yield, colony formation, and plant regeneration

Cultivar	Protoplast yield[a]	Colony formation[b] (%)	Regeneration frequency[c] (%)
Gracia*	4.0±2.1	1.4±0.6	66.8±6.2
Boro*	2.0±0.9	1.4±0.4	49.5±18.4
Desirée*	3.6±2.0	1.2±0.5	47.9±15.9
Gülbaba**	4.75±0.5	0.6±0.2	4.0***
Somogy-gyöngye**	1.9±0.7	no colonies	no shoots

*Data from four independent experiments.

**Data from three independent experiments.

***Regeneration was observed only in one experiment.

[a]See Table 1.

[b]Percentage of protoplasts forming colonies (1-2 mm in diameter) on greening medium (med. "C"), counted after six weeks of culture.

[c]Percentage of those calli that formed shoots whithin three month of culture on soot inducing medium "D" at monthly subculture

medium for culture. For this reason we used the "agarose-bead" type culture system described first by Shillito et al. /20/ for other plant species. Protoplasts were embedded in 0.6% low melting point agarose containing V-KM medium at 4-7 days of culture. After another two days agarose was cut into segments and put into liquid V-KM medium. Half of this was weekly changed (see Materials and Methods). In this system cells divided quickly and formed microcolonies at a high frequency (Fig. 1/e, f).

Plant regeneration

When cell colonies reached 1-2 mm in diameter (three-four weeks) they were plated on top of agar solidified medium "C" after careful smashing the agarose blocks. On this medium which contains 1 mg/l NAA and 0.5 mg/l BAP colonies continued their growth and became green. Counting the green colonies (Fig. 2/a) after one week on this medium, 1-2% of the iso-

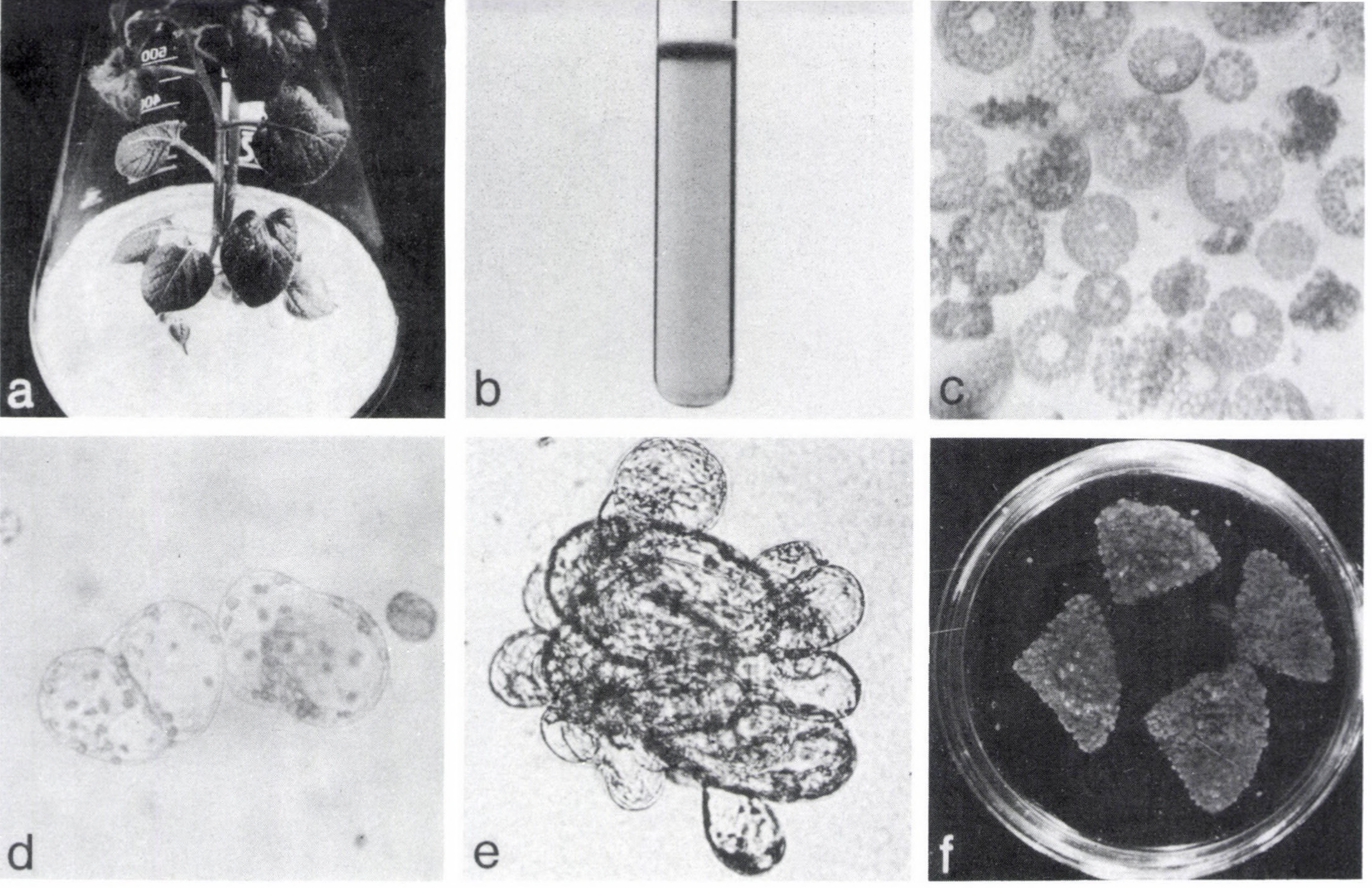

Fig. 1. Isolation and culture of leaf protoplasts of *Solanum tuberosum* (cv. Gracia) plants. a *In vitro* shoot culture explants; b Separation of healthy protoplasts from the debris by centrifugation in a high density sucrose solution; c Freshly isolated protoplasts; d First division of protoplasts at 2-5 days of culture; e Ten days old cell colony embedded inagarose; f Microcolonies developed in "agarose-beads"

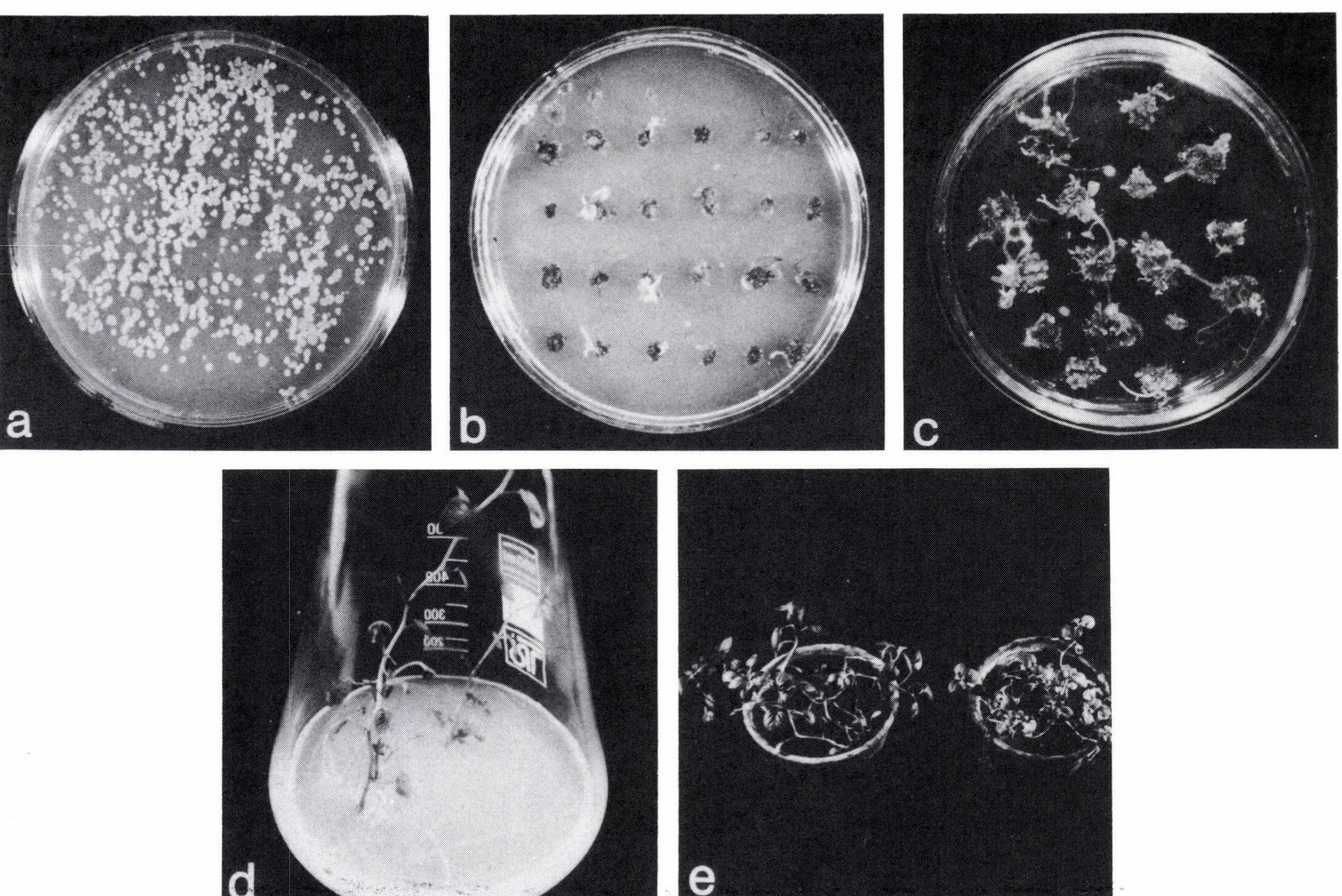

Fig. 2. Plant regeneration from microcolonies of potato cultivar "Gracia". a Small green calli on medium "C"; b Shoot formation after transfer to medium "D"; c Appearance of high number of shoots transfering colonies to medium "E"; d Small plants regenerated from protoplasts; e Protoplast derived plant in the greenhouse

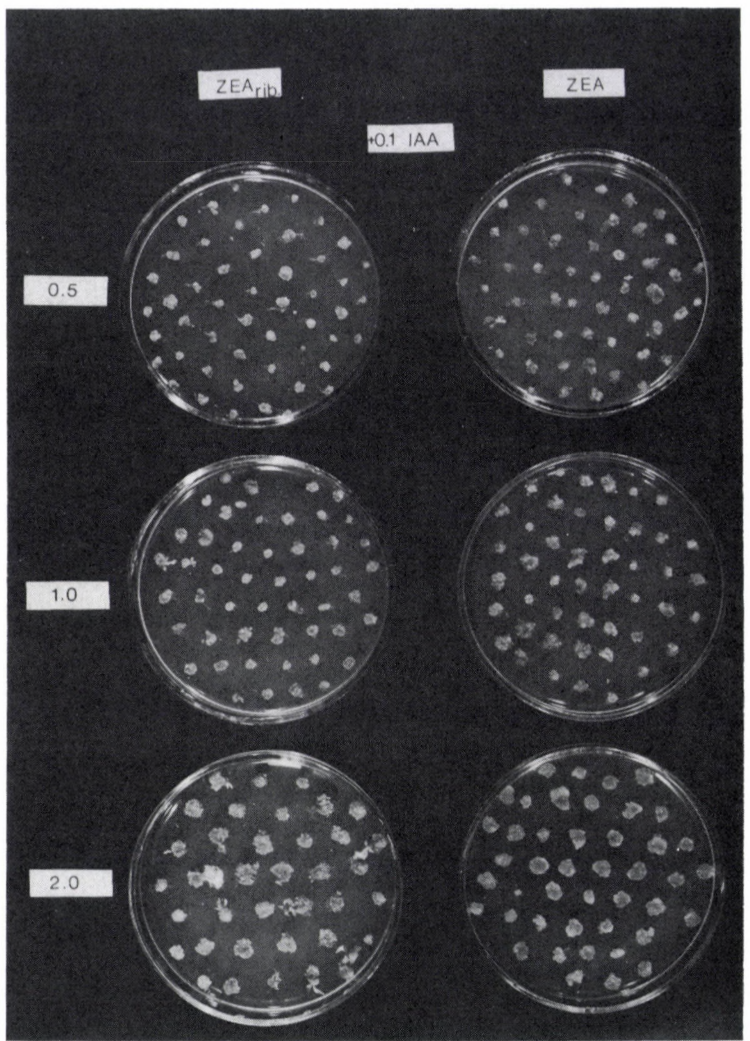

Fig. 3. Beneficial effect of zeatin-riboside (ZEA_{rib}) as compared to zeatin (ZEA) on shoot redifferentiation of potato cell colonies.
At the end of the second month of culture protoplast derived microcolonies of potato (cv. Gracia) were transferred from the conditioning medium (medium "C") to shoot differentiation inducing medium (medium "D"; 40-50 colonies per plate at three repetitions) containing 0.1 mg/l indoleacetic acid (IAA) and 0.5; 1.0; and 2.0 mg/l ZEA_{rib} or ZEA, respectively, as growth regulators. Three weeks later the beneficial effect of the increasing cytokinin concentrations on colony growth and ZEA_{rib} as compared to ZEA on shoot regeneration could be observed

lated protoplasts were able to form green calli in the case of all of the four cultivars investigated (Table 2). Colonies were kept on medium "C" for 2-4 weeks and than were transferred individually onto shoot inducing medium "D" supplemented with 0.1 mg/l IAA and 0.5 mg/l ZEA. Calli were subcultured monthly on this medium and become dark green followed by shoot redifferentiation (Fig. 2/b) 6-8 weeks after transfer onto medium "D". At regular subculture shoot regeneration continued for several months, and most of the calli regenerated several shoots. Among the four colony forming cultivars three showed reasonable regeneration frequency (about 50% or more) on this medium. For shoot induction J_1 medium of Austin et al. /1/ also were tested but colonies turned light green and failed to regenerate.

Haberlach et al. /8/ reported that 2 mg/l instead of 0.5 mg/l ZEA in the regeneration medium provided better response for numerous genotypes of potato. However we could not observe high differences using 0.5 or 2 mg/l ZEA in medium "D" concerning regeneration frequency (data not shown). It turned out, however, that 2 mg/l ZEA-riboside in shoot induction medium resulted in earlier shoot redifferentiation starting at 2-3 weeks after transfer of the colonies onto shoot induction medium (Fig. 3).

Shoots reaching 0.5-1 cm in high were excised and put onto rooting and elongating medium (medium "E"). After 2-4 weeks rooted shoots were further cultured in hormon free MS-P medium containing flasks (Fig. 2/d). Alternatively whole calli or parts of them were transferred to medium "E" following the appearance of the first shoots. In this case unvisible shoot meristems differentiated earlier on "D" medium started to develop and within some weeks numerous new shoots appeared on the surfaces of calli (Fig. 2/c). These can be rooted on hormon free MS-P medium.

After one month further in vitro culture plants could be planted into soil in the greenhouse and they developed into normal plants (Fig. 2/e).

In conclusion, the investigation of different factors affecting plant regeneration from potato leaf protoplasts has made possible to reach high regeneration frequencies for three different cultivars. It provides us the possibilities to use cell culture techniques (somatic hybridization, genetic transformation) in genetic manipulation of this potato genotypes having agronomical importance in Hungary.

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ORGAN-SPECIFIC AND PLOIDY-DEPENDENT SOMACLONAL VARIATION; A NEW TOOL IN BREEDING

L.E. HESZKY, LI SU NAM, I. SIMON-KISS*, K. LŐKÖS
G. GYULAI, E. KISS

Department of Genetics and Plant Breeding, University of Agriculture
Gödöllő, Hungary
*Research Institute of Irrigation, Szarvas, Hungary

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The organ-specific somaclonal variation means the differences between the variability of somaclones originated from different somatic tissue of plant. Significant differences in some agronomical characters were achieved among somaclones of seed and plumule meristem origin.

The ploidy-dependent somaclonal variation means the differences between the variability of somaclones originated from different ploidy-level tissue. Increased variation among regenerated plants was postulated by origin from cultured cells of reduced ploidy level. The comparison of somaclonal variation in the progenies of diploid plants regenerated from callus of haploid and diploid origin supported the ploidy dependent theory.

The pollenhaploid somaclone method (PHS-method) was developed and tested for utilization somaclonal variation in rice breeding. The PHS-method comprises the two well-known and widely applied in vitro methods which are the androgenesis (another culture) and genetic instability of cultured haploid somatic cells (callus cultures). Developmental varieties produced by this breeding sheme are under certification in Hungary.

Keywords: Oryza sativa, rice, pollenhaploid, somaclonal variation

Send offprint requests to: Dr. L. Heszky, Department of Genetics and Plant Breeding, University of Agriculture, H-2103 Gödöllő, Hungary

Introduction

Somaclonal variations are apparently the results of genetic changes that occur during the cell and callus culture.

In rice, the presence of regenerated variant plants has been documented since the first successful plant regeneration, e.g. morphological variants from seed callus /12/ albinos /2/ and polyploids /13/ from pollen callus. Variations in plant height and panicle length were also observed in regenerated haploid plants obtained from a single pollen callus /14/. Oono /15, 16/ observed that regenerated plants from haploid and diploid tissues carried some mutated characters and concluded that they were not preexisting in the original tissues but were repeatedly induced in the callus. Schaeffer /18/ has confirmed that somaclonal variation occurs in doubled haploid regenerants of rice.

Ramulu et al. /17/ invesitgated the origin and basis of somaclonal variation in potato. Their data suggested that the explant source and the initial ploidy level of the genotype play an important role in influencing the degree of somaclonal variation.

Our assumption was that the phenotypic manifestation of molecular and chromosomal changes (somaclonal variation) depends on the origin and the ploidy level of the initial explants and primary callus. Consequently the rate of manifestation and in this way the variation of the somaclones can be increased at lower ploidy level of initial explants. Therefore we planned to classify the variation of somaclones derived from different somatic tissues of different ploidy level in rice for developing in vitro techniques for broadening somaclonal variation.

Materials and Methods

1. Plant materials

Hungarian rice cultivar Nucleoryza (cv. Nuc.) was the plant material in our investigations. Seeds (A), plumule meristems (B) of diploid and immature inflorescences (C) of haploid plants were used as explants.

2. Production of somaclones (2n) of diploid origin (Fig. 1)

Seeds were husked manually and then surface sterilized by immersing in 96% ethanol for 1 min. followed by 0.2% $HgCl_2$ for 10 min. After several rinses with sterilized water, the whole kernels were used for callus induction.

To prepare plumule meristems sterilized kernels were allowed to germinated by submerging them for 48-72 h in sterile distilled water at 25 ^{o}C. Plumule meristems of 0.5-1.5 mm in length were carefully dissected out from the germinating embryos after separating both coleoptile and radicle.

Calli were induced and subcultures on the modified Murashige-Skoog (MS) medium /10/ consisting of MS salts /11/ and the following organic constituents per liter: 1.0 mg thiamine, 200 mg inositol, 3.0 g casein hydrolysate and 3% sucrose. The medium was supplemented with 2.0 mg/l 2,4-dichlorophenoxyacetic-acid (2,4-D) alone or 1.0 mg/l 2,4 D + 0.4 mg/l kinetin /3, 8, 9/.

Regeneration medium was the same as that used for callus induction and subculture, except that 2,4-D was substituted for kinetin (10 mg/l) and concentration of sucrose was increased to 4%. The calli were subcultured three times at 30-day intervals.

Plants of 1-4 cm in height were removed individually from the callus mass and transferred to a medium containing MS mineral salts, 2.0 mg/l kinetin, 0.2 mg/l indole-3-acetic acid (IAA), 2% sucrose and 0.6% agar. When the roots were well developed, plantlets were placed in commercial potting soil, acclimatized in mist chambers for two weeks, and grown to maturity in a greenhouse.

Abbreviations of somaclones were followed according to Larkin et al. /7/: SC_1 represents the regenerants, and SC_2, SC_3 etc. represent subsequent generations.

3. Production of somaclones (2n) of haploid origin (Fig. 1)

Shoots bearing panicles with uninucleate pollen were cut and pretreated for 7 days at 10 ^{o}C before another isolation. Shoots were surface-sterilized in 0.2% $HgCl_2$ for 10 min and rinsed 3 times with sterilized water. Whole panicle was excised out aseptically and anthers inoculated in Petri dishes with medium.

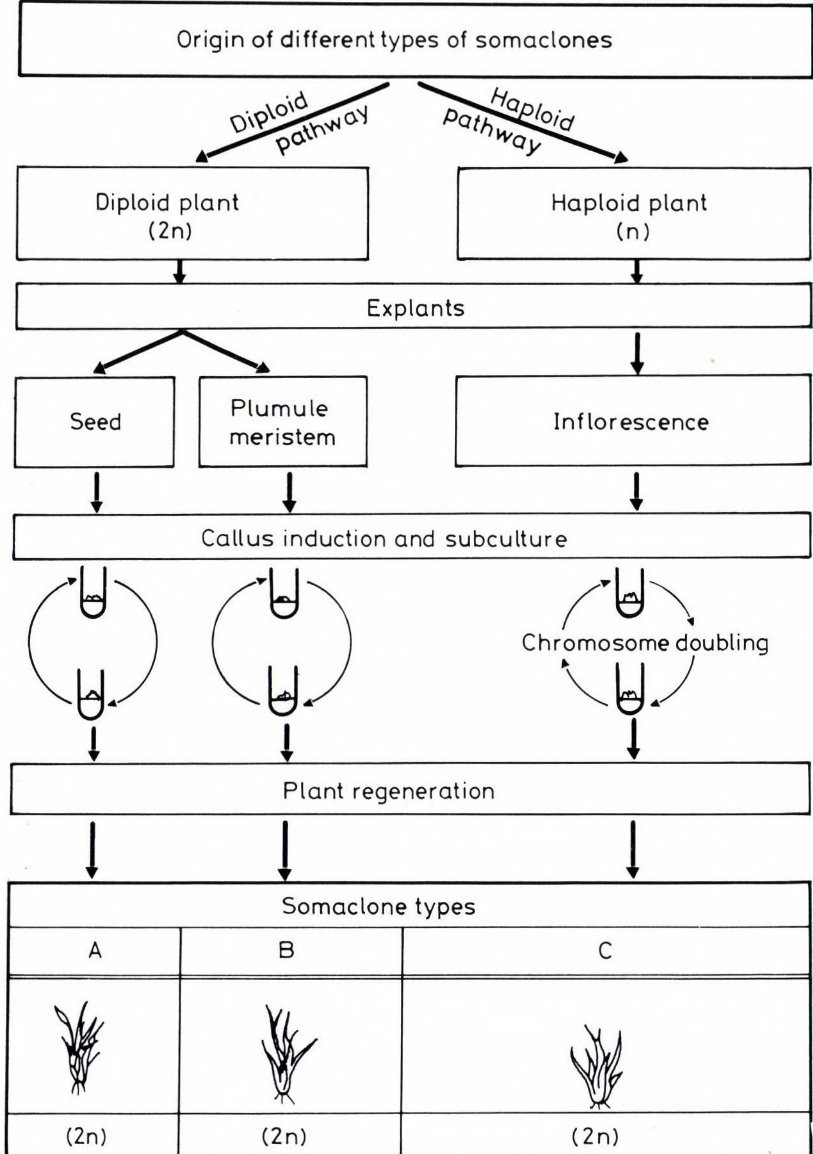


Fig. 1. Scheme of production of ploidy level dependent and tissue-specific somaclones

Anther calli were obtained by culturing anthers on N_6 medium /1/ containing 2.0 mg/l 2,4-D and 3% sucrose. The pH of the medium was adjusted to 5.6-5.8 before adding agar (0.8%) and then sterilized for 15 min at 121 ^{o}C. Procedure for plant regeneration was as previously described in the selection of diploid origin.

Immature inflorescences were excised from pollenhaploid plants propagated vegetatively in a greenhouse. The stem was selected prior to emergence of the flagleaf and was cut below the peduncular node and surface sterilized in the same way as the seeds. The whole immature inflorescence, 3-10 mm in length, was isolated.

Callus from haploid inflorescence was induced, cultured and plants were regenerated according to the above-mentioned protocol for seed and meristem callus /3, 8, 9/.

After three passages of the primary haploid callus, nearly hundred per cent of regenerants were diploid /5/. These have been termed as pollen-haploid somaclones referring to the gamete origin and to distinguish them from the somaclones of zygote and doubled haploid origin.

4. Field performance

Selfed seeds (SC_2) were harvested from individual regenerants (SC_1) grown in a greenhouse betwen 1983-1984 in the Research Centre for Agrobotany. A total of 105 lines of second and third generations (SC_2 and SC_3) were sown in rice field tirals of the Research Institute of Irrigation (Szarvas) in 1985-86.

Earliness (number of days between sowing and flowering) and tillering ability (number of tillers per plant) were examined under upland conditions. On the other hand, yield components such as panicle length, grain number/panicle and number of branching/panicle were observed in lowland field-trial. The data were statistically evaluated and compared by F-and t-test.

Results

The effect of origin and ploidy level of explants on the variability of different traits (flowering date, fillering and morphological features) of 105 somaclones (SC_3 plants numbering 1960) were tested in field trials.

Table 1

Distribution of earliness and tillering ability in somaclones (SC_3) of different origin

Origin	No. of lines	Earliness			Tillering ability		
		Early^{+} %	Medium^{++} %	Late^{+++} %	Low^{x} %	Medium^{xx} %	High^{xxx} %
A: Seed callus (2n)	40	0	50	50	100	0	0
B: Meristem callus (2n)	45	0	11	89	0	100	0
C: Inflorescence callus (n)	20	25	25	50	25	50	25

Number of days from sowing till flowering:

$^{+}$less than 81; $^{++}$82-88; $^{+++}$more than 88

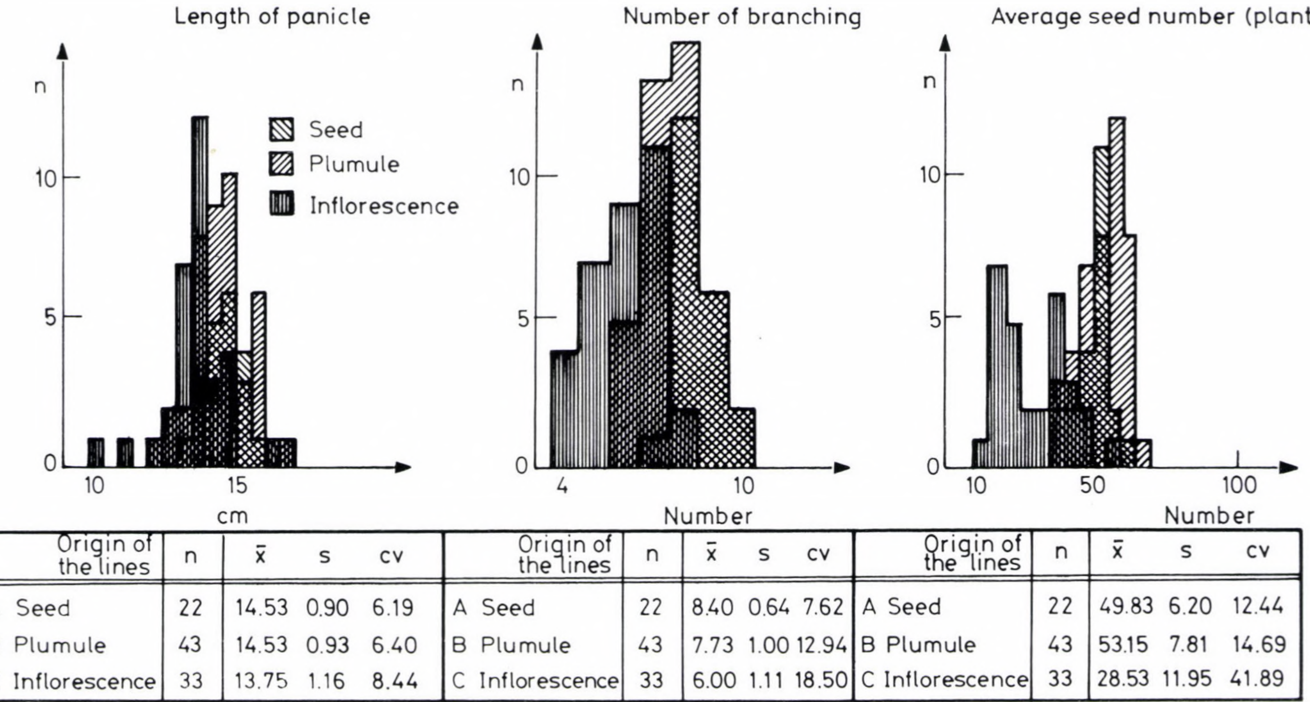
Number of stems per plant:

^{x}3-5; ^{xx}5-10; ^{xxx}10

Hungary is situated in the northest part of the rice production area therefore early maturity is a very important trait. The results show that the earliness of somaclones of three origins are different (Table 1). Highest variation was observed in the case of somaclones of haploid origin. Likewise shortest vegetation period has been obtained in pollenhaploid somaclones.

Tillering capacity (number of productive tillers/plant) was tested under irrigated field conditions (Table 1), the results indicate that not only the tillering capacity but also the drought resistance, that is stress resistance of the somaclonal lines, as well. Table 1 shows the tillering distribution of 105 somaclone lines, that differ obviosuly according to their origin. "Seed" and "meristem" origin have low and medium tillering, respectively, but somaclones derived from young inflorescences possess a rather wide variation in the range of low, medium and high tillering.

The analysis of several yield components has not always given significant differences to the advantage of somaclonal lines of haploid origin. Table 2 demonstrates the deviations and coefficients of variance of the three tested traits.



Origin of the lines	n	$\bar{x}$	s	cv	Origin of the lines	n	$\bar{x}$	s	cv	Origin of the lines	n	$\bar{x}$	s	cv
A Seed	22	14.53	0.90	6.19	A Seed	22	8.40	0.64	7.62	A Seed	22	49.83	6.20	12.44
B Plumule	43	14.53	0.93	6.40	B Plumule	43	7.73	1.00	12.94	B Plumule	43	53.15	7.81	14.69
C Inflorescence	33	13.75	1.16	8.44	C Inflorescence	33	6.00	1.11	18.50	C Inflorescence	33	28.53	11.95	41.89

Fig. 2. Frequency distribution and caracteristic values of three yield components in somaclones derived from different organs

Table 2

Comparison of somaclones of different origin in pairs by F-test and t-test

Origin	FG	p%	Table value	Calculated value		
				length of panicle	branching	seed No./ plant
			F-test			
A-B	42;21	10 2	1.96 2.63	1.07	2.44	1.59
		result		$S_A \approx S_B$	$S_A \sim S_B$	$S_A \approx S_B$
A-C	32;21	10 2	1.91 2.51	1.66	3.01	3.72
		result		$S_A \approx S_C$	$S_A < S_C$	$S_A < S_C$
B-C	32;42	10 2	1.73 2.17	1.56	1.23	2.34
		result		$S_B \approx S_C$	$S_B \approx S_C$	$S_B < S_C$
			t-test			
A-B	63	5 1	2.00 2.66	0	2.82	1.71
		result		$\overline{X}_A = \overline{X}_B$	$\overline{X}_A > \overline{X}_B$	$\overline{X}_A \approx \overline{X}_B$
A-C	53	5 12	2.01 2.68	26		
		5	2.05=t		10	8.66
		result		$\overline{X}_A > \overline{X}_C$	$\overline{X}_A > \overline{X}_C$	$\overline{X}_A > \overline{X}_C$
B-C	74	5 1	1.99 2.65	3.21	7.03	
		5	2.06=t			10.25
		result		$\overline{X}_B > \overline{X}_C$	$\overline{X}_B > \overline{X}_C$	$\overline{X}_B > \overline{X}_C$

The deviation of panicle length of haploid somaclones has not been significantly higher (Fig. 2).

The variation of number of branching in lines of seed origin has been essentially smaller than in pollenhaploid somaclones (Fig. 2).

The variability of seed number/panicle of pollenhaploid somaclones has surpassed the variation of somaclones of diploid origin significantly (Fig. 2).

Table 2 also calls attention to that in addition to variation depending on ploidy level there probably exists an organ-specific somaclonal variation, as well, which seems to be proved by differences between two somaclones of diploid origin (seed and meristem).

Discussion

Genetic diversities observable among plants regenerated from *in vitro* cultures termed somaclonal variation were summarized by Larkin and Scowcroft (1981). In the following years experiments were directed on the one hand to observe and describe the phenomenon in different plant species and to prove the heritability of the variation, on the other hand the goal of the experiments was to focus on the molecular and chromosomal changes resulting in somaclonal variation. However, there are few reports in connection with the dependence of somaclonal variation on the type of isolated tissue and its ploidy level.

As a first step the effect of ploidy level of isolated tissue was tested. It was assumed that phenotypic manifestation of the molecular changes decreases with increase of ploidy level, that is somaclonal variation can be enhanced by the decrease of ploidy level. Otherwise if we regard the genetic changes taking place in haploid and diploid primary calli as identical, in that case, a higher percentage of the changes will be phenotypically manifested in the homozygous diploid plants regenerated from haploid cultures, consequently the variation will be wider.

The quantity and quality of genetic variation induced in haploid somatic tissues of rice has been studied /16/. Haploid, diploid and tetraploid plants regenerated from callus of spontaneous haploid clones were examined and compared.

Agronomic characters in haploids and diploid varied greatly. The frequency of diploids was more than 50%. These results supported our theory

but the diploids were only compared to haploids and to diploid somaclones of zygotic origin.

Therefore proving our assumption comparative experiments were performed. Primary calli were induced, on the one hand from the somatic tissue (young inflorescences) of pollenhaploid plants maintained vegetatively, and from diploid embryos and shoot meristems of zygotic origin in cultivar Nucleoryza on the other. Haploid calli suffered from spontaneous rediploidization during several passages so diploid plants were regenerated from them in the same way as from calli of embryogenic and meristematic origin. Differences in somaclonal variation were investigated in the second and third generations of diploid regenerants in order to eliminate the effect of the possible non-heritable changes.

Results have proved and supported our supposition, because the variation in agronomic characters tested was greater in the somaclonal lines of haploid origin. Based on these convincing results a new method was elaborated being given the name of pollenhaploid somaclone method, abbreviated PHS method.

Pollenhaploid somaclone method (PHS-method)

The scheme of the PHS-method is shown in Figure 3. It consists of the following main steps.

A) Reduction of ploidy level

Haploid plants are produced from the desired breeding material (hybrid, line etc.) by anther (androgenesis) or ovary (gynogenesis) cultures.

B) Maintenance and propagation of reduced ploidy level

Haploid plants are grown, propagated and maintained in vitro and/or in greenhouse, depending on a given species. As a result of meiotic recombination these plants have already possessed variation of a certain level called gametoclonal variation in literature.

C) Production of somaclones from somatic tissue of reduced ploidy level

Callus is induced from the somatic tissue of haploid plants (flower, meristem, leaf etc.) and after several passages diploid plants are regenerated. A part of genetic changes taking place at haploid cell level - also in the case of recessive genes - during diploidization gets into

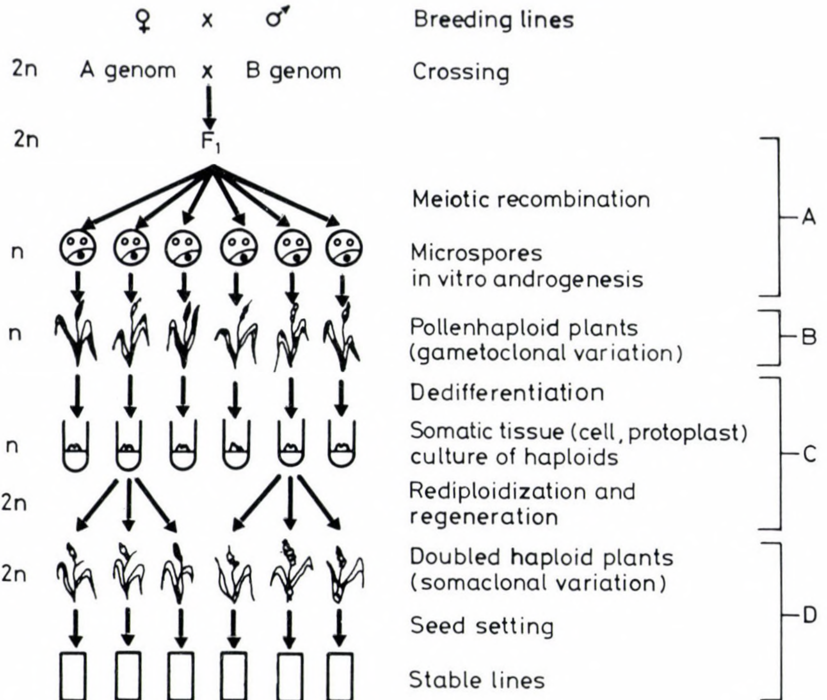


Fig. 3. Scheme of PHS-method (pollenhaploid somaclone) for increasing genetic variability in breeding materials

homozygous status and phenotypically manifested in regenerated diploid plants.

D) Field test of pollenhaploid somaclones

Seeds are obtained from diploid regenerants (SC_1). The variability of seccessive generations can be considered as genetic changes and the somaclonal lines contain the changes new character in homozygous form because of haploid level and diploidization, that is further segregation cannot be excepted. So these lines can directly be used for producing new variety (cultivar) and the time requirement of breeding can be reduced.

Introduction of the new term of pollenhaploid somaclone requires to revise relevant terminology. Such a terminology is necessary, which can help to define the concepts and the origin of the variation (Fig. 4).

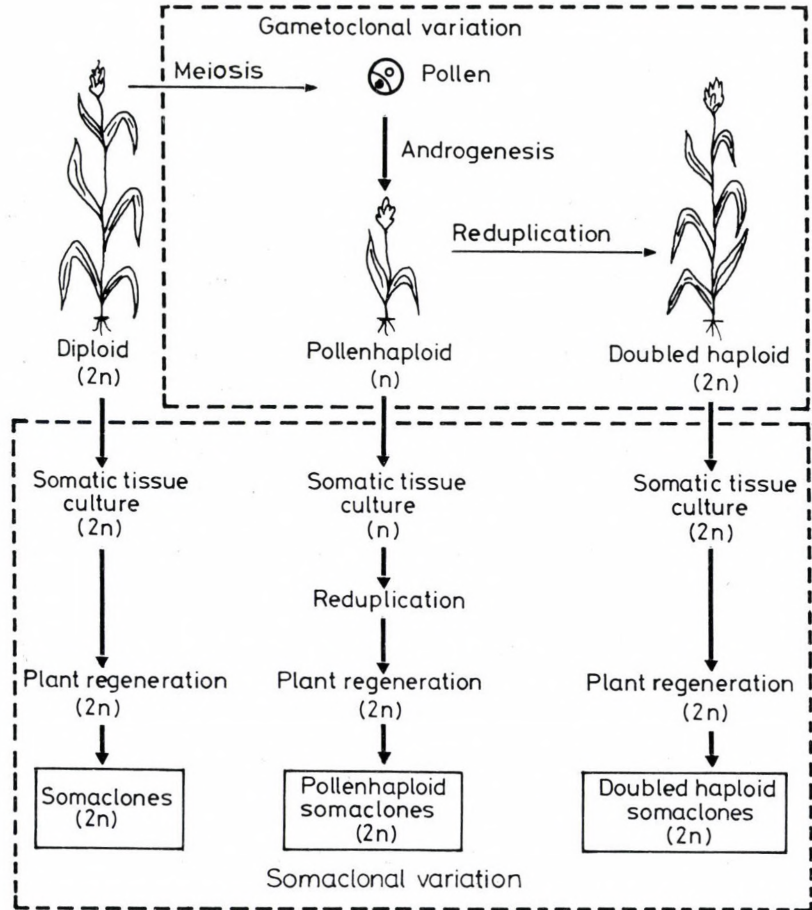


Fig. 4. Scheme showing the origin and production of different somaclone types

Pollenhaploid: haploid (n) cell, tissue or plant of male gamete origin. Variation among pollenhaploids originates from meiotic recombination of the parents and due to the stress during culture named gametoclonal variation.

Doubled haploid: diploid (2n) cell, tissue or plant of haploid (n) origin, produced by doubling the haploid genom. Variation among doubled haploids originates from meiotic recombination, named gametoclonal variation.

Somaclone (classic): diploid (2n) plants regenerated from cultures somatic tissue of diploid plants of zygotic origin. Variation among somaclones originates from the genetic instability of cultured diploid cells, named somaclonal variation.

Pollenhaploid somaclone: diploid (2n) plants regenerated from cultured somatic tissue of pollenhaploid plants (n) of androgenic origin. Variation among pollenhaploid somaclones originates from the genetic instability of cultured haploid cells.

Doubled-haploid somaclone: diploid (2n) plants regenerated from cultured somatic tissue of doubled-haploid (homozygous) plant (2n) or their progenies. Variation among doubled haploid somaclones originates from the genetic instability of cultured homozygous diploid cells.

The concept of pollenhaploid somaclone can exactly be interpreted and significant differences distinguishing it from gametoclones and doubled-haploid somaclones can well be appreciated by means of the above listed definitions.

The PHS-method has been tested /8, 9/ with cv. Nucleoryza and Carolina. The results have proved its applicability in rice. The PHS-somaclones were introduced in breeding and the new somaclone originated varieties (HSC-1, HSC-2) are being tested in the state controlled field certification trials /4/.

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EFFECT OF AGE, SEX, AND SMOKING ON SERUM CATALASE ACTIVITY

L. GÓTH

Department of Laboratory, Municipal Hospital, Sümeg, Hungary

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In selected hospitalized patients (9294) with normal serum catalase activity, 7.58 per cent higher ($p < 0.001$) serum catalase activity was detected in males than in females. With age, serum catalase activity decreased (slope - 0.0683) in males, increased (slope 0.101) in females and did not change in all patients (slope 0.049). Cigarette smoking did not influence serum catalase activity ($p > 0.05$).

Keywords: Serum catalase, selected hospitalized patients, age, sex, smoking

Introduction

Enzyme catalase (EC 1.11.1.6) decomposes hydrogen peroxide into oxygen and water. It can be found in human tissues with its highest activity in erythrocytes, liver and kidney while its activity in sera is much lower /1, 3/.

Serum catalase activity increases in some diseases /2, 6, 11/ but a diagnostic importance can be attributed to it in acute pancreatitis /4/, haemolytic disease /5/ and some liver diseases /7/. Its elevated serum activity was derived from erythrocytes in haemolytic diseases /8/, acute pancreatitis /9/ from liver peroxisomes in liver diseases while in normal circumstances the main source of serum catalase is the erythrocyte pool /8/. Serum activity of some enzymes depends on age (alkaline phosphatase), sex (GOT, gamma-GT) and in some cases on smoking i.e., angiotensin converting

Send offprint requests to: Dr. L. Góth, Department of Laboratory, Municipal Hospital, H-8330 Sümeg, Hungary

Akadémiai Kiadó, Budapest

enzyme /10/, alanin aminopeptidase /12/. Similar data for serum catalase including a large number of catalase assays have not been known.

Therefore, the aim of this paper is to investigate the effect of age, sex and smoking on serum catalase determined by our rapid and cost-effective method /6/ in a large number of selected hospitalized patients.

Patients and Methods

4402 males and 4802 females between 14 and 98 years were selected from patients of department of internal medicine. Two criteria were used for selection of patients. First, they did not have had any diseases which could influence their serum catalase activity. This requirement was fulfilled with help of our earlier findings /6/ which revealed most of the internal diseases which could influence serum catalase activity. Second, serum catalase of the selected patients was in the normal range.

1042 subjects were considered to be smokers. The mean of smoked cigarettes was 16.9 in a day and they have smoked for more than two years. They were divided into four groups depending on the number of smoked cigarettes. 2819 non-smokers have never smoked.

Serum catalase activity was determined by decrease in concentration of its hydrogen peroxide substrate /6/. Hydrogen peroxide concentration was measured by a programmable polarograph (OH 106, Radelkis, Hungary) at 37 ^{0}C. The range and the mean value of serum catalase was 14.1 - 99.3 kU/L and 56.7 kU/L in 111 healthy subjects.

Statistical analyses were performed by microcomputer and two paired Student's t-test was used for calculation of significance (p) and least squares method for determination of correlation (r) as well as the slope (b) and intercept (a) of the regression curve.

Results

Our results are summarized in Tables 1 and 2. They showed higher (7.58%, $p < 0.001$) serum catalase activity in males (64.81 $\pm$ 19.08 kU/L, n = 4402) than in females (60.24 $\pm$ 18.77 kU/L, n = 4892).

In males the serum catalase activity decreased with age (b=-0.0683, a=69.18, r=0.886) and it was 6.83% lower ($p < 0.01$) in elderly patients (aged between 50 and 90 years) than in young ones (aged between 20 and 30

Table 1

Effect of age and sex on mean and SD of serum catalase

Catalase activity kU/L									
Age, years	Male Mean	Male (SD)	Male n	Female Mean	Female (SD)	Female n	Male + Female Mean	Male + Female (SD)	Male + Female n
10-19	68.25	(18.25)	108	56.20	(19.08)	143	60.97	(19.47)	251
20-29	68.56	(17.96)	266	55.48	(18.93)	293	61.71	(19.58)	559
30-39	66.85	(18.52)	432	56.44	(17.38)	418	61.22	(18.57)	850
40-49	65.56	(18.66)	608	58.27	(19.33)	702	61.55	(19.36)	1310
50-59	64.05	(19.06)	915	60.43	(17.91)	863	62.29	(18.60)	1778
60-69	64.93	(19.23)	873	62.37	(18.30)	934	63.60	(26.33)	1807
70-79	62.35	(19.55)	858	61.82	(19.14)	1104	62.44	(31.82)	1962
80-89	63.31	(19.89)	268	62.60	(18.80)	362	63.49	(29.39)	630
90-99	64.53	(18.71)	14	61.50	(17.60)	21	63.86	(26.60)	35
All subjects	64.81	(19.08)	4402	60.24	(18.77)	4892	62.41	(19.06)	9294

Table 2

Serum catalase activity in smokers and non-smokers

Smokers cigarettes a day	Serum catalase activity kU/L Mean	(SD)	n
0 - 10	62.97	(18.72)	251
11 - 20	63.79	(18.80)	644
21 - 30	64.52	(17.45)	71
above 31	67.32	(19.38)	76
All smokers	63.57	(18.77)	1042
Non-smokers	63.19	(19.06)	2819

years). In contrast to this, serum catalase in females increased with age ($b = 0.101$, $a = 53.93$, $r = 0.941$) and it was 11.29% higher ($p < 0.001$) in elderly subjects than in young patients but it always remained below the serum catalase activity of males.

Serum catalase activity of all patients showed a weak increase with age ($b = 0.0349$, $a = 60.42$, $r = 0.901$) and yielded a non-significant (2.3%, $p > 0.1$) change between elderly and young patients.

The mean of serum catalase of selected hospitalized patients (62.41 ± 19.06 kU/L, n = 9294) was higher (10.1%, p 0.001) than those of the healthy persons (56.7 ± 21.3 kU/L, n = 111).

We found no significant change ($p > 0.5$) in serum catalase activity of smokers (63.57 ± 18.77 kU/L, n = 1042) and non-smokers (63.19 ± 19.06 kU/L, n = 2819). In smokers the relationship between serum catalase and number of smoked cigarettes also revealed a non-significant ($p > 0.5$) change with a weak increase ($b = 0.1234$, $a = 61.73$, $r = 0.946$).

Discussion

Our results showed a significant difference in serum catalase of males and females. Furthermore, we detected the age dependence of serum catalase which increased with age in females and decreased in males. These two opposite effects yielded smaller sex difference for elderly patients compared to the young ones and no age dependence for all of the patients. Similar differences were not detected in large number of patients.

Earlier we found a relationship between blood haemoglobin concentration and serum catalase activity /8/. In accordance with this effect, the lower blood haemoglobin of females could explain their lower serum activity.

In males the decrease of serum catalase with age also could be explained by the lower blood haemoglobin level of the elderly patients.

Contrary to these, in females the cessation of periodical loss of blood might be reflected in the increasing serum catalase which is significantly higher in elderly patients than in young ones. We have been screening serum catalase activity for some years. From these hospitalized patients we selected our patients by two criteria. We found the mean of serum catalase in selected hospitalized patients higher (10.1%, $p < 0.001$) than in healthy subjects. Against the very careful selection of the hospi-

talized patients this difference was to be expected and reflected further diseases and circumstances might be supposed which could cause a very weak influence on serum catalase.

In accordance with the age and sex differences detected in selected hospitalized patients similar conclusions might be expected for healthy subjects as well.

Chronic effect of cigarette smoking can influence some serum enyzme activities /10, 12/. Our results showed no significant change ($p > 0.5$) of serum catalase in smokers. In heavy smokers with more than 30 cigarettes a day serum catalase activity yielded a non-significant ($0.05 < p < 0.1$) increase. This effect may be attributed to the elevated erythrocyte catalase of smokers /13/ which could be reflected in their serum catalase activity.

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CENTROMERE SEPARATION SEQUENCE IN HUMAN CHORIONIC CELLS

KATALIN BAJNÓCZKY[1] and K. MÉHES[2]

[1]Center of Clinical Genetics, County Hospital, Győr, Hungary;
[2]Department of Paediatrics, University Medical School of Pécs, Hungary

The sequence of centromere separation in human chorionic cells was analyzed in two laboratories. Despite of certain interexaminer variations, in both series chromosomes 18, 2, 12, 4, 5, 17 and X proved to be early dividing and the acrocentrics were the last to separate. The pattern was very similar to those observed in other human tissues, which reflects a species-specific sequence of centromere separation.

Keywords: Cell division, centromere, chorion biopsy

Introduction

The existence of a "normal sequence" of centromere separation in human mitoses and a possible relation of "out-of-phase" centromere separation to aneuploidy have often been discussed /3, 5, 7, 12/. Most of these reports are based on observations on lymphocyte mitoses and only a few studies on bone marrow preparations /1, 6, 13/, fibroblast cultures /5/, and amniotic cells /2/ are known to us.

Here we report on the centromere separation sequence of chorionic cells.

Send offprint requests to: Dr. Katalin Bajnóczky, Center of Clinical Genetics, County Hospital, P.O.Box 92, H-9002, Győr, Hungary

Akadémiai Kiadó, Budapest

Materials and Methods

The material was put together from two parts:

1. In Győr a total of 151 chorionic cells from 41 diagnostic biopsies were studied, i.e. in average 3.7 mitoses per subject were evaluated. In the majority of the cases the bipsy was performed because of higher maternal age; the mean age of the mothers was 38.1 years. The data were pooled, individual variations could not be analyzed.

2. In Pécs, preparations of 6 induced abortions were studied. The number of mitoses per sample examined varied from 13 to 20. The centromere separation sequence was determined in each sample separately, but since no significant variation was found, only the mean values are given here.

In both laboratories late metaphase, mainly G-banded mitoses of "direct" chorionic villi preparations were selected. Only cells with normal 46,XX or 46,XY karyotypes were analyzed in parallel under the microscope and from karyotypes.

The sequence of centromere divisions was characterized by the centromere separation index (CSI) introduced by Vig /11/ and also applied in our earlier works /2, 3/. By this method, the centromeres that had not divided at all were assigned a score of 0, those that had just begun separation scored 1, and those showing clear separation, i.e. no connection between the sister chromatids, were scored as 2 (Figs 1, 2). The scores obtained were pooled for individual pairs of chromosomes. These totals divided by the largest number in the series provided relative values (CSI) against a given value of 1 for the earliest separating pair. The higher the CSI for a chromosome, the earlier was its position in the separation sequence.

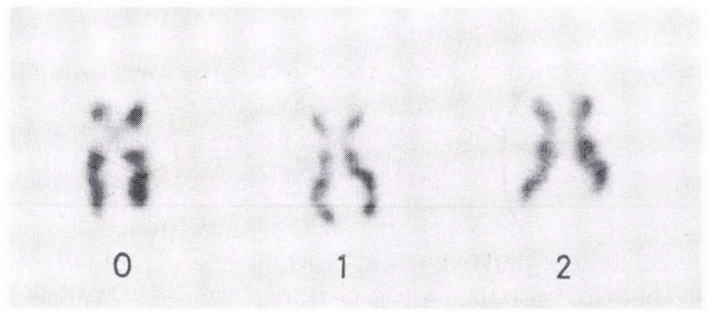

Fig. 1. "Degrees of separation"

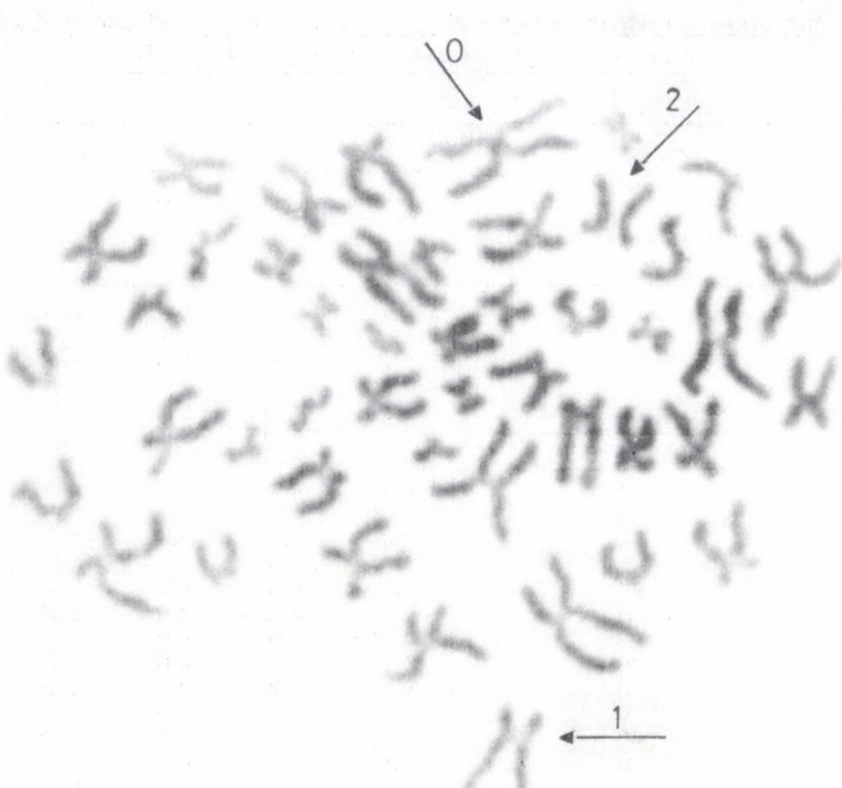

Fig. 2. Example of metaphase-anaphase cell showing centromeres in various phases of separation

Results

The mean CSI values of chorionic cells obtained independently in the two laboratories are compared in Table 1. As shown by the figures, the distribution of CSI values by chromosome pairs was clearly non-random in the two laboratories. Chromosomes 6, 10, 12, 20 and X were found to have significantly higher values in Győr than in Pécs, however, this was a merely quantitative difference. The main tendency was fairly similar in the two laboratories: chromosomes 18, 2, 12, 4, 5, 17 and X belonged to the early separating ones, while chromosomes 1, 7, 8, 9, 11, 16 and the acrocentrics were the last to separate.

Table 1

Centromere separation index (CSI) values for individual chromosomes of chorionic cells obtained in two laboratories

	46,XX cells		46,XY cells	
	Győr	Pécs	Győr	Pécs
No. of chorionic villi samples	22	3	19	3
No. of mitoses analyzed	73	45	78	42
Chromosome No. 1	0.06	0.13	0.00	0.17
2	0.85	0.94	0.95	0.70
3	0.55	0.56	0.48	0.40
4	0.70	0.54	0.71	0.57
5	0.62	0.52	0.70	0.53
6	0.24	0.02	0.29	0.05+
7	0.08	0.02	0.10	0.01
8	0.00	0.05	0.00	0.09
9	0.08	0.07	0.00	0.05
10	0.68	0.30+	0.87	0.33+
11	0.00	0.00	0.05	0.03
12	1.00	0.46+	0.61	0.59
13	0.00	0.02	0.00	0.05
14	0.00	0.00	0.00	0.03
15	0.00	0.03	0.00	0.11
16	0.00	0.02	0.02	0.03
17	0.36	0.48	0.58	0.49
18	0.72	0.81	1.00	0.90
19	0.18	0.02	0.16	0.03
20	0.36	0.11+	0.43	0.17+
21	0.00	0.03	0.00	0.05
22	0.00	0.02	0.00	0.03
X	0.81	0.55+	0.88	0.59+
Y	-	-	0.00	0.10

+$p < 0.05$

Discussion

The present methods of determining centromere separation sequence cannot be free of subjectivity. Thus, the slight differences between the two series in the present study may rather be attributed to interexaminer errors than to real cytological events. Anyhow, the sequence of centromere separation in human chorionic cells seems to be fairly similar to that repeatedly observed in other human tissues.

This may have three important implications:

1. The very early centromere division of chromosomes 18 and X and the late separation of chromosomes 13 and 21 are interesting from the point of view of human pathology. Aneuploidies of these four chromosomes are the most frequent chromosome aberrations in liveborn infants, and the relation of being at the two ends of separation sequence to non-disjunction seems to be a promising subject of research /3/.

2. Considering the association of errors of sequential separation of centromeres with some human disorders /4, 8, 10/, the knowledge of "normal sequence" in chorionic cells may be useful in prenatal diagnosis.

3. Various tissues of Chinese hamster have almost identical sequence of centromere separation /9/. In harmony with this finding, the similar sequences described by various authors in human lymphocytes, bone marrow, fibroblasts, amniotic cells, and now in chorionic villi suggest that the separation sequence is a species specific, genetically controlled phenomenon.

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BOOK REVIEWS

RECENT ADVANCES IN NERVOUS SYSTEM TOXICOLOGY

C.L. Galli, L. Manzo and P.S. Spencer (eds)

Plenum Press, New York and London, 1988
ISBN 0306-42209-3

A collection of rather divergent 18 papers is presented in this Volume, linked with the common characteristics that they deal with the actions of chemical agents on the nervous system of animals and man. The wide variety both of the methods and of the types of chemicals represented by the articles gives inevitably the impression of contingency, but on the other hand, with this selection the necessity of interdisciplinary and multidisciplinary approach is emphasized.

There are three main chapters: Neurotoxic effects of prolonged exposure to toxic agents; Neurotoxic agents in the human environment; Experimental models and assessment of neurotoxic mechanisms.

Biochemical contributions deal with molecular, enzymological and metabolic approaches, including hormonal and transmitter regulation partly in relation to clinical practice. Electrophysiological papers describe membrane and channel effects of some toxicants, namely of pyrethroid insecticides and neurotoxic amino acids. Clinical and pathological studies include the effect of toxic metals and organic compounds, referring to the mechanism of toxicity at different levels.

Most of the papers is much more then presentation of own results which is of great value for readers who wish to obtain a deeper insight into the problems and recent results in environmental neurobiology, neurotoxicology. The reference lists following each paper increase the value of the Volume in this respect.

Because of the mutlilateral influence of environmental toxicants on animal and human behaviour neurotoxicological research will certainly expand in the future, and the results presented in this Volume serve as good examples both for methodical and methodological handling of the problem.

J. Salánki (Tihany)

BIOLOGICAL ROLE OF PLANT LIPIDS

P.A. Biacs, K. Griuz and T. Kremmer (eds)

Akadémiai Kiadó, Budapest, 1989, pp 626

The book collects the lectures and posters presented at the "8th International Symposium on the Biological Role of Plant Lipids" held in Budapest, July 25-28, 1988. The 140 papers are divided into 7 chapters, such as: "Lipid metabolism", Structural and functional organisation of plant lipids", Biosynthesis and function of prenyl lipids", "Carrier proteins and genetics of plant lipids", Biocydes, interaction with lipids", "Biotechnology of plant lipids", "Development and environmental stress". Up-to-date information about the present state of lipid and membrane research can be obtained from the book. Those, for instance, interested in molecular organization of photosynthetic membrane or membrane lipids as well as in the role of lipids in signal transduction in plant systems will find the most recent results from the most authentic authors. Molecular biology is represented by several highly interesting papers such as data on cloning and sequence determination of a complementary DNA for glycerol-3-phosphate acyltransferase. Similarly, detailed information on the role of plant lipids in targeting biocydes and on the role of lipid metabolism in development of herbicide resistance will make the book attractive to the readers. An exiting chapter deals with responses given to different environmental stresses with special emphasis on temperature and water stress. Data on homeoviscous adaptation of plant membranes and membrane lipids subjected to temperature stress or artificial modification by catalitic hydrogenation of unsaturated fatty acyl chains are also presented. The book is recommended for scientists as well as to those teaching lipid biochemistry or plant physiology.

T. Farkas (Szeged)

SCANNING MICROSCOPY OF VERTEBRATE MINERALIZED TISSUES

L. Martin, A. Boyde, F. Grine and Sh. Jones (eds)

Scanning Microscopy International, 1988, pp 384

The papers included in this book were originally published in the earlier volumes of J. Scanning Microscopy and J. Scanning Electron Microscopy. They are arranged into five chapters the first of which is devoted to hard tissue structure and remodelling. The reviews and articles of this chapter deal with the scanning microscopical characterization of bones, teeth and cartilages. The next section contains three papers on bone pathology and dental/enamel caries. The following two chapters provide an extensive coverage of the present investigations on dental enamel structure and microwear carried out by the use of scanning electron microscope. Finally, the last chapter of the book is devoted to vertebrate egg-shell structure.

The book as a whole gives a clear overview to the reader on recent problems, trends and achievements in the research of mineralized tissues by

the application of scanning electron microscope. The reports are written by leading experts in the field. The superior quality of illustrations and the excellent layout of the book deserves special notion. Thanks to the policy of the Editors and Publisher, place was available to present the questions and comments of reviewers to each article. These discussions greatly facilitate the understanding of the problems investigated by the authors.

I recommend the book to all those interested in the research of bones, teeth and other mineralized tissues and to researchers using SEM and microanalysis for studying biological specimens.

J. Kovács (Budapest)

PROTOZOA AND OTHER PROTISTS

M. Sleigh

Edward Arnold, Div. Hodder and Stoughton Publishers, London, New York, Melbourne, Auckland, pp 342, 158 figures, 13 tables

ISBN 0-7131-2943-3

Research using protozoa has flourished in recent years not only because of studies directed at increasing our knowledge of the protozoa themselves, but also because it has been recognized that these organisms are excellent objects for studies of general biological phenomena at the cellular level. The flourishing of this research activity has principally been directed towards elucidating the structure and understanding the functioning of protozoa as cells.

The author has written in the preface: "It is the main aim of this book to emphasize new insights into cell functioning and ultrastructure as they apply to protozoa, and to illustrate the wealth of diversity in organization and physiology that has been revealed among the various groups of organisms classed as Protozoa."

The realization of this aim presents many difficulties because of the extent of knowledge in protozoology exceed a single volume. I think that the author solved this problem successfully. All the important fields of protozoological knowledges are proportionately discussed. After a very good outline of the origins, evolution and cellular organization of protists follows an outline of their nutrition, metabolism and growth. The chapter in which the nuclear and genetic aspects of reproduction and the division of the cell and morphogenesis of protists is described, is perhaps the best part of this volume. Additional chapters take a clear survey of the main phyla of flagellated, ciliated and spore-forming parasitic protozoa, and the ecology of protists.

The amount of information is growing rapidly, often faster than what we can keep pace with, and this volume provides a welcomed review of some of the most important advancing fields. This book should be in the library of every biologist who uses protists for experiments.

P. Kovács (Budapest)

CONSERVATION AND MANAGEMENT OF LAKES - SYMPOSIA BIOLOGICA HUNGARICA 38.

J. Salánki and S. Herodek, (eds)

Akadémiai Kiadó, Budapest, 1989, $ 64

The book contains 50 selected papers presented at the Third International Conference on the Conservation and Management of Lakes, held in Keszthely (Lake Balaton, Hungary) 11-17 September 1988, and hosted by the Hungarian Academy of Sciences and the Ministry for Environment and Water Management. The International Lake Environment Committee Foundation (Otsu, Shiga Prefecture, Japan) and the United Nations Environment Programme (Nairobi, Kenya) supported the organization of the Conference and also the publication of this volume.

156 experts from 31 countries took part in the conference. The volume consists of 6 chapters, with 645 pages with 177 figures and 98 tables. Finally, the list of participants and the subject index are presented.

The main topics of the conference were as follows: Eutrophication and its control; Acidification and toxic pollutants; Lake protection over the world; Management of shallow waters; Lake management as an ecological, economic and jurisdictional complex; and Modelling in lake management; all these conformed to the titles of the six chapters.

The above mentioned topics are discussed in 229 water bodies, among others as Laurentian Great Lakes, Lake Biwa, Lake Viktoria, Lake Baikal, Lake Ladoga, and in little, shallow fish ponds, too. The great amount and the variability of lakes discussed make the book a bit mozaik-like, but at the same time, opportunity is given to compare the different lakes and to synthetize the results of such multi-dimensional research activities as the elaboration of eutrophication-models, solution of acidification-, and lake management problems.

The introduction of socioeconomic, institutional and jurisdictional aspects of hydrobiology into the topics of a conference dealing with conservation and management of lakes was really an up-to-date approach, and perhaps therefore experts with different qualification (hydrobiologists, decision makers, managers, students) will find this volume useful.

Abaffyné Bothár Anna (Göd)

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