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METABOLISM OF PROGESTERONE IN THE BRAIN AND IN THE ANTERIOR PITUITARY: SIGNIFICANCE FOR THE CONTROL OF GONADOTROPIN SECRETION*

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Introduction

It has recently been reported that in several of its target structures (uterus, anterior pituitary, hypothalamus, etc.) progesterone (P) is metabolized into the following 5α-reduced metabolites: 5α-pregnan-3,20-dione (dihydroprogesterone, DHP) and 5α-pregnan-3α-ol-20-one (3α-ol) [1, 8, 9, 12, 15, 17]. The hypothesis has been put forward that the formation of these compounds represents an essential step for progesterone to exert its biological effects. Such a hypothesis is similar to the one proposed for explaining the mode of action of testosterone [13].

The experiments here to be described have been planned in order to verify whether the formation of DHP and of 3α-ol in the anterior pituitary and in the central nervous system plays a role in the expression of the central effects of P. The following two approaches have been adopted: A) study of the metabolism of progesterone in the anterior pituitary and in the central nervous system in different conditions; and B) study of the effects of progesterone metabolites on gonadotropin secretion.

Results and discussion

Metabolism of progesterone in the anterior pituitary and in the central nervous system

The ability of the anterior pituitary, of the hypothalamus, of the amygdala and of the cerebral cortex to convert P into its 5α-reduced metabolites has been studied by incubating "in vitro" fragments of the different tissues in the presence of labelled P and by identifying, at the end of the incubation period, the different metabolites formed. Samples of the various structures

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were collected from adult female rats in different physiological conditions or following different types of experimental manipulations. The uterus was used as the control tissue. In order to have, for each tissue examined, a quantitative and separate estimate of the activity of the enzyme involved in the transformation of P into DHP and 3α-ol the following two parameters have been selected: (1) the total amounts of 5α-reduced metabolites formed by each tissue (i.e., the sum of the amounts of DHP plus those of 3α-ol); this figure provides a clear indication of the activity of the 5α-reductase present in the different tissues; and (2) the per cent quantities of 3α-ol contributing to the total amounts of 5α-reduced metabolites; this index provides a satisfactory estimate of the efficiency of the enzyme 3α-hydroxysteroid-dehydrogenase which converts DHP into 3α-ol.

Figure 1 shows that the uterus of normal female animals is able to convert P into the corresponding 5α-reduced metabolites with rather elevated yields. The uterine 5α-reductase exhibits conspicuous changes in activity during the different phases of the estrus cycle. The highest amounts of 5α-reduced metabolites are formed by the uterus of animals killed in the morning of estrus. After that time, a progressive decline of the 5α-reductase activity occurs, the lowest values being detected in animals sacrificed in the morning of proestrus. The major 5α-reduced metabolite formed in the uterus is 3α-ol (about 80% of the total) (not shown in the Figure). Figure 1 also indicates that the anterior pituitary of normal female rats is able to convert P into the 5α-reduced metabolites. The 5α-reductase activity of this gland is much lower than that of the uterus. During the different phases of the estrus cycle, changes similar (although quantitatively smaller) to those found at uterine level have been

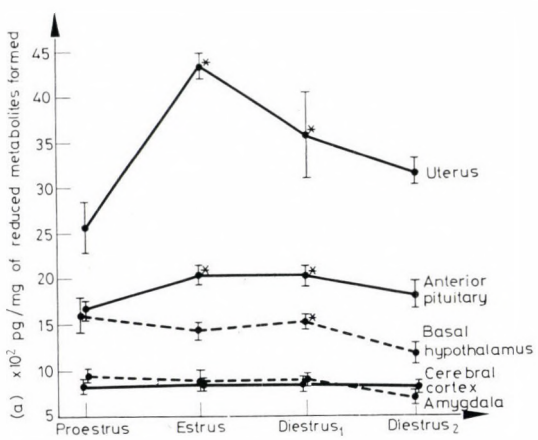

Fig. 1. Conversion of progesterone to 5α-reduced metabolites (5α-pregnan-3,20-dione, DHP; 5α-pregnan-3α-ol-20-one, 3α-ol) by the uterus, the anterior pituitary, the basal hypothalamus, the amygdala and the cerebral cortex of normal adult female rats in the different phases of the estrus cycle

recorded. In all phases of the estrus cycle, 45—50% of the 5α-reduced metabolites made by the gland are in the form of 3α-ol (not shown in the Figure).

The hypothalamus of normally cycling female rats is able to convert P into DHP and 3α-ol. The 5α-reductase activity of this structure is smaller than that of the anterior pituitary, and does not show a clear cyclicity during the estrus cycle (Fig. 1). The hypothalamus has a very limited 3α-hydroxysteroid-dehydrogenase activity, 3α-ol only representing 20—30% of the reduced metabolites found at the end of the incubation period (not shown in the Figure). The cerebral cortex and the amygdala of adult female rats possess some 5α-reductase activity (Fig. 1). Such an activity is quantitatively similar in the two structures, is lower than that found in any of the tissues previously considered, and does not show any estrus-linked type of cyclicity. In these two structures the formation of 3α-ol varies between 30 and 40% (not shown in the Figure).

At uterine level, castration induces a significant decrease of the 5α-reductase activity (Table 1). A minimum is reached two days after the opera-

Table 1

Conversion of progesterone to its 5α-reduced metabolites (5α-pregnan-3,20-dione, DHP and 5α-pregnan-3α-ol-20-one, 3α-ol) by the uterus of normal, castrated and estrogen (estradiol benzoate, EB, 50 μg/rat/day) pretreated castrated female rats[1]

Groups[2]	5α-reduced metabolites pg/mg[3]	% of 3α-ol
Estrus (4)	4 378.5 ± 117.5	83.62 ± 1.73
Diestrus$_2$ (3)	3 200.7 ± 134.9	78.80 ± 2.87
Castrated		
2 days (3)	1 559.7 ± 160.5[4,5]	78.46 ± 3.84
7 days (3)	2 215.8 ± 326.5[6,7]	79.53 ± 1.13
14 days (6)	2 130.6 ± 295.9[4,7]	72.22 ± 4.19
21 days (6)	2 597.8 ± 494.6[6]	76.20 ± 2.60
Castrated + EB		
7 days (4)	3 669.1 ± 326.0[8]	76.55 ± 2.27
14 days (6)	4 363.0 ± 750.3[9]	66.66 ± 6.04
21 days (4)	3 212.8 ± 414.0	63.00 ± 7.46

[1] Values are means ± S.E.
[2] Number of experiments performed in parentheses
[3] Picograms of 5α-reduced metabolites formed per mg of wet tissue following a 3-hour incubation with 150 nanograms of (4-C^{14}) progesterone (specific activity: 60.0 mCi/m mol)
[4] $p < 0.0005$ *vs* Estrus
[5] $p < 0.0025$ *vs* Diestrus$_2$
[6] $p < 0.0025$ *vs* Estrus
[7] $p < 0.025$ *vs* Diestrus$_2$
[8] $p < 0.025$ *vs* Castrated 7 days
[9] $p < 0.01$ *vs* Castrated 14 days

tion. At later intervals, the activity of the enzyme tends to rise again but without approaching the levels found at diestrus (i.e., the lowest values recorded before castration). These data agree with and extend the observations made by ARMSTRONG and KING [1]. In agreement with the findings of these authors, the rate of conversion of DHP into 3α-ol is not significantly altered by castration. Like in normal animals, 3α-ol represents about 80% of the total 5α-reduced metabolites formed. Contrary to what happens in the uterus, castration induces a progressive increase in the ability of the anterior pituitary gland to transform P into DHP and 3α-ol (Table 2). The levels found 7 days after the operation are twice as high as those found at estrus. They double again at 14 days after castration and subsequently seem to stabilize at levels which are four times those of non-castrated animals. An increase of the formation of 3α-ol is observed 2 days after castration. On the contrary, a decrease

Table 2

Conversion of progesterone to its 5α-reduced metabolites (5α-pregnan-3,20-dione, DHP and 5α-pregnan-3α-ol-20-one, 3α-ol) by the anterior pituitary of normal, castrated, and estrogen (estradiol benzoate, EB, 50 μg/rat/day) pretreated castrated female rats[1]

Groups[2]	5α-reduced metabolites pg/mg[3]	% of 3α-ol
Estrus (5)	2 057.0 ± 99.5	46.98 ± 4.32
$Diestrus_2$ (4)	1 856.5 ± 132.2	44.01 ± 2.30
Castrated		
2 days (3)	2 144.7 ± 69.9	51.50 ± 0.32[4]
7 days (6)	4 625.2 ± 444.2[5,6]	45.50 ± 2.51
14 days (6)	8 104.8 ± 451.4[5,6]	27.38 ± 3.56[7]
21 days (5)	8 043.3 ± 1 234.4[5,6]	33.18 ± 4.45[8,9]
Castrated + EB		
7 days (4)	2 513.7 ± 321.9[10]	46.00 ± 5.58
14 days (6)	2 459.4 ± 127.7[11]	54.50 ± 1.98[11]
21 days (6)	1 722.1 ± 122.6[12]	55.55 ± 4.25

[1] Values are means ± S.E.
[2] Number of experiments performed in parentheses
[3] Picograms of 5α-reduced metabolites formed per mg of wet tissue following a 3-hour incubation with 150 nanograms of (4-C^{14}) progesterone (specific activity: 60.0 mCi/m mol)
[4] $p < 0.025$ *vs* $Diestrus_2$
[5] $p < 0.005$ *vs* Estrus
[6] $p < 0.0025$ *vs* $Diestrus_2$
[7] $p < 0.005$ *vs* $Diestrus_2$
[8] $p < 0.05$ *vs* Estrus
[9] $p < 0.05$ *vs* $Diestrus_2$
[10] $p < 0.05$ *vs* Castrated 7 days
[11] $p < 0.0005$ *vs* Castrated 14 days
[12] $p < 0.0025$ *vs* Castrated 21 days

in the proportions of this steroid is observed at later intervals following ovariectomy. Castration does not exert any relevant effect on the 5α-reductase and 3α-hydroxysteroid-dehydrogenase activities of the hypothalamus, of the amygdala and of the cerebral cortex (data not shown).

As reported by ARMSTRONG and KING [1], the "in vivo" administration of estradiol to ovariectomized female rats induces an increase in the 5α-reductase activity of the uterus (Table 1). This reaches levels similar to those found in the morning of estrus.

In contrast with these observations, "in vivo" treatment with estrogen significantly decreases the 5α-reductase activity of the anterior pituitary of ovariectomized animals (Table 2); after treatment, the amounts of 5α-reduced metabolites formed by the gland revert to the levels found in normally cycling animals. Estrogen pretreatment brings back to normal (or even increases above physiological levels) the per cent formation of 3α-ol in long-term ovariectomized rats. The administration of estrogen to ovariectomized females does not exert any significant effect on the 5α-reductase activity of the hypothalamus, of the amygdala and of the cerebral cortex (data not shown).

The observation that the anterior pituitary and the hypothalamus are able to convert P into DHP and 3α-ol is confirmatory of previous findings of this [15] and other laboratories [8, 9, 12, 17], and may bring to postulate a physiological role of these two metabolites in the expression of the feedback activities P exerts on the hypothalamic–pituitary complex. This hypothesis is reinforced by the observation that the 5α-reductase activity of the anterior pituitary is influenced by estrus cyclicity, and can be modified in opposite directions by castration and by the administration of exogenous estrogens, two manipulations which have a deep impact on gonadotropin secretion. The fact that castration and estrogen treatment do not influence the enzymatic activity of the hypothalamus seems to indicate that the feedback effects of P are mainly exerted at anterior pituitary level [15].

The results obtained at pituitary level following ovariectomy are similar to previous data of this and other laboratories [3, 4, 10, 11, 14] which have shown that, in male rats, orchidectomy is followed by an elevation of the 5α-reductase activity of the anterior pituitary. Also the observation that ovariectomy does not exert significant effects on the 5α-reductase levels of the hypothalamus, of the amygdala and of the cerebral cortex agrees with previous findings in males [3, 10, 14].

It is not clear why castration and estrogen administration should exert opposite effects at anterior pituitary and at uterine level. It is possible that the post-castration increase of the 5α-reductase activity of the anterior pituitary reflects the changes in the composition of pituitary cell populations which follow the operation. It is known that gonadotrophs increase in size and proliferate after gonadectomy [5]. Should this hypothesis be correct,

the data might be taken as providing evidence for a specific localization of the 5α-reductase in the gonadotrophs. This hypothesis is supported by the observation that the 5α-reductase activity of the pituitary of ovariectomized animals reverts to normal after the administration of estrogens which restore normal pituitary histology. The hypothesis that the 5α-reductase might be specifically associated with the gonadotrophs has been recently corroborated by LLOYD and KARAVOLAS [12], who have found the enzyme to be present in high concentrations in gonadotrophs-enriched pituitary cell preparations. With regard to the uterus, it is possible that the decrease of the 5α-reductase activity observed immediately after castration is linked to the disappearance of estrogens from the circulation, and that the subsequent tendency to return towards normal levels as post-castration time progresses might be correlated with a compensatory rise of estrogens of non-ovarian origin (e.g., adrenal gland? peripheral conversion from androstenedione?). Exogenous estrogens have been shown in this and in other studies [1, 6, 18] to increase the 5α-reductase activity of this structure.

Effects of progesterone metabolites on gonadotropin secretion

P is known to influence the secretion of anterior pituitary gonadotropins. Depending on the circumstances, P may exert either inhibitory (negative feedback effect) or stimulatory (positive feedback effect) actions [16]. In order to clarify whether these activities of P might be mimicked by DHP and/or 3α-ol, the effects exerted by these two steroids on gonadotropin secretion have been evaluated in adult female rats, using two different experimental models, designed to test respectively their facilitatory and their inhibitory activities. In order to analyze whether DHP and 3α-ol are able to stimulate gonadotropin release, these steroids have been administered to castrated estrogen-pretreated female rats. It is a well documented phenomenon that P and several physiological or synthetic progestogens exert a positive feedback effect on LH and/or FSH secretion in animals so prepared [7, 19, 20]. In order to test their inhibitory activity on gonadotropin secretion, DHP and 3α-ol were administered in rather large amounts to castrated female rats without any estrogen pretreatment [see 21 for details].

Table 3 summarizes the data obtained in the experiments aimed at evaluating the possibility that DHP and 3α-ol might facilitate the release of pituitary gonadotropins. It appears that castrated oil-treated female rats have rather elevated levels of serum LH. In agreement with previous data of this and of other laboratories these are significantly depressed by the injection of ethinyl estradiol (EE) [19, 22]. The injection of P on the fifth day of estrogen priming is followed by a significant increase of serum levels of LH. DHP and 3α-ol are both able to significantly elevate serum levels

Table 3

Effect of 100 μg/rat of progesterone (P) 5α-pregnan-3,20-dione (DHP) and 5α-pregnan-3α-ol-20-one (3α-ol) on serum levels of LH and FSH of castrated female rats pretreated for 5 days with 0.4 μg ethinyl estradiol (EE) and sacrificed 8 hours after progestogen administration[1]

Treatment[2]		ng LH/ml (NIH-LH-S17)	ng FSH/ml (NIAMD-RAT-FSH-RPI)
1 Oil	(24)	27.36 ± 1.73	1 027.24 ± 40.00
2 EE (0.4 μg)	(18)	3.43 ± 0.39[3]	283.87 ± 7.95[3]
3 EE + P (100 μg)	(20)	8.49 ± 0.82[4]	1 209.46 ± 31.75[4]
4 EE + DHP (100 μg)	(18)	5.87 ± 1.06[5,6]	566.74 ± 35.01[4]
5 EE + 3α-ol (100 μg)	(16)	8.45 ± 1.16[4]	603.11 ± 19.84[4]

[1] Values are means ± S.E.
[2] Number of animals in parentheses
[3] $P < 0.0005$ *vs* 1
[4] $P < 0.0005$ *vs* 2
[5] $P < 0.0125$ *vs* 2
[6] $P < 0.025$ *vs* 3

Table 4

Effect of 2 mg/rat of progesterone (P), 5α-pregnan-3,20-dione (DHP) and 5α-pregnan-3α-ol-20-one (3α-ol) on serum levels of LH and FSH of female rats castrated since 3 weeks and sacrificed 24 hours after progestogen administration[1]

Treatment[2]		ng LH/ml (NIH-LH-S17)	ng FSH/ml (NIAMD-RAT-FSH-RPI)
1 Oil	(23)	21.95 ± 0.83	916.89 ± 27.15
2 P (2 mg)	(26)	21.06 ± 0.97	939.99 ± 26.82
3 DHP (2 mg)	(21)	15.21 ± 0.79[3]	647.03 ± 17.78[3]
4 3α-ol (2 mg)	(25)	11.48 ± 0.97[3,4]	545.28 ± 12.75[3]

[1] Values are means ± S.E.
[2] Number of animals in parentheses
[3] $P < 0.0005$ *vs* 1
[4] $P < 0.0005$ *vs* 3

of LH over those observed in animals given EE alone. In this test, 3α-ol appears to be at least as effective as P. DHP, on the contrary, is less effective than P. Table 3 summarizes also the data obtained in the same groups of animals by measuring plasma FSH. It is apparent that the elevated levels of FSH found after ovariectomy can be depressed by the administration of EE. This is confirmatory of previous data of this and other laboratories [19, 22]. It is also clear that under the experimental conditions selected P is able to exert a very strong positive feedback effect on the release of FSH. DHP and 3α-ol also exert a stimulatory effect on FSH release; however, such an effect is less pronounced than that of P, and is quantitatively similar for the two steroids.

The data of the experiments in which the negative feedback effects of P, DHP and 3α-ol have been evaluated are reported in Table 4. It is evident that, in the absence of estrogen pretreatment and under the experimental conditions selected, P does not inhibit either LH or FSH release. On the contrary, DHP and 3α-ol exert a rather strong blocking effect on both gonadotropins.

The observation that DHP and 3α-ol display a positive feedback effect on LH and FSH secretion qualitatively similar to that of P in castrated estrogen-primed female rats is compatible with the hypothesis that P exerts its facilitatory effects on gonadotropin secretion after the local (intrapituitary?) conversion into DHP and 3α-ol [8, 9, 12, 15, 17]. The fact that the activity of 3α-ol is quantitatively similar to that of P in releasing LH suggests that this metabolite might be the intracellular mediator for the positive feedback P exerts on LH release. The finding that DHP and 3α-ol are able to inhibit gonadotropin release in castrated female rats even if P is ineffective is puzzling. There are several possible interpretations of these results. One possibility is that P operates on the negative feedback system only after conversion into DHP and 3α-ol. If one accepts such an interpretation, the inactivity of P here reported might be explained by the fact that the short interval used in the experiments did not allow a sufficient conversion of the steroid into its 5α-reduced metabolites. However, such a possibility appears to be a rather remote one on the basis of the observations previously reported that after castration the 5α-reductase activity of the anterior pituitary increases severalfold. Consequently in the present experimental conditions a rather large conversion of P into DHP and 3α-ol could be expected. Another possibility is that the negative feedback effect of P (in the experimental conditions in which it appears) [2] is a property of the steroid as such. For the reasons just mentioned, the authors would favour such a view, and would consider the inhibitory activity exerted on LH and FSH secretion by the 5α-reduced metabolites as an inherent property of these steroids. If such an interpretation will be proven valid by subsequent experiments, the data here presented might provide a preliminary answer to the question of the opposite effects P may exert on gonadotropin secretion. Apparently, the positive effect seems to be due to the conversion into the 5α-reduced metabolites, while the negative feedback effect appears to be a property of P as such.

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BRAIN CATECHOLAMINES AND PITUITARY-OVARIAN FUNCTION[1,2]

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Results and discussion

Our interest in catecholamines and pituitary-ovarian function dates back 30 years to the time when, with MARKEE and HOLLINSHEAD at Duke University, we were able to induce ovulation in the rabbit by infusing a little adrenalin into the pituitary gland approached parapharyngeally [24]. Ovulation was a good index of stimulation of release of gonadotropin in this species because the rabbit does not ordinarily ovulate spontaneously but only after coitus, and ruptured ovarian follicles are easily seen with the naked eye at laparotomy 48 hours after treatment. Our original intrapituitary infusions were made with Parke—Davis Adrenalin, an extract of the adrenal medulla containing both epinephrine and norepinephrine. At that time, in the late 1940's, pure epinephrine and norepinephrine became available, and we found [33] that they were about equally effective in inducing ovulation when infused by stereotaxic cannula into the third ventricle of the brain (Fig. 1). These were relatively acute experiments performed under ether anesthesia, as were the intrapituitary infusions. These findings raised the question as to the site of the catecholamine's stimulatory action, i.e., brain or pituitary gland?

In results hitherto published only in part [33] we discovered that the ovulation-inducing intraventricular action of epinephrine could be blocked by agents which came later to be known as "α-blockers" (phentolamine and the β-chlorethylamine product, SKF-501) but not by pentobarbital or large doses of atropine sulfate (Table 1). On the other hand, norepinephrine's effectiveness was blocked by weak pentobarbital and by a large dose of atropine sulfate as well as by SKF-501. Blockade by pentobarbital would

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[2] Lecture delivered at the inauguration of the author as Honorary Member of the Hungarian Society of Endocrinology and Metabolism. July 14, 1976.

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suggest that norepinephrine's ovulatory stimulus was exerted centrally rather than at the pituitary level. Phentolamine was less potent than SKF-501 in blocking the ovulatory action of norepinephrine.

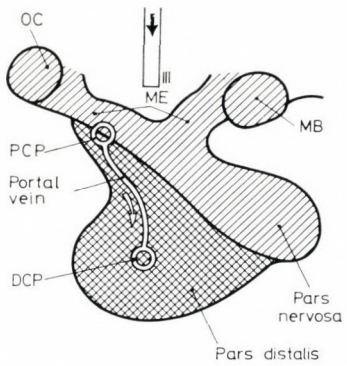

Fig. 1. Ovulatory effects of infusing catecholamines into the third ventricle of the brain of the estrus rabbit (cf. Table 1). Data from SAWYER [33]

We also tested these newly developed adrenergic blocking agents against the natural coital stimulus for ovulation. Rapid postcoital intravenous injections of Dibenamine (a β-chlorethylamine α-blocking derivative like SKF-501 and phenoxybenzamine) blocked ovulation (Fig. 2), but injections delayed over a minute did not [36] even though the pituitary requires at least half an hour to release enough gonadotropin to induce ovulation [9]. Phentol-

Table 1

Effects of various blocking agents on the induction of ovulation in the rabbit by intraventricular injections of epinephrine and norepinephrine (stereotaxic surgery under local procaine anesthesia)

Agent	Dosage µg base	Blocking agent	Dosage and route	Ovulation Animals ovulated/tested
l-epinephrine	100	—	—	6/10
	100	Phentolamine	15 mg/kg iv	1/10
	100	SKF-501	10 mg/kg iv	0/5
	500	Pentobarbital	15 mg/kg iv	3/4
	500	Atropine-SO_4	300 mg/kg sc	4/5
l-norepinephrine	100	—	—	7/10
	100	Phentolamine	15 mg/kg iv	3/10
	100	SKF-501	10 mg/kg iv	1/10
	500	—	—	8/8
	500	Pentobarbital	15 mg/kg iv	1/10
	500	Atropine-SO_4	300 mg/kg sc	0/4

amine was ineffective in blunting the coital stimulus but rapid post-coital injections of atropine sulfate did block ovulation, suggesting a cholinergic synapse in the system [35, 37]. These findings pointed to a rapidly completed noradrenergic central nervous process as a component of the coital stimulus for ovulation in the rabbit. As a sequel to these experiments we discovered with EVERETT that "spontaneous" cyclic ovulation in the rat could be blocked by administering Dibenamine or atropine sulfate prior to 2 P.M. on the day of proestrus, a finding which led to the concept of a proestrus "critical period" in the neurogenous activation of LH release in this cyclic ovulator [8].

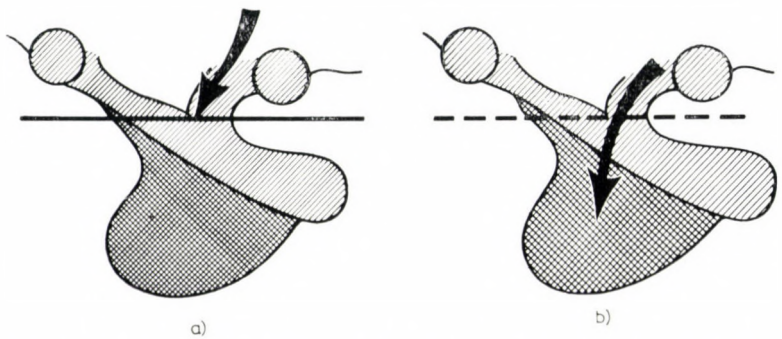

Fig. 2. A rapid post-coital intravenous injection of the α-adrenergic-blocking agent Dibenamine prevents the coital stimulus (arrow) from activating pituitary LH release and ovulation. Data from SAWYER et al. [36]

In the early 1950's DONOVAN and HARRIS repeated our initial rabbit experiments, with very slow infusions of epinephrine into the pituitary gland approached by a stereotaxic cannula through the brain. Under these conditions they failed to induce ovulation and they reached the correct conclusion that the neurohumoral mediator from brain to pituitary was not epinephrine [7, 12]. However, they ignored their positive cases which might have suggested a central mechanism. Meanwhile VOGT [47] had demonstrated high concentrations of norepinephrine (NE) in the dog hypothalamus, and a few years later CARLSSON [2] described large amounts of dopamine in the brain, especially in the caudate nucleus. In the early 1960's an exquisite fluorescence technique for visualizing amines in histological sections of the brain was developed by the Swedish investigators HILLARP and FALCK and applied extensively by FUXE, HÖKFELT, BJÖRKLUND and others [see 13 for references]. Very high concentrations of catecholamines were found in the median eminence, and much of the fluorescence in the external zone was traced to dopaminergic neurons in the arcuate nucleus and paraventricular region — a tuberoinfundibular tract. Elaborate maps were plotted of the distribution of noradrenergic and dopaminergic fibers [46], and it appeared that noradrenergic fibers ending in the inner zone of the median eminence were ascending from the brain stem.

Using the deafferentation technique introduced by HALÁSZ and PUPP [11] in Hungary, WEINER and associates [50] in our laboratory showed that the hypothalamic island lost all of its NE within three weeks of deafferentation, whereas dopamine was not significantly changed (Fig. 3). The results were consistent with a brain stem origin of NE whereas many dopaminergic neurons lay completely within the island.

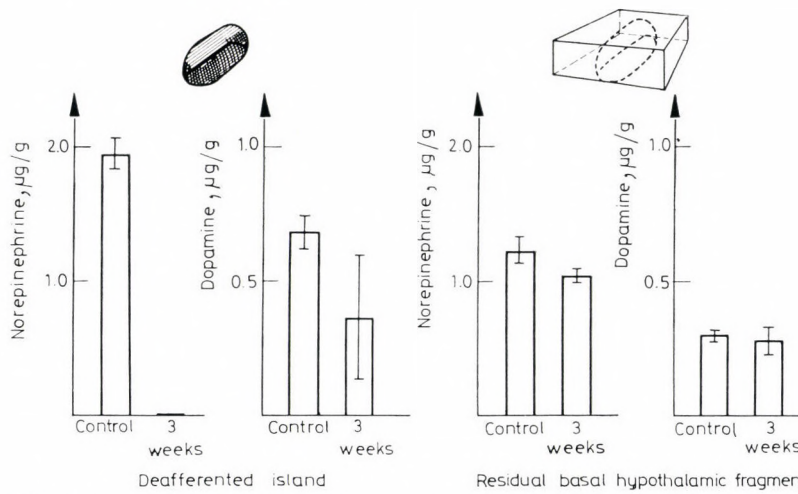

Fig. 3. Effects of complete deafferentation of the hypothalamus on the norepinephrine and dopamine concentrations in the deafferented island itself, left, and in the residual basal hypothalamic fragment, right. From WEINER et al. [50]

Another technique introduced by a Hungarian anatomist has been used to study the catecholamine distribution in the hypothalamus — the "punch out" method of PALKOVITS [27]. Tiny cylinders of tissue were cut out of frozen sections of the brain and assayed for catecholamines by an enzymatic–isotopic micro method. Dopamine was found to be most highly concentrated in the median eminence and arcuate nucleus, with somewhat lesser amounts in the retrochiasmatic area, paraventricular and dorsomedial nuclei, and NE was concentrated in the paraventricular and suprachiasmatic nuclei, perifornical and retrochiasmatic areas and the ventral part of the dorsomedial nucleus; in the median eminence it was about half as concentrated as DA and surprisingly small amounts were found in the medial forebrain bundle [28].

SELMANOFF et al. [39] have recently employed the PALKOVITS microdissection technique to study changes in DA and NE in selected hypothalamic nuclei between 1000 and 1700 hr on diestrus-1 (D1) and proestrus. They found no changes in either catecholamine on D1 but on proestrus NE in the suprachiasmatic nucleus (SCN) rose 3× between 1000 and 1700. There was no change in other nuclei and no change in DA, even in the SCN, which suggests that NE terminals in SCN may be involved in the proestrus ovulatory

surge of gonadotropin, and supports the prior suggestion of STEFANO and DONOSO [41] reached with less sensitive techniques.

One of the problems of the enzymatic–isotopic method used by PALKOVITS, SELMANOFF and their colleagues [28, 39] is that it does not distinguish between epinephrine (E) and norepinephrine (NE), an item of potential importance inasmuch as the enzyme phenylethanolamine-N-methyl transferase (PNMT) which converts NE to E has been found to be widely distributed in the brain [31]. With an immunohistochemical technique for PNMT, HÖKFELT et al. [14] found the enzyme in a distribution roughly paralleling what had been earlier considered noradrenergic pathways. This raises the possibility that mechanisms heretofore attributed to NE may be exerted in part by brain epinephrine.

The immunohistochemical demonstration of the enzymes synthesizing catecholamines has largely replaced the nonspecific fluorescence method of mapping adrenergic pathways in the brain [15, 42]. Immunofluorescence preparations of tyrosine hydroxylase and dopa-decarboxylase have been used to localize dopaminergic cells and fibers and dopamine-β-hydroxylase (DBH) as a marker for noradrenergic neurons. The proximity of dopaminergic fibers to projections of luteinizing hormone releasing hormone (LH—RH) in the median eminence is shown in Fig. 4. SWANSON and HARTMAN [42] have remapped the rat brain for noradrenergic pathways with DBH immunofluorescence and have described a principal adrenergic pathway from the locus coeruleus, subcoeruleus and dorsal vagal nucleus projecting rostralward ventrolateral to the central grey in the midbrain, through the zona incerta in the thalamus and through the bed nucleus of the stria terminalis. They could not confirm the localization of UNGERSTEDT's [46] ventral noradrenergic pathway in the medial forebrain bundle and they suggested that the latter might be a dopaminergic projection.

With some disagreement on the anatomical distribution of catecholamines in the hypothalamus, there has been even more controversy about the functional roles of dopamine (DA) and NE in controlling gonadotropic secretion. From their fluorescence studies during the rat estrus cycles FUXE and HÖKFELT [10] concluded that DA is inhibitory to LH—RH release and their claims have been confirmed indirectly by UEMURA and KOBAYASHI [45] and CRAVEN and MCDONALD [6]. However, SCHNEIDER and MCCANN [38] and KAMBERI et al. [18] have reported that DA is more effective than NE in triggering LH release. On the other hand, in MCCANN's laboratory the KALRAS have blocked LH release by inhibiting DBH synthesis of NE and restored gonadotropic function by resynthesizing NE from dihydroxyphenylserine (DOPS) which bypasses DBH inhibition [16, 17].

These procedures did not block DA synthesis and they reaffirmed the importance of NE in controlling LH release. In the estrus rabbit we [34]

found that intraventricular NE triggered LH release in amounts and timing comparable to the response to coital stimulation but that intraventricular DA not only failed to stimulate LH release but actually inhibited the release which would have followed the injection of NE (Fig. 5). PRZEKOP et al. [29] also reported the induction of LH release and ovulation in rabbits as well as in sheep by intraventricular NE but not by DA.

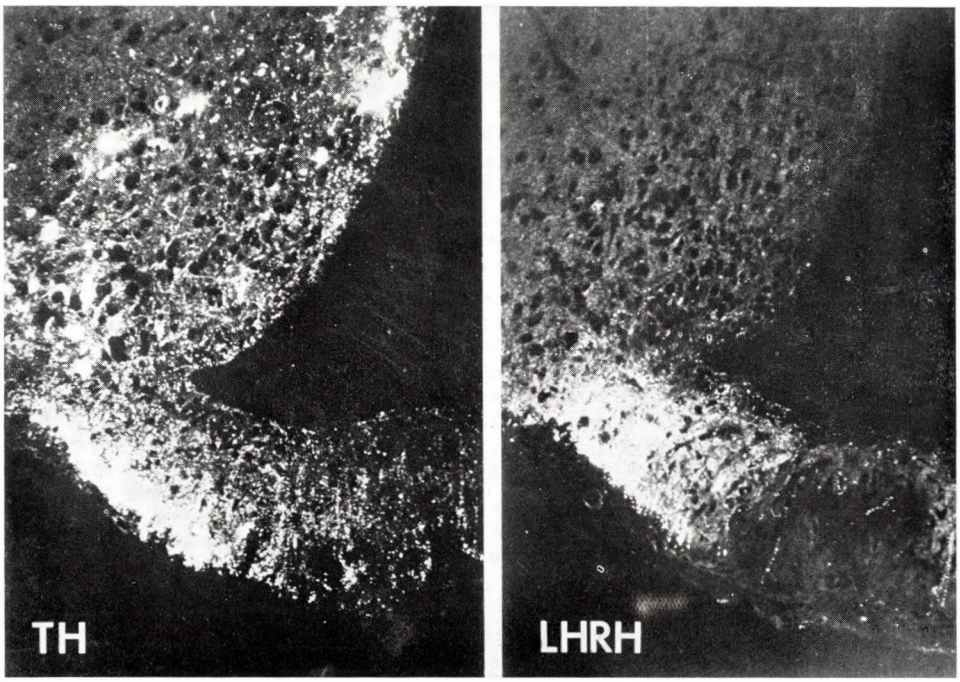

Fig. 4. Immunofluorescence preparations of adjacent sections through the median eminence and arcuate nucleus of the rat hypothalamus showing overlapping distributions of tyrosine hydroxylase (TH) and luteinizing hormone–releasing hormone (LH—RH) in the lateral external zone of the median eminence. Figure from HÖKFELT et al. [15]

In the female rat several investigators have recently studied the effects of intraventricular infusions of catecholamines on LH release and/or ovulation. RUBINSTEIN and SAWYER [30] found that intraventricular epinephrine or norepinephrine but not dopamine would induce ovulation when administered to the proestrus pentobarbital-blocked rat. TIMA and FLERKÓ [44] have reported that intraventricular infusions of 100 μg NE but not DA induced ovulation in rats which had been rendered constant estrus and anovulatory by subjecting them to continuous illumination. KIMURA et al. [21] have observed elevated blood LH levels in rats following intraventricular administration of NE but not DA. Quite recently KRIEG and SAWYER [22] studied the effects of varying doses of NE and DA administered intraventricularly to

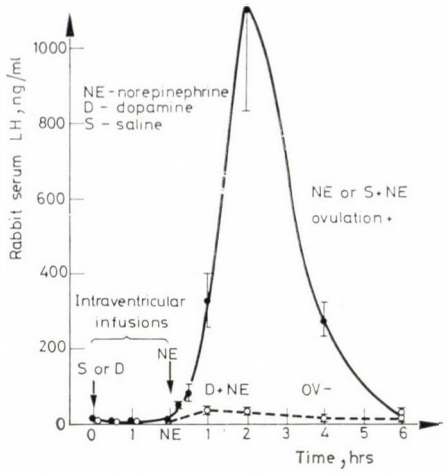

Fig. 5. Stimulatory effect of 50 μg of intraventricular norepinephrine on LH output in estrogen—primed rabbits and its blockade by treatment 2h earlier with intraventricular dopamine. From SAWYER et al. [34]

unanesthetized freely moving ovariectomized estrogen-progesterone primed rats with chronically implanted infusion and intraatrial cannulae. Doses of DA at 4 μg and 15 μg base in 2 μl saline at pH 5.5 infused intraventricularly over 2 minutes failed to alter the plasma LH level over the next 100 minutes. However, NE at increasing dosages up to 20 μg (Fig. 6) induced increasingly higher plasma LH values, with peaks at 20 min and a return to preinfusion levels by 60

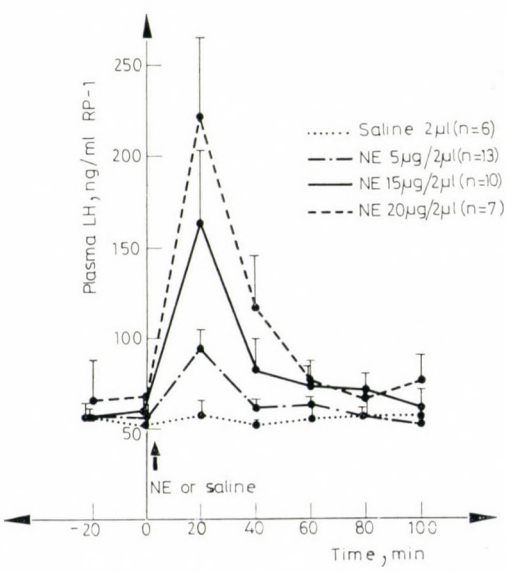

Fig. 6. Effects of intraventricular norepinephrine on LH release in the ovariectomized estrogen-progesterone primed rat. Responses to saline and three different doses of NE are shown. From KRIEG and SAWYER [22]

minutes. The amplitude and timing of the plasma LH response to 20 μg NE infused intraventricularly was almost identical to the effect of 1.25 μg LH—RH given intravenously. In studies in which multi-unit spiking electrical activity was recorded from the arcuate nucleus the infusion of 20 μg NE depressed the spiking activity for periods up to an hour [22].

These results recalled earlier experiments of WEINER et al. [48] in which an intraventricular infusion of epinephrine or NE, but not DA, had caused a biphasic elevation and subsequent depression of integrated multiple unit activity (MUA) in the median eminence (Fig. 7). Those experiments also registered a depression of MUA when the electrode tip was in the arcuate nucleus as indicated in the Figure. On the other hand, integrated MUA of

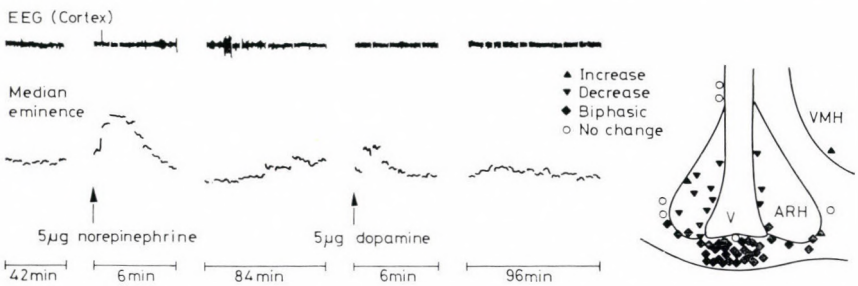

Fig. 7. Changes in multiple unit activity (MUA) in the rat median eminence induced by infusing norepinephrine and dopamine into the third ventricle. Epinephrine and norepinephrine induced biphasi effects on MUA in the median eminence and depressed activity in the arcuate nucleus (ARH), summarized in drawing at right. Dopamine exerted very little, if any, effect. From WEINER et al. [48]

the arcuate nucleus had been consistently elevated by intravenous injections of LH [43]. It was decided to compare the effects of intraventricular infusions of LH—RH with those of NE on amplitude discriminated spike activity of the same population of neurons in the arcuate nucleus of the proestrus rat. A typical sequence of responses in this study by KRIEG et al. [23] is seen in Fig. 8. Intraventricular saline did not affect the spike count or integrated MUA level. However, 0.5 μg LH—RH stimulated a dramatic elevation in firing rate and integrated MUA, and the effect lasted for an hour or more. About an hour and a half after recovery 20 μg NE was administered and it consistently depressed multi-unit spiking and integrated MUA; again the effect lasted for an hour or more.

Results of single unit-iontophoresis studies which appear to have a bearing to the interpretation of these multi-unit results have recently been reported by MOSS et al. [25] and KAWAKAMI and SAKUMA [19, 20, 32]. MOSS et al. described neurons in the arcuate nucleus identified antidromically as projecting to the median eminence whose firing rate was stimulated by iontophoretically applied DA and depressed by NE and *vice versa*. None of these

units was stimulated or depressed by both catecholamines. Similar findings were reported by KAWAKAMI and SAKUMA [20, 32]. These investigators also found that a large number of antidromically identified neurons in the arcuate nucleus were stimulated by iontophoretically administered LH—RH [19].

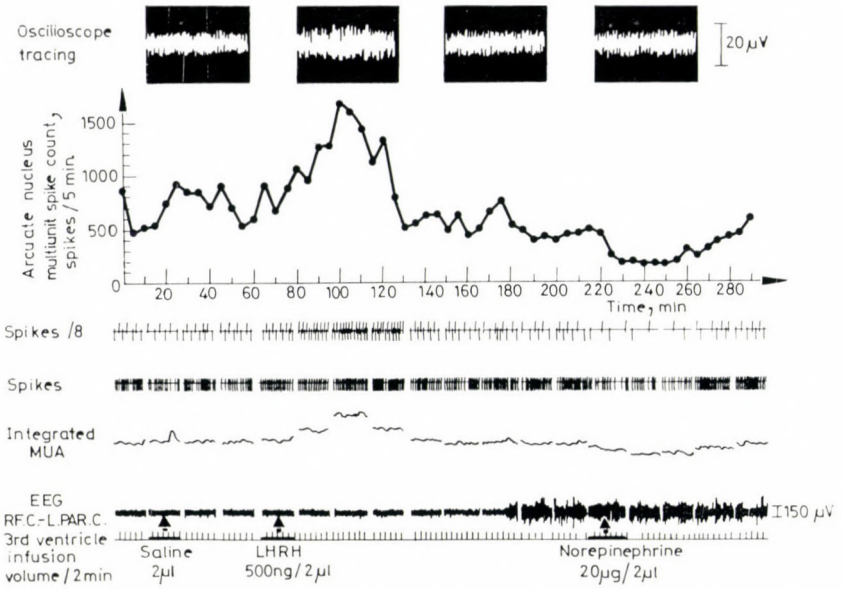

Fig. 8. Effects of LH—RH and NE on amplitude-discriminated multiple unit spike activity in the arcuate nucleus of the proestrous rat. Multi-unit spike counts at 5 min intervals are graphed beneath representative 100 millisecond samples of the oscilloscopic raw multiunit activity. Corresponding 30 sec samples of multi-unit spike activity, integrated multiunit activity (MUA) and cortical EEG are shown in the lower channels with the times of saline, LH—RH and NE infusion marked. From KRIEG et al. [23]

The arcuate nucleus has a large number of dopaminergic neurons projecting to the median eminence as shown by both fluorescence [10] and immunofluorescence [15] studies (Fig. 4). If the firing of such neurons is stimulated by dopamine it should be depressed by NE according to the studies cited above [20, 25, 32]. Intraventricular NE would then inhibit the firing of a population of these cells and this could depress the multi-unit spike activity (Figs 8 and 9). Depressing a neuron which is inhibitory to the release of LH—RH should facilitate that release. By activating the same neurons LH—RH could be functioning as an ultrashort loop feedback agent to inhibit its own secretion. The median eminence projections whose firing was *stimulated* by NE [48] probably represent peptidergic neurons whose perikarya lie rostrally in, for example, the region of the suprachiasmatic nucleus. SÉTÁLÓ and colleagues [40] have presented evidence that little of the median eminence's LH—RH comes from neurons in the arcuate nucleus; anterior deafferentation of the hypothalamus severely depletes the LH—RH content of the median eminence [1, 49].

CARRER and TALEISNIK [4] reported that electrochemical stimulation of the dorsal midbrain tegmentum precisely in the region described later by SWANSON and HARTMAN [42] as the principal adrenergic pathway, resulted in ovulation in constant estrus rats. CARRER and SAWYER [3] have recently observed that such stimulation inhibits the MUA firing of preoptic neurons in much the same manner as NE depresses MUA in the arcuate nucleus, i.e., depression of inhibitory interneurons (Fig. 10). Activation of the firing of

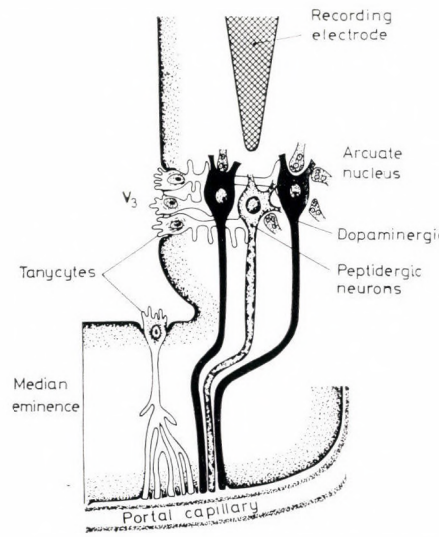

Fig. 9. Diagrammatic representation of the tip of a multiple unit recording electrode near a population of predominantly dopaminergic neurons in the arcuate nucleus. It is proposed that the firing of these cells may be stimulated by intraventricular LH—RH and inhibited by norepinephrine. Figure adapted from OKSCHE et al. [26]

these same neurons was induced by electrochemical stimulation of midbrain raphe nuclei [3], which inhibited ovulation in the cycling rat [5]. Ultimately, LH—RH neurons must be activated by an LH-releasing ovulatory stimulus, either directly or *via* depression of an inhibitory mechanism.

Figure 11 is a simple diagram of relationships of hypophysiotropic area dopaminergic neurons (in black), peptidergic neurons producing LH—RH (in white) and the principal adrenergic pathway bringing noradrenergic input from the region of the locus coeruleus (stippled). Intraventricular NE appears to inhibit DA neurons while stimulating LH—RH neurons passing through the median eminence. Stimulating the principal adrenergic pathway to induce LH release and ovulation probably affects DA and peptidergic neurons in the same manner as intraventricular NE. Whereas DA neurons may inhibit LH—RH and LH release, their principal function is probably related to prolactin secretion, acting as PIF, the prolactin inhibiting factor, or stimulating its production or release.

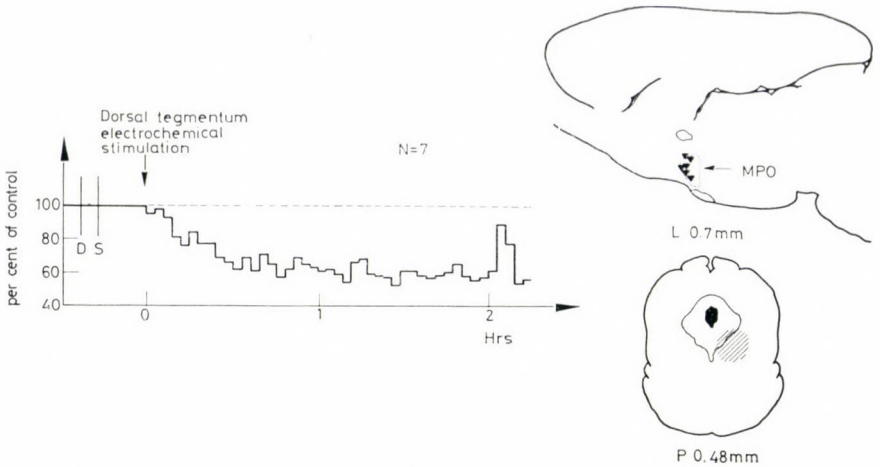

Fig. 10. Effect of electrochemical stimulation of the dorsal midbrain tegmentum (shaded area) on multi-unit spike activity in the medial preoptic area (MPO). Results are presented graphically as percentage of the activity (spikes/min) recorded during the control period. Recording sites for this group of animals are projected onto the sagittal brain. From CARRER and SAWYER [3]

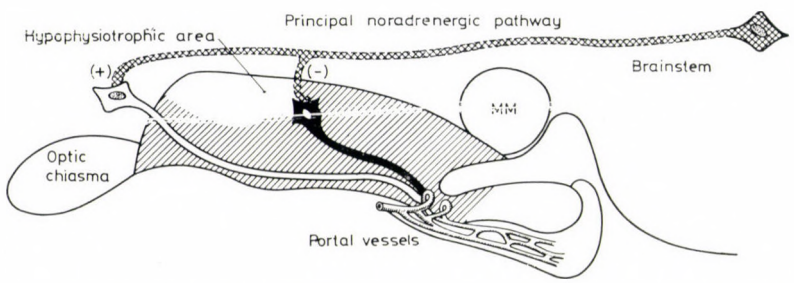

Fig. 11. Suggested relationships of noradrenergic, peptidergic and dopaminergic neurons. The perikaryon of the peptidergic LH—RH neuron is pictured as lying rostral to the hypophysiotrohic area. Further explanation in text

Acknowledgements

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INVESTIGATION ON LICHEN PRODUCTION OF GRASSLAND COMMUNITIES OF SANDY SOIL II.

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Abstract

Lichen production was investigated on two types of sandy Grasslands over more than two years. Two maxima of production were observed, a minor one in spring and a major one in autumn. The degree of these depended on climatic factors. Path analysis was applied for determining (1) the order of importance of the climatic factors and (2) the role of these factors in the changes of the total lichen production of associations and their influence on the productivity of the two determinant species, viz., *Cladonia magyarica* and *Cl. furcata*. Lichen production should be investigated for a period longer than the vegetation period.

Introduction

The results [7] of our investigations performed solely in the period of vegetation in 1968 and 1969 prompted us to initiate studies lasting throughout a year. Due to the slow growth rate and the variable life rhythm of lichens, their production must be investigated for a time longer than the vegetation period. My investigations were started in November 1970 and were continued till December 1972. Samples were collected monthly up to May 1972 and hereupon in June, September, October and December 1972.

Material and method

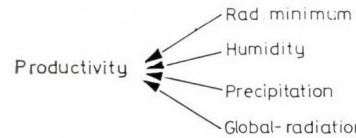
Samples were collected in the reservation area of Csévharaszt from two grassland communities of sandy soil: from the one year old *Brometum tectorum secaletosum* and from the perennial open calciphil *Festucetum vaginatae danubiale*. Ten monoliths of 20×20 cm each were taken monthly from each community. The lichens were cleaned from sand and dry grass, classified by species and weighed in an air-dry condition. The total lichen production and the ratio of the contribution by individual species were both expressed in grams. The total lichen production and, within this, the distribution of the monthly production by species the relations of the monthly productions, furtheron, the relation between the productivity and the climatic factors were evaluated by statistical methods. Correlations were calculated between productivity on the one hand and the mean radiation minimum, the average humidity at 7 o'clock a.m., the monthly precipitation and global radiation on the other. Productivity was calculated according to the Květ—Ondok formula, viz.,

$$\text{Productivity} = w_2 - w_1 / t_2 - t_1$$

where w_1 = dry weight at sampling time t_1, w_2 = dry weight at sampling time t_2, $t_2 - t_1$ = number of days between the two samplings.

Path analysis was applied for determining the order of importance of the single climatic factors and the effect of their seasonal changes on, the productivity of the two dominant species (*Cladonia magyarica, Cl. furcata*). Path analysis is suitable for examining systems in which the relation between the variables is unidirectional, i.e., one of the variables determines in additive way, the value of the other, but not *vice versa* [5, 6].

Formula of Path analysis:



Results and discussion

Seasonal changes of the total lichen production in the studied associations

The seasonal changes of lichen production were similar in the two associations. In 1970, a high autumnal (November) maximum was followed by a winter decrease, and a minor spring minimum. In the dry and hot summer (June + July + August 1971), when the global radiation was: 48.226 g/cal/cm^{-2} and simultaneously the total precipitation was only 103 mm, the phytomass in *Festucetum vaginatae* was found to be similar to our findings in the same season of the year 1968, while in *Brometum* it was much lower. In 1971, no autumnal maximum appeared, due to the unfavourable weather. Instead a slow rise was observed which reached a minor peak in March, 1972. This maximum higher than the peak in March 1971 was identical in *Brometum* and *Festucetum* associations In summer, 1972, a minimum occurred, followed in October by a high maximum (Table 1, Figs 1—2).

Table 1

Total lichen production
(g/m^2)

Year	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
In *Festucetum vaginatae* assecation. Months												
1970											363	249
1971	232	172	147	159	88	69	116	77	118	76	102	Nt
1972	144	168	127	102	N.t.	75	N.t.	N.t.	56	299	N.t.	154
In *Brometum tectorum* association												
1970											447	334
1971	290	143	187	156	84	66	65	91	91	77	93	N.t.
1972	134	156	181	147	N.t.	125	N.t.	N.t.	173	258	N.t.	268

N.t. = not tested

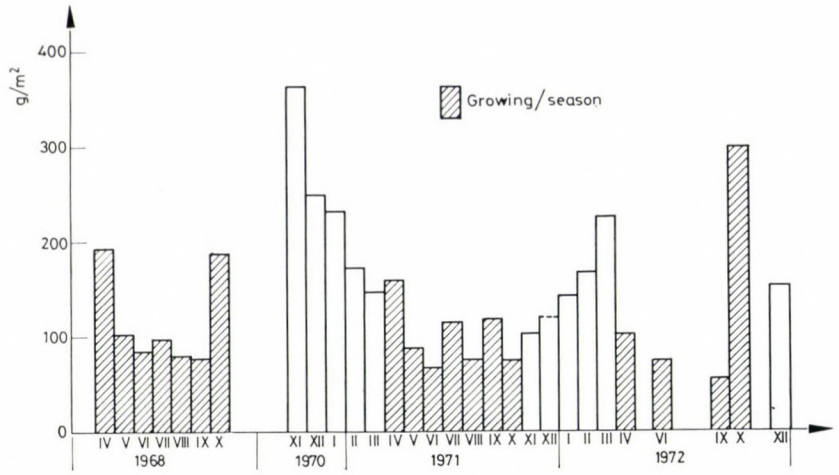


Fig. 1. Total production of lichens in *Festucetum vaginatae* association, g/m²

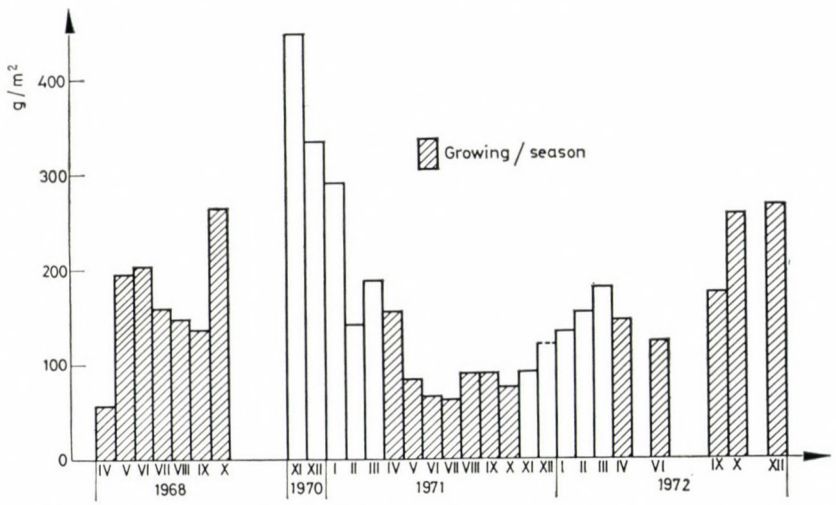


Fig. 2. Total production of lichens in *Brometum tectorum* association, g/m²

Seasonal changes of the production of single lichen species

In different associations the seasonal changes in the production of the same lichen species were different. The mass of *Cladonia magyarica* was dominant nearly throughout the year in *F. vaginatae*, while in *Brometum* it was subordinant. The course of productivity was similar in both associations.

Generally, two maxima occurred, one in the spring and the other in the autumn. This was so even in September 1971, when the other lichen species

of the community were at a minimum, due to the unusually dry and hot summer (Figs 3–4).

Cladonia furcata is the dominant lichen species of *Brometum*. The seasonal changes in its production are more balanced compared to the other species and the periods of production maxima are similar. The great maximum in November, 1970, was followed by a low minimum in the winter and hereupon the next maximum appeared as late as in February, 1972. This was followed by a summer minimum, and another maximum in December, 1972. A similar seasonal change of production was observed in *Festucetum vaginatae* (Figs 3 and 4).

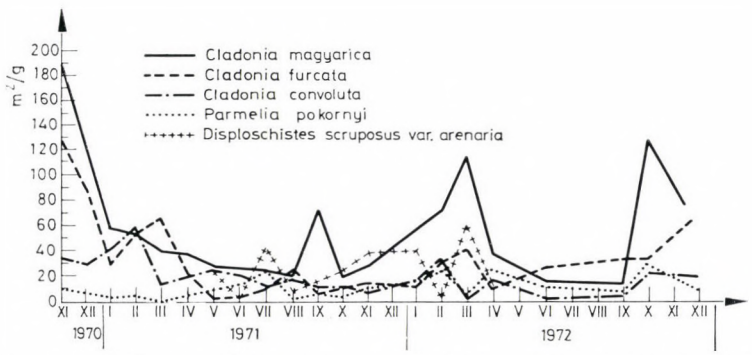


Fig. 3. The seasonal changes of the phytomass of different lichen species in *Festucetum vaginatae* association

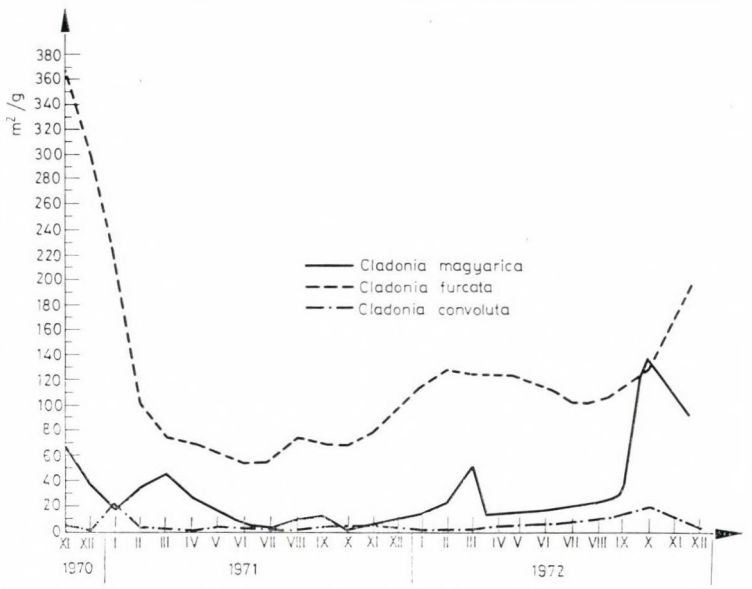


Fig. 4. The seasonal changes of the phytomass of different lichen species in *Brometum tectorum* association

The productivity of species *Cl. convoluta* has its maximum in the winter months. In *Brometum* its phytomass was very poor (Figs 3 and 4).

A considerable amount of *Parmelia pokornyi* only occurs in *F. vaginatae* association with two maxima in February and in summer (Figs 3, 4).

Diploschistes scruposus var. *arenaria* lives in *F. vaginatae* association but not in *Brometum* one. Its phytomass was so little that it was only measured from May 1971 to June 1972. In this period owing to the higher specific weight of its thallus, the phytomass exceeded those of any of the other species (Fig. 3).

Effect of climatic factors on the productivity of lichens

BESCHEL [1, 2] was the first to attach importance to climatic factors. The growth of lichens is proportional to the daily time of activity. The active life functions are regulated by humidity and temperature. Air humidity is more important than precipitation. After rainfall the lichen quickly dries in the sunshine, and only a high humidity slackens down the rate of withering, which is quicker when the temperature rises. In withered state the life functions are latent. We searched the correlations between (1) radiation minimum. (2) humidity, (3) precipitation, (4) global radiation and the lichen productivity. Path analysis was applied for determining the order of importance of these factors in the productivity of (a) the total lichen and (b) the two dominant species (*Cl. magyarica* and *Cl. furcata*, Table 2).

In both associations the winter and summer periods can be sharply separated. In winter, in autumn, and in the early spring season, the productivity was positive. In the *F. vaginatae* association at this time the primary factors influencing productivity are global radiation, air humidity and in further order of importance precipitation and radiation minimum. In summer however, the climatic factors are of minor importance, "other" factors not investigated by us seem to be more important. These may be related to the growth and development of flowering plants. Lichens are influenced in a positive way by precipitation, whereas, humidity has a negative effect. Of three summers viz., 12 summer months, 8 displayed negative influences on productivity, i.e., in these months destruction exceeded production.

In the one-year old open *Brometum* association in winter the effect of "other" factors is decisive, while in summer these are the fourth in order of importance. In winter the lichens may dominate grass unlimitedly, their productivity is primarily determined by some unknown factors not investigated by us. The radiation minimum stands in the first place in winter and in the second in summer, global radiation in the third in winter and the second in summer. An interesting phenomenon is that humidity assumed to be of great importance for lichens in grass communities stands in the 4th or 5th place of importance throughout the year.

Table 2

Results of Path analyses

	Winter period						Summer period					
	radiation minimum	relative humidity	precipitation	global radiation	"other"	total direct indirect effect	radiation minimum	relative humidity	precipitation	global radiation	"other"	total direct indirect effect
	per cent											
Total productivity of lichens in *F. vaginatae* association	10.23 **4**	120.01 **2**	34.43 **3**	286.18 **1**	**5**	159.3	0.35 **5**	8.13 **3**	20.16 **2**	1.22 **4**	87.00 **1**	2.65
direction of effect	+	—	—	—	+		+	—	+	—	+	
Total productivity of lichens in *Brometum tectorum* association	7.35 **2**	8.50 **4**	0.02 **5**	1.86 **3**	82.79 **1**	17.21	35.22 **2**	0.93 **5**	6.59 **3**	2.03 **4**	46.17 **1**	53.83
direction of effect	—	—	—	+	+		+	—	—	—	+	
Total productivity of *Cladonica magyarica* in *F. vaginatae* ass.	1.09 **3**	13.42 **2**	0.33 **5**	0.52 **4**	93.33 **1**	6.67	40.61 **4**	137.78 **2**	19.01 **5**	140.39 **1**	44.26 **3**	55.74
direction of effect	—	—	+	—	+		+	—	+	—	+	
Total productivity of *Cladonia furcata* in *F. vaginatae* ass.	76.49 **1**	48.30 **3**	2.04 **4**	0.07 **5**	52.37 **2**	47.63	24.05 **3**	129.14 **2**	7.64 **4**	195.80 **1**	**5**	129.6
direction of effect	—	—	—	—	+		+	+	—	+	+	
Total productivity of *Cladonia furcata* in *Brometum* association	15.15 **3**	133.12 **1**	0.15 **5**	1.50 **4**	27.22 **2**	72.22	71.35 **1**	3.42 **5**	26.47 **2**	8.71 **4**	18.97 **3**	81.03
direction of effect	—	—	+	—	+		+	—	—	—	+	

The order of importance of the factors affecting the two dominant lichen species (*Cl. furcata, Cl. magyarica*) of the two communities is as follows: for *Cl. furcata* the order of importance in winter is: radiation minimum, humidity and "other" factors in both associations. In summer in the *Brometum* the radiation minimum, and in the *Festucetum* association the energy of global radiation promotes the productivity of *Cl. furcata.*

Cl. magyarica was only analysed in the *F. vaginatae* association. In winter the "other" factors were primary, while in summer global radiation. Humidity is the second factor throughout the year, and precipitation is the last in the order of importance.

Conclusions

(1) The very slow and periodical growth of lichens, differing from the flowering plants in life rhythm prompted us to examine their phytomasses throughout the year and even for a longer period.

(2) In both sandy soil grassland associations (*Festucetum vaginatae, Brometum tectorum*) a minor spring and a major autumn maximum occurred, which were influenced by climatic factors, unfavourable weather prevented these maxima from appearing.

(3) The productivity and the seasonal changes of the single species were variable. The productivity course of *Cl. magyarica* and *Cl. furcata* was similar in both associations. The productivity maximum for *Cl. convoluta* occurred in winter, *Parmelia pokornyi* displayed a maximum in winter and another in summer.

(4) Of the environmental factors, the order of importance of several climatic factors (radiation minimum, humidity, precipitation and global radiation) and the role of their seasonal changes in affecting lichen productivity were determined by a method which had not been used in lichenology before, i.e., path analysis.

(5) The role of climatic factors differs whether it is related to total lichen productivity or to the productivity of the individuals.

(6) Productivity and the effect of climatic factors are sharply separated regarding the one-year-old *Brometum* and the perennial open *Festucetum vaginatae* association.

(7) The order of importance of the examined factors is different in winter and summer.

(8) Studying the trend of influence of the single factors we have concluded that in all associations total lichen productivity and the single species are influenced positively by the "other" factors, not studied by us throughout the year, and by the radiation minimum in summer. The trend of influence of the remaining factors is variable. For explaining this, further studies are necessary.

(9) Seasonal changes in the phytomass of lichens are not entirely explained by climatic changes. The anatomical changes of the individual and the development of some phenologic phases are related to productivity.

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IN VIVO CUMULATION AND DISCHARGE OF AZINE, THIAZINE AND XANTHENE DYES AND THEIR EFFECTS ON THE CHLORAGOGEN CELLS OF LUMBRICIDAE (OLIGOCHAETA)

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Abstract

The *in vivo* cumulation of neutral red, methylene blue and acriflavine in the body wall, midgut epithelium and chloragosomes of *Lumbricus terrestris* L. and *Octolasium transpadanum* Rosa, and the discharge of these dyes by chloragosomes was investigated by spectrophotometry. The chloragosomes cumulated the largest amount of the ingested dyes. Electron microscopy displayed the activation of the chloragogen cells and an increased chloragosome formation on the first days of dye intake. In dye-free environment, the dye content of the chloragosomes decreased rapidly. The above properties of the chloragogen cells indicate the role of these cells in the defence mechanism against the toxic effect of certain cationic xenobiotics ingested *via* the intestinal tract.

Introduction

Several authors have studied the electron microscopic structure of chloragogen cells [5, 8, 10], however, only Winkler [10] has studied the relation between experimental feeding and chloragosome formation. Valembois and Cazaux [9], using an autoradiographic technique, has proved that amino acids and carbohydrates introduced into experimental animals are cumulated first in the chloragogen cells, thus providing evidence of the food storing activity of these cells. Roots and Johnston [6] demonstrated in the chloragosomes stored hydroxycoumarins and chromones (flavons and flavonols) toxic substances ingested from vegetable food. However, experimental data on the importance and kinetics of xenobiotic storage are yet lacking.

According to our own experiments *in vitro*, the chloragosomes of *Lumbricidae* have cation exchanger [1], electron acceptor [3] and complex forming [2] properties. These physicochemical properties of chloragogen cells may explain the ability of these cells to store both tropic [4, 5, 9] and toxic [6, 8] compounds.

In the present work, we investigated the uptake, storage and discharge activities of chloragogen cells *in vivo*. We selected compounds which

(1) are stable and do not occur in the organism under natural circumstances;

(2) are rapidly absorbed and cumulated in large quantities, and can be determined even when present in small concentrations in tissue extracts;

(3) are of low toxicity, not affecting the metabolism and the structure of the organism except when present in extremely large doses.

The purpose of our work was (1) to determine the effectiveness of cation exchanging and complex forming actvities of the chloragosomes *in vivo*, and (2) to determine how they are involved in the circulation of xenobiotics. in the organism. Finally, (3) we studied the response of chloragogen cells to the cumulation of xenobiotics.

Material and method

Approximately 200 *Lumbricus terrestris* L. and 200 *Octolasium transpadanum* Rosa specimens were used for our experiments.

Feeding of animals. The animals were placed on a moist filter paper, which, in the absence of any other food, was eaten by the worms. The dyes were introduced by placing the worms on filter paper moisted with a 0.01% solution of dye. The dyes did not affect the intensity of ingestion. Animals began to die only after 6—8-week treatment. The following dyes were used: acriflavine (Hoechst AG) (a xanthene), neutral red (USSR) (an azine) and methylene blue (Merck) (a thiazine).

Comparison of the dye uptake by different tissues. From the worms, kept on stained filter paper for 2 weeks, 4×5 specimens were examined. Preparations of body wall, endothelium and chloragosomes were made, each weighing 100 mg. The chloragogen tissue was removed from a 1 cm piece, excised from the centre of the midgut. Rinsed midgut pieces were blotted on filter paper and 100 mg portion was weighed. The remaining midgut pieces were homogenized and from them chloragosomes were isolated by filtration, centrifugation and repeated washings [2]. 100 mg portions were weighed from the chloragosome fraction and from the section behind the clitellum of the body wall. Depending on the quantity of ingested dye, the tissue samples weighing 100 mg were extracted with 5 or 10 ml acid alcohol (90 ml 96% ethanol + 10 ml 0.1 N-HCl). For comparison, tissue extracts were similarly prepared from animals fed with dye-free filter paper. The dye contents of the extracts were determined in a Spektromom Type 361 spectrophotometer.

Investigation of the neutral red cumulation and discharge by chloragosomes. The dye ingestion by animals kept on neutral red soaked filter paper was investigated in chloragosomes isolated as described above from animals killed on days 1, 2, 3, 4, 6, 10 and 14. Fifty worms were placed on garden soil on the 3rd day after the onset of feeding with neutral red and the dye content of the chloragosomes was followed until the 14th day. The examinations were run duplicate.

Electron microscopic examination of chloragogen tissue. Control animals kept on moist filter paper, animals fed with methylene blue for 2—42 days, furtheron, animals fed with acriflavine or neutral red for 3—28 days were examined. The gut pieces from the part behind the clitellum were fixed in glutaraldehyde (3% with cacodylate buffer of pH 7.4). This was followed by postfixation in isotonic osmium tetroxide (1% in veronal acetate buffer of pH 7.4). The materials were embedded in Durcupan ACM. Semi-thin and ultra-thin sections were cut, the latter were counterstained with uranyl acetate and lead citrate. Samples were examined in TESLA 613 type electron microscope; accelerating voltage: 80 KV.

Results

Comparative study of dye cumulation in different tissues

A few days after the onset of dye feeding the chloragosomes contained considerable amounts of dye. The extinction of the acid alcohol extract of the control chloragosomes rapidly decreased when the wave length was grown over 400 nm. After 4 days of dye uptake, even the slowly absorbed acriflavine (absorption maximum, 455 nm) was already well detectable in the chloragosome extract. The amounts of neutral red (absorption maximum, 540 nm) and of methylene blue (absorption maximum, 665 nm) were well over the threshold of detectability (Fig. 1) in both species. The dye cumulation was

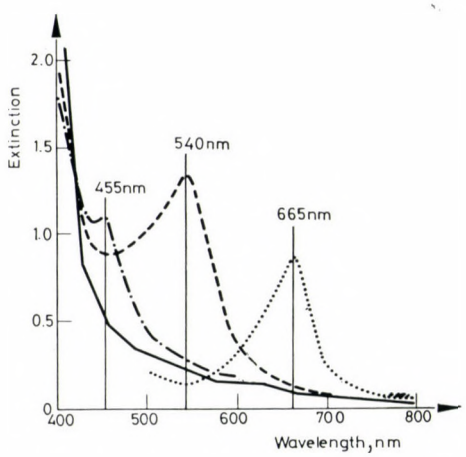


Fig. 1. The absorption curves of acid alcohol extracts of chloragosomes of controls and worms fed for 4 days with dyes. 100 mg chloragosome fraction was extracted with 5 ml acid alcohol. Control: ———; fed with acriflavine: —.—.—; fed with neutral red: — — — —; fed with methylene blue: . . . . . .

significantly lower in the body wall, hence, comparison of the dye amounts in the different tissues was performed on the 14th day of experimental feeding. The chloragosomes cumulated all three dyes in the highest concentrations both in *L. terrestris* and *O. transpadanum*. The epithelium of the midgut cumulated significantly less and the body wall cumulated the least (Fig. 2). Tenfold neutral red was cumulated by the chloragosomes compared with the uptake of the midgut epithelium and 40-fold compared with the body wall. The differences were much smaller in methylene blue-treated worms. Acriflavine reached the lowest concentration in each tissue sample.

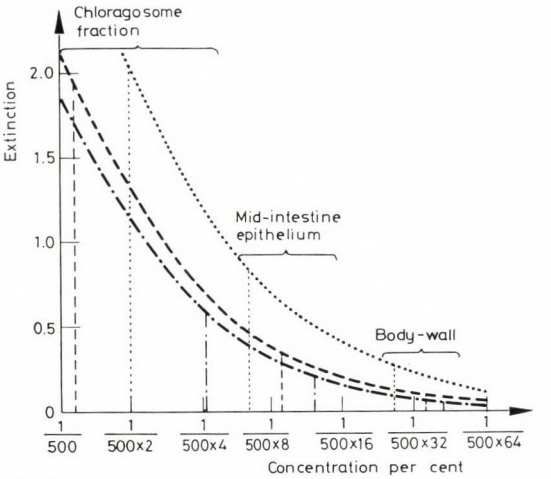


Fig. 2. Comparison of dye uptake of chloragosomes, midgut-epithelium and body wall. The worms were kept for 2 weeks on filter paper moisted with 0.01% dye solution. 100 mg tissue samples were extracted with 10 ml acid alcohol. The extinctions of the extracts are projected on to the standard extinction curves of the dye. Acriflavine (measured at 455 nm): —.—.—.; neutral red (at 540 nm): — — — — —; methylene blue (at 665 nm): . . . . . .

The kinetics of the uptake and discharge of neutral red by the chloragosomes

The cumulation of neutral red was slow on the first two days of dye-feeding, but intensely increased until the sixth–seventh day; from the 10th day on, there was hardly any further increase (Fig. 3). The dye concentration in the chloragosomes of worms that had been fed with dye for 3 days and subsequently placed in a dye-free soil initially decreased rapidly, later decreased at a gradually slowing rate (Fig. 4). After two weeks, the neutral red content of chloragosomes was still measurable and remained detectable in traces for a long time.

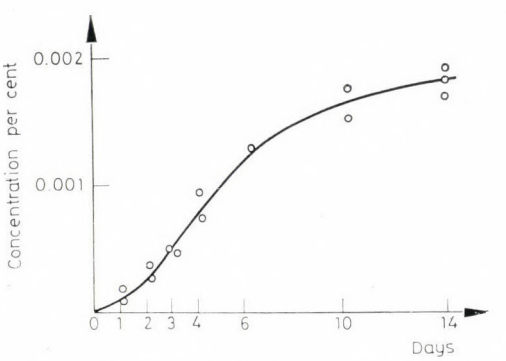


Fig. 3. In vivo kinetics of neutral red uptake by chloragosomes. The concentration values refer to 100 mg chloragosomes/10 ml extracting solution

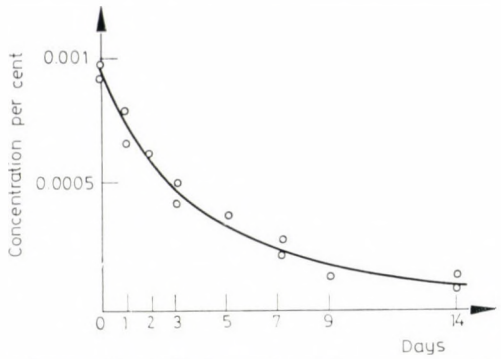


Fig. 4. In vivo kinetics of neutral red discharge by chloragosomes. The concentration values refer to 100 mg chloragosome/5 ml extracting solution

The effect of dyes on the chloragogen cells

All three *in vivo* ingested dyes induces significant, in many respect similar changes in the chloragogen cells. Comparison of the chloragogen cells in *L. terrestris* fed with methylene blue for two days with those in the control (Figs 5 and 6) displayed a striking change in the cytoplasm and a definite structural development of the chloragosomes. The tissular bond of the chloragogen cells was loosened, the intercellular space enlarged. Often processes were ejected by these cells into the intercellular space, or narrow plasma margins surrounding empty vacuoles appeared through which chloragosomes could pass on to the intercellular space or to the coeloma. Methylene blue induced an enlargement of, and a numerical increase in mitochondria and an increase of endoplasmic reticulum, particularly near the cell surface. In the majority, the chloragosomes of the control animals appeared homogeneously osmiophil, except an occasional indistinctly lighter central area. After 2 days of methylene blue feeding the density of the chloragosomes decreased and a definitely demarcated internal nucleus of low density and a darker cortical zone were observable. In many chloragosomes, a cumulation of tiny vesicles was observable above the cortical zone, indicating a more intensive growth rate of the chloragosomes. In the cytoplasm small, dense clumps consisting of tubules or vesicles were often visible. These were not demarcated from the cytoplasmic matrix. Hereafter, a narrow space was gradually formed around larger corpuscles of similar structuture, which at this time resembled the chloragosomes. As all transient forms between the cytoplasmic vesicle clumps and the chloragosomes were visible, former were considered as prechloragosomes. After the ingestion of methylene blue, the tubular–vesicular structure characteristic of prechloragosomes was well observable in chloragosomes, too.

On the 14th day of methylene blue feeding (Fig. 7A), the number of mitochondria in the chloragogen cells was lower again, the endoplasmic reti-

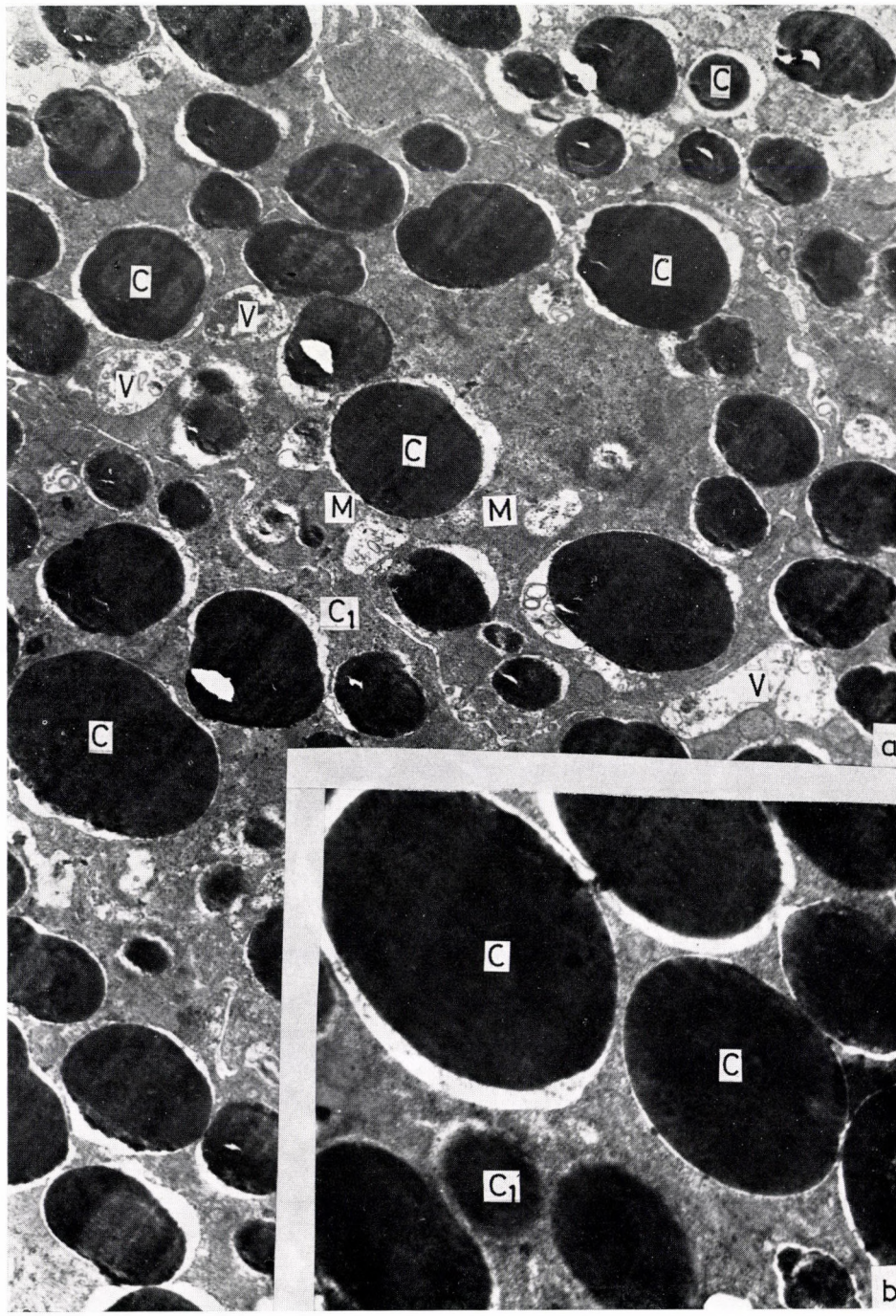


Fig. 5. A section of the chloragogen cells of control *L. terrestris*. A = × 12 000; B = × 24 000; C = chloragosome; C_1 = prechloragosome; V = vacuole

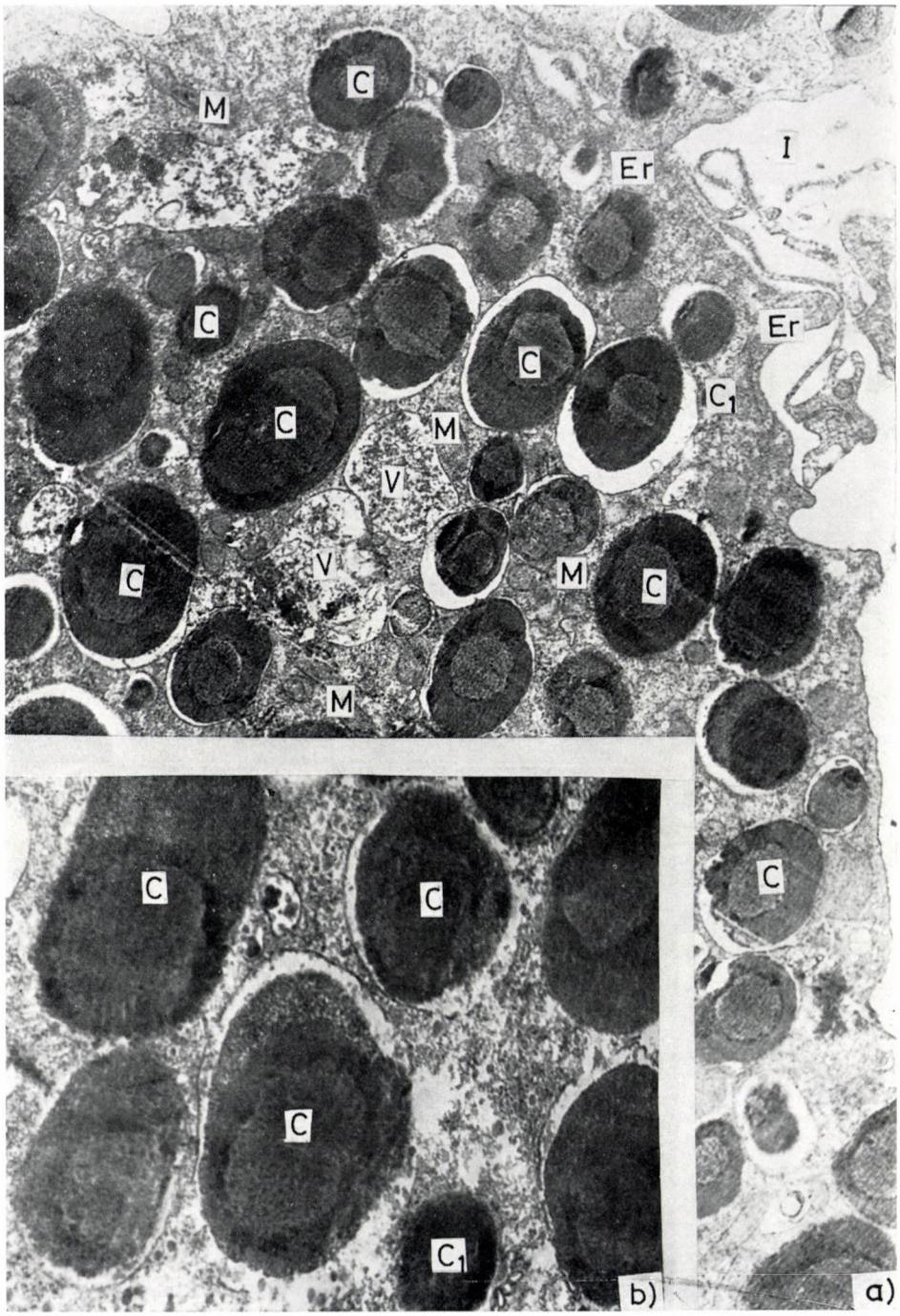


Fig. 6. A section of chloragogen cells of *L. terrestris* after two days of methylene blue ingestion. A = × 12 000; B = × 24 000. C = chloragosome, C_1 = prechloragosome, M = mitochondrion, Er = endoplasmic reticulum, V = vacuole, I = intercellular space

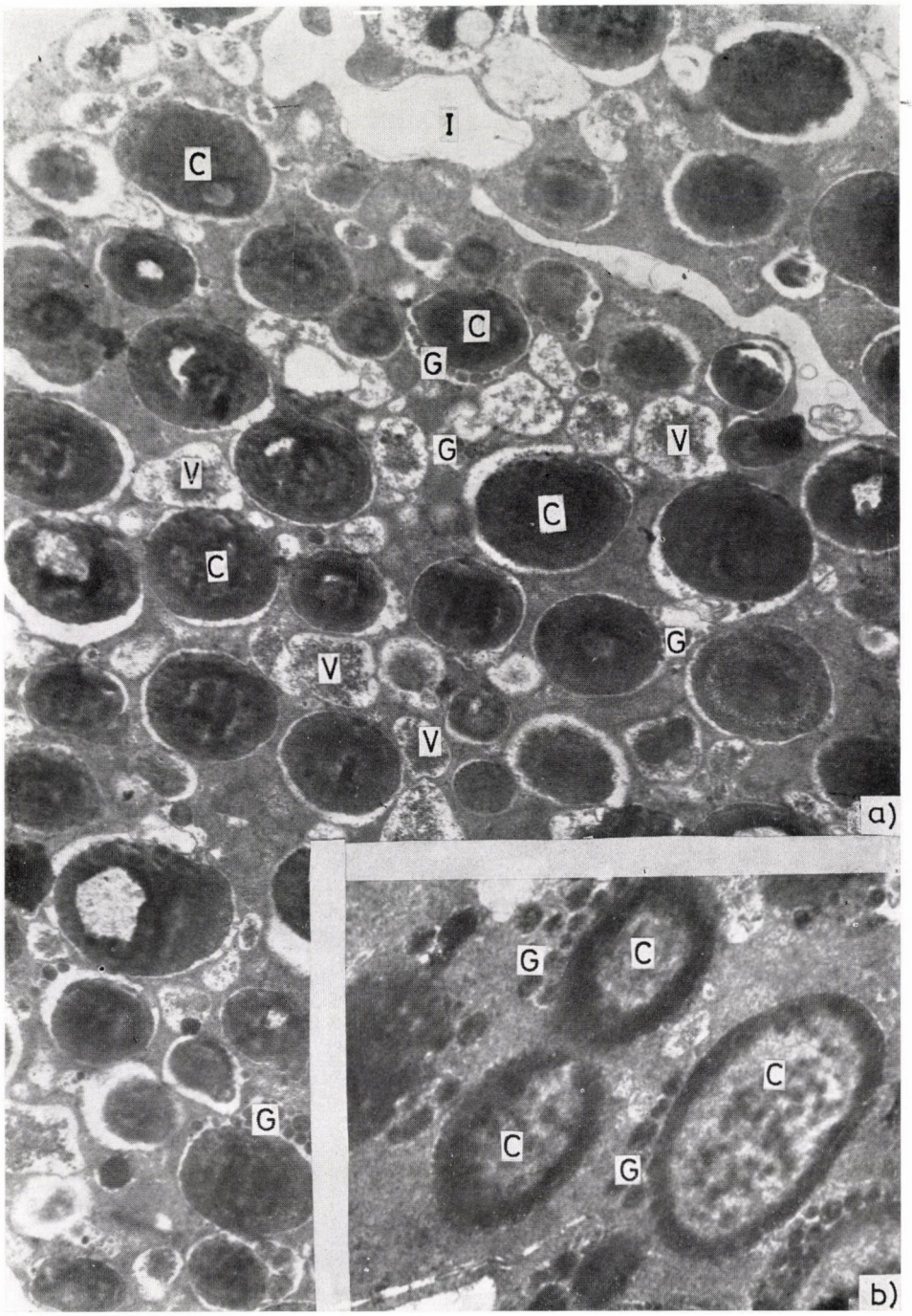


Fig. 7. A section of chloragogen cells of *L. terrestris* A = after 2 weeks of methylene blue ingestion, × 12 000; B = after 6 weeks of methylene blue ingestion, × 24 000. C = chloragosome, G = chloragogenic granules, V = vacuole, I = intercellular space

culum was less developed. The chloragosomes were more dense than after two days, and numerous vacuoles containing finely granulated substance were seen. The most striking at this period were the approximately 0.1 μm-sized dark compact granules appearing around the chloragosomes or in the vacuoles. In some cases these were fused with the chloragosomes, hence, these may be regarded as chloragogenic granules. On the 42nd day of methylene blue feeding, the cytoplasm of the chloragogen cells was degraded and the structure of the chloragosomes was often strikingly changed (Fig. 7B). Chloragogenic granules occurred in high numbers around the chloragosomes and incorporated in the chloragosome matrix.

The effect of 3-day neutral red uptake on the chloragogen cells of *O. transpadanum* was well observable compared to the control (Figs 8 and 9). The changes in the cytoplasm were similar to those observed after 2 days of methylene blue uptake in *L. terrestris*. The nucleus visible in Fig. 9 is extremely large, however, we have been unable to prove any relation between dye uptake and the enlargement of the nucleus, so far. Neutral red initially induced a rise in the number of prechloragosomes, the concentric array of the chloragosomes was not characteristic, however, the tubular–vesicular structure persisted for a long time in the chloragosomes. On the 28th day of neutral red ingestion the number of mitochondria was reduced and signs of cytoplasmic degradation appeared. Only few chloragogenic granules were present, the chloragosomes of tubular–vesicular structure were replaced by chloragosomes of compact or of concentric structure.

Acriflavine induced an increase of the endoplasmic reticulum, which was still visible on the 28th day (Fig. 10) in the chloragogen cells of *O. transpadanum*. At this time the majority of the chloragosomes were concentric in structure, and a relatively high number of chloragogenic granules appeared.

Discussion

In an earlier paper [2], we reported our findings related to the dye-binding capability of chloragosomes *in vitro*. Cationic dyes are bound most intensely and in highest amounts. In our present work, cationic dyes were introduced into the worms through the intestinal tract. This work has proved the cationic dye binding capability of chloragosomes *in vivo*. The composition of chloragosomes is significantly influenced by environmental factors, the amount of ingested cationic dyes may reach $0.1-0.2\%$ of the total weight of the chloragosomes.

The *in vivo* neutral red accumulation in chloragosomes was slow on the first two days of the experiment. The entire filling of the intestinal tract took at least one day, this partly explains the slow rate of dye cumulation. On the other hand, chloragosomes formed newly during the experiment may cumulate

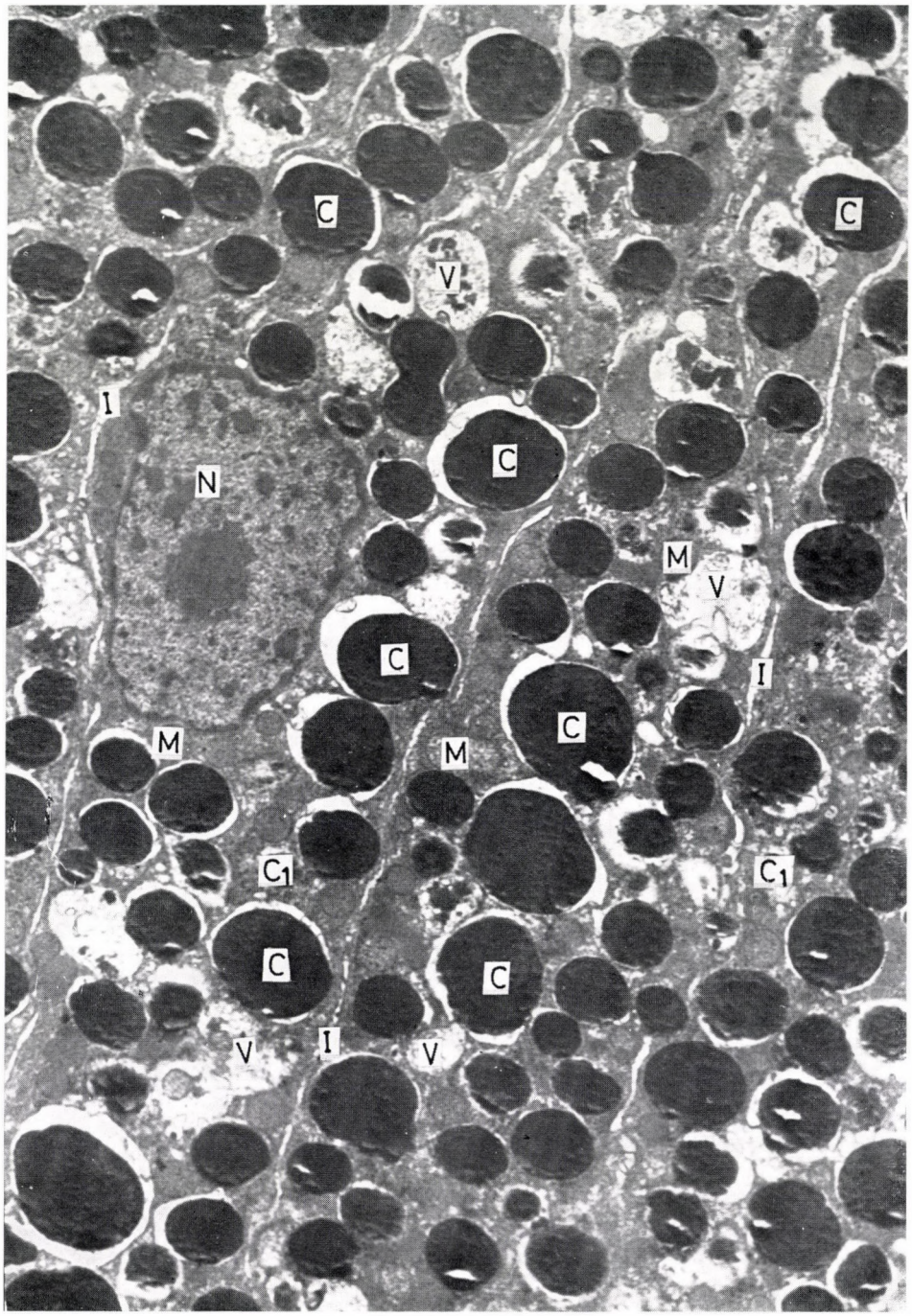
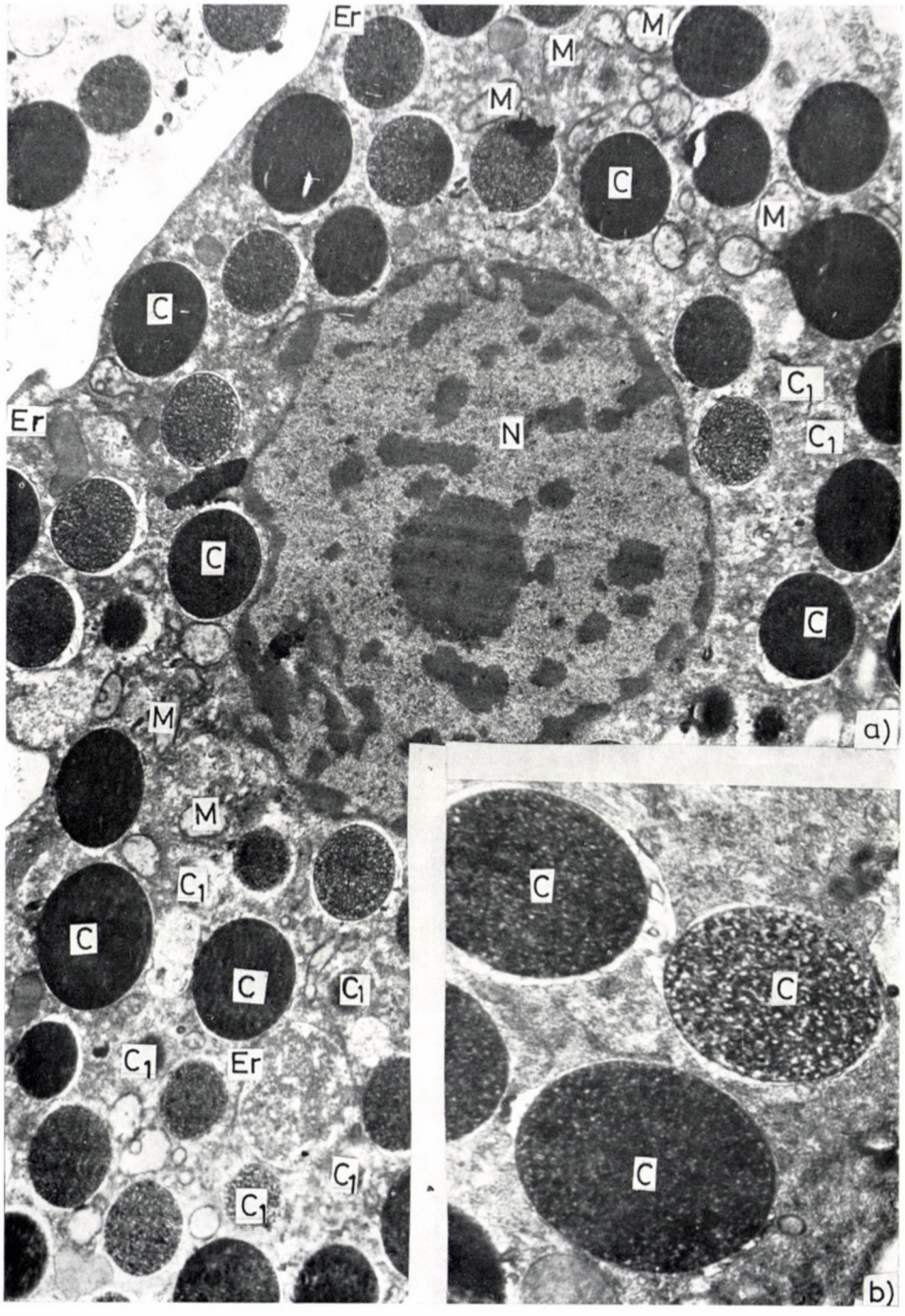


Fig. 8. A section of chloragogen cells of control *O. transpadanum*; × 12 000. N = nucleus, V = vacuole, C = chloragosome, C_1 = prechloragosome, M = mitochondrion, I = intercellular space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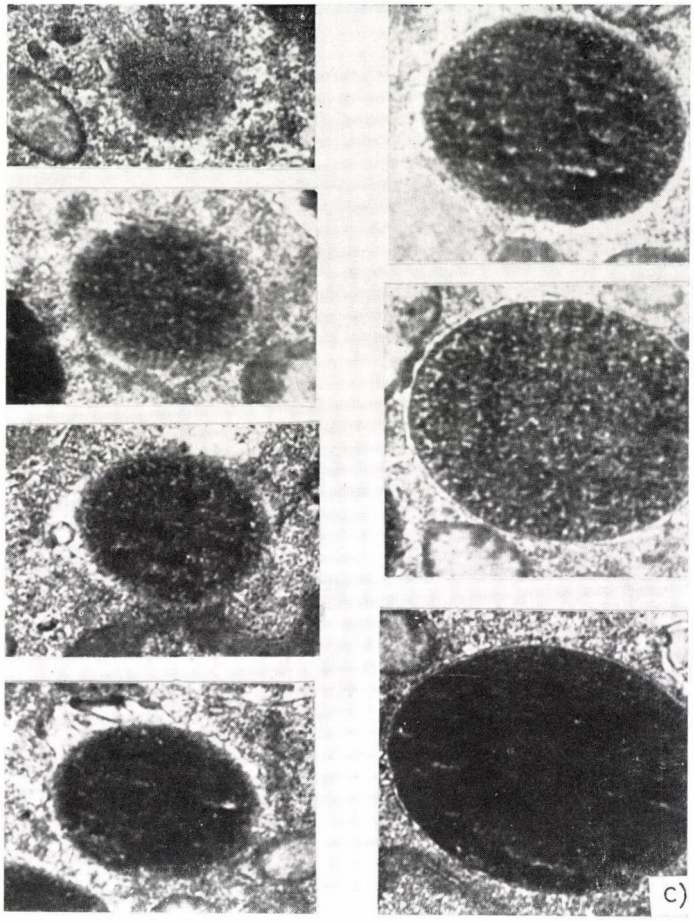

Fig. 9. A section of chloragogen cells of *O. transpadanum* after 3 days of neutral red treatment. A = × 12 000; B = × 24 000. N = nucleus, C = chloragosome, C_1 = prechloragosome, M = mitochondrion, Er = endoplasmic reticulum, I = intercellular space, C = × 24 000. The development of chloragosomes from prechloragosomes after 3 days of feeding with neutral red

a higher quantity of dye compared to those present there before. We have shown (1) that the chloragosomes bind cationic dyes partly by a cation exchange mechanism and partly by complex formation (2). The ratio of dye bound by the two mechanisms was approximately 1:1. Presumably, the chloragogen cells are only capable of discharging dyes bound by complex formation, together with the chloragosomes. Hence, the kinetics of chloragosome formation and discharge may be conclude from the kinetics of dye discharge. Our data and electron-microscopic observations indicate that the intensity of chloragosome formation and release increased during the experiments until the half-life of chloragosomes was shortened to 6—8 days. In spite of the rapid

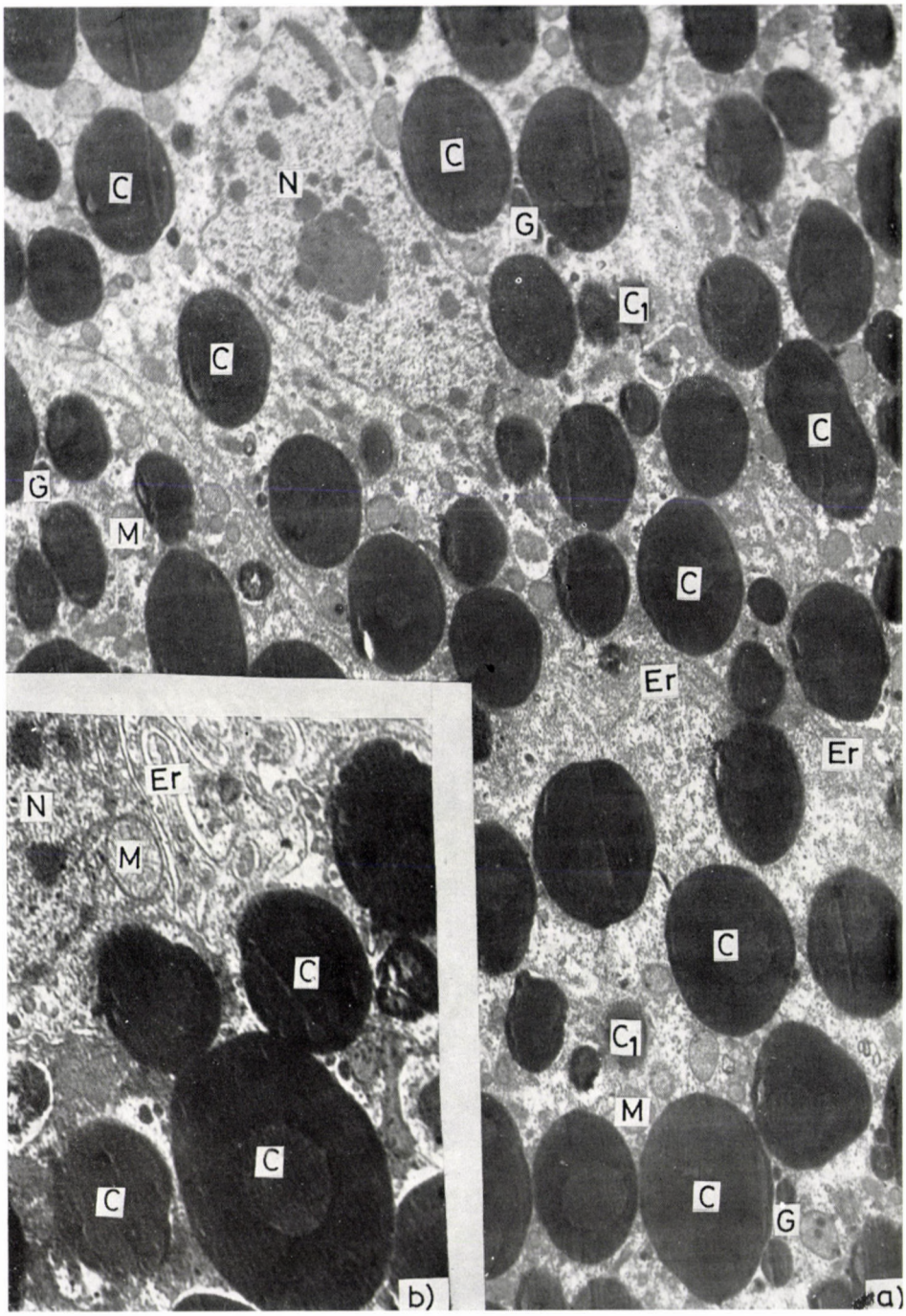

Fig. 10. A section of chloragogen cells of *O. transpadanum* after 4 weeks of acriflavine treatment. A = × 12 000; B = × 24 000. N = nucleus, M = mitochondrion, Er = endoplasmic reticulum, G = chloragogenic granules

exchange of the chloragosomes, the dye-discharge curve is protracted. Presumably the dye molecules released continously from the various organs are rebound to newly formed chloragosomes.

On the first days of dye uptake the changes in the structure of the chloragogen cells display an increasing activity. The endoplasmic reticulum is enlarged, the mitochondria increase in number and chloragosome formation accelerates. Literary data on the mechanism of chloragosome formation are scanty. WINKLER [10] was able to stimulate chloragosome formation by glucose and lipid feeding, while starvation induced the opposite changes. In his opinion, chloragosomes develop from cytoplasmic vacuoles and tiny vesicles transport the components involved in chloragosome formation. LINDNER [5] differentiates two types of chloragosomes, such as small and large, and assumes a developmental relation between these.

In the present studies, we observed in the cytoplasm of chloragogen cells characteristic tubular–vesicular clumps which were not distinctly demarcated from the cytoplasmic matrix. A narrow space appeared around larger bodies of similar structure, which gradually demarcated the dense tubular–vesicular structure from the surrounding cytoplasmic matrix. In this state, these bodies may be regarded as to be small chloragosomes. The cytoplasmic clumps of tubular–vesicular structure are considered the initial phase of chloragosome formation and termed prechloragosomes. The mode of chloragosome formation as described by WINKLER [10], viz., that the vacuoles are formed initially and the chloragosomes are formed in these, cannot be refuted.

Initially dye ingestion increased the number of prechloragosomes, indicating an accelerated chloragosome formation. The incorporated dyes reduce the density of the chloragosomes and thus make their structure easier to recognise. Similar changes were not found in control animals, kept on filter paper.

When dye ingestion was prolonged for 2—4 weeks, the cytoplasmic organelles were reduced again and, presumably, the chloragosome formation was also disturbed. The latter is indicated by the cumulation of chloragogenic granules around the chloragosomes and in the vacuoles, viz., lesser amount of chloragogenic material is built in the chloragosomes.

The majority of authors involved with the functions of chloragogen cells assume [5] that the chloragogen cells may discharge the chloragosomes into the intercellular space or the coeloma. We also observed an expansion of the intercellular space and the dendritic transformation of the cell surfaces, which may be related to the accelerated chloragosome discharge.

Degenerative changes in the chloragogen cells were only observed after 4—5 weeks of dye ingestion. It may be concluded that in cases of low doses or a short period of intoxication, the chloragogen cells may play a fundamental role in the defence mechanism against the toxic effect of cationic xenobiotics absorbed through the intestinal tract.

Acknowledgement

Grateful acknowledgements are due to Professor J. Tigyi, Rector of the University Medical School, Pécs, for making electron microscopic examinations available.

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OPTICAL POLARIZATION REVEALS DIFFERENT ULTRASTRUCTURAL MOLECULAR ARRANGEMENT OF POLYSACCHARIDES IN THE YEAST CELL WALLS

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Abstract

The topo-optical aldehyde bisulfite–toluidine blue (ABT) reaction of vicinal OH and amino-OH groups offers new ways to study the ultrastructure of polysaccharides in different biological substrates. Through oriented dye binding on the reacting groups, the ABT reaction induces strong birefringence on the linearly ordered polysaccharides, which is negative with respect to their chain length. Using this method, two types of molecular order of the polysaccharides could be distinguished in the cell walls and capsules of yeasts. (1) The optically *negative spherulitic* character of the yeasts after the ABT reaction indicated that the toluidine blue molecules were bound tangentially (in a surface-parallel pattern) while the polysaccharide chains of the cell walls and capsules were oriented mainly radially. This structural pattern may be explained as resulting from a helicoid conformation of the polysaccharide component. (2) Acid or alkali hydrolysis removed the radially oriented polysaccharide component of the cell wall. The remaining, resistant polysaccharides showed up in the form of optically *positive spherulites* indicating radially oriented dye molecules on a circularly ordered, micellar polysaccharide texture.

Introduction

The polysaccharide structure of the yeast cell wall have been extensively studied by means of biochemical methods [12, 13, 14]. About 31% mannan 29% glucan and smaller amounts of protein (13%) and lipid (8.5%), were found in the cell wall of yeasts, whereas the dominant cell wall polysaccharide of molds proved to be chitin.

Acid and alkali treatment at high temperatures [11, 14] removed the mannans and partly, the glucans from the yeast cell wall, which showed a starch-like structure in chemical and X ray diffraction investigations [7, 9].

Electron microscopic studies after acid treatment revealed fibrillary structure of the cell walls [5, 8] and a double membrane which was identified by MUNDKUR [11] as chitin. X-ray diffraction studies also suggested the presence of chitin [8]. Recently it has been demonstrated that N-acetylglucosamine is an activator for forming the hyphae of *Candida albicans* [18].

So far only limited information has been available about the ultrastructure of the polysaccharide components in the complex cell walls of yeasts.

The PAS reaction or its modifications for electron microscopy have been used for morphological studies of the polysaccharide components of the yeast cell wall [8]. However, those methods provided evidence only for the presence of polysaccharides, but no information about their ultrastructural orientation. X-ray diffraction studies were useful only after acid treatment of the cell wall [5, 8], or on the extracted solubilized cell wall polysaccharides [9].

In the present study we used the selective topo-optical reaction of linearly ordered vicinal OH and amino-OH groups (the aldehyde bisulphite–toluidine blue (ABT) reaction), which is characterized by strong birefringence caused by oriented binding of toluidine blue molecules on micellar polysaccharide textures [16]. This optical effect is very sensitive, and the birefringence induced by the ABT reaction of polysaccharide chains is negative with respect to their chain length [16] (Fig. 1a). We have reported on the molecular arrangement of polysaccharide chains of microbial cell walls, using the topo-optical ABT reaction [1]. We distinguished two types of molecular orientation of the vicinal OH groups of polysaccharide chains:

(1) tangential (circular) arrangement of cellulose in the cell walls of plants as well as of chitin in the cell wall of fungi; accordingly, these cell walls appeared as optically *positive spherulites* in the polarization microscope after the ABT reaction (Fig. 1b);

(2) yeasts, some bacteria and starch granules appeared as optically *negative spherulites* after ABT, indicating a surface-parallel arrangement of the dye molecules and in turn, radially oriented vicinal OH groups of the polysaccharides (Fig. 1c).

The present paper is a report on our ultrastructural observations on the yeast cell wall and capsule made by polarization optical analysis by means of the ABT reaction.

Material and method

Yeasts, such as *Saccharomyces cerevisiae*, *S. ellipsoideus*, *Candida albicans*, and *Cryptococcus neoformans* were investigated. Smears from cultures were fixed in 5% glutaraldehyde in phosphate buffer at pH 7.2, or in formaldehyde vapour. For studies in semi-thin sections, yeasts were embedded in Durcupan. Durcupan was removed by keeping the sections for 30—60 min in saturated alcoholic solution of NaOH. Since *Cryptococcus* forms large capsules only *in vivo* [19, they were studied in paraffin section (4—5 μm) of infected tissues from human cases of generalized cryptococcosis.

Selective topo-optical ABT reaction of vicinal OH groups [16]. The molecular mechanisms of the ABT reaction is shown in Fig. 1. The vicinal OH and amino OH groups of polysaccharides are oxidized to dialdehydes with periodic acid (1%) treatment for 30 min. Then, by treatment with a saturated Na-bisulphite solution for 30 min, the dialdehydes bind sulphite residues and are rendered negatively charged and strongly basophilic after staining with toluidine blue at pH 1.0. Staining with 0.05% toluidine blue at pH 1.0 (toluidine blue in 0.1 N-HCl) for 2—3 min was followed by post-staining stabilization with 2% potassium ferricyanide [15]. The smears were covered with 20% gum arabic containing 0.1% potassium ferricyanide and, after drying, with Canada balsam.

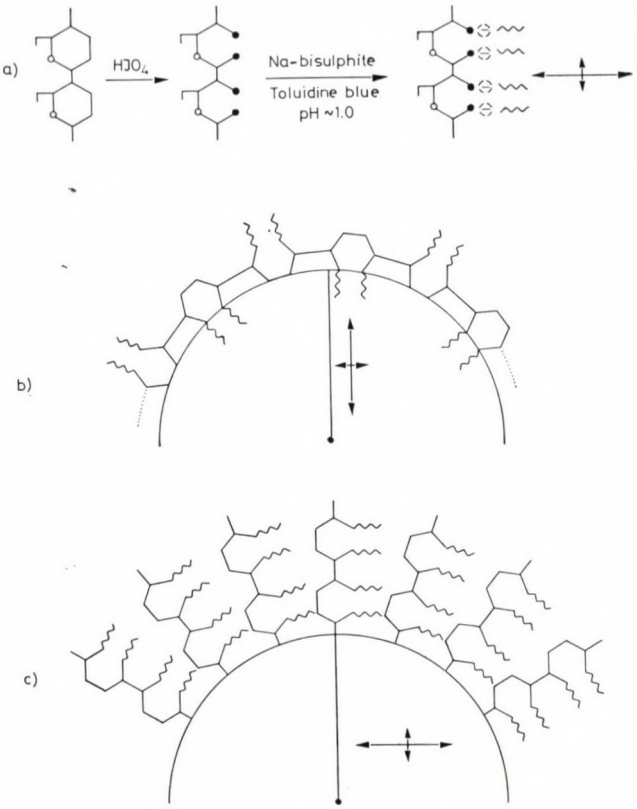


Fig. 1. Schematic demonstration of the molecular — structural mechanism of the birefringence effect of polysaccharide chains as induced by ABT. *a* = The dialdehydes formed by periodic acid oxidation of vicinal OH groups are rendered negatively charged by addition of bisulphite and able to bind toluidine blue at pH 1.0 in an oriented pattern. Oriented dye binding is manifested by strong birefringence, negative with respect to the length of the polysaccharide chains [16]. *b* = Radially positive birefringence (optically positive spherulites) of the cell walls is indicative of radially oriented dye molecules and in turn of surface-parallel orientation of the reacting polysaccharide chains. *c* = Radially negative birefringence (optically negative spherulites) induced by ABT is indicative of surface-parallel dye molecule orientation and of mainly radially oriented, linear OH groups which are probably fixed to a circular structure possibly resulting from radial or helical polysaccharide chains

Extraction and digestion experiments. Mannan components were removed from the yeast cell walls [5, 11] by treating sections in 3% NaOH at 56 °C for 2 h and in 2N HCl at 56 °C for 3—4 h. After extraction, the slides were washed in distilled water and the ABT reaction was performed.

In order to remove protein components, we treated sections in other experiments with trypsin (Serva 4 mg/ml) in phosphate buffer at pH 8.2, and with papain (Serva 8 mg/ml) in pH 5.4 acetate buffer at 37 °C for 1—2 h. Diastase (Serva) treatment (2 mg/ml) in distilled water at 37 °C for 2 h liberated non-structural polysaccharide components. Polarization-optical investigations were made with a Leitz Ortholux microscope equipped with compensators [1, 15, 16].

Results

In an unstained state *Saccharomyces cerevisiae*, *S. ellipsoideus* and *Candida albicans* show neither form, nor intrinsic (Figs 2a—b) birefringence. They remain unstained also with toluidine blue within the pH range of 1.0 to 3.0, indicating the absence of strongly acidic (sulphate) groups in the cell wall structure. The cell walls of *Saccharomyces* show basophilic staining with toluidine blue at pH 4.0 and even then they reveal no toluidine blue-induced birefringence, indicating isotropic staining with the dye molecules, associated at random (Figs 3a—b).

However, after ABT reaction (toluidine blue at pH 1.0) a very strong basophilic staining effect was seen (Figs 4a—b) with intensive birefringence and a retardation of about 50—70 nm. This optical effect is the result of oriented binding of toluidine blue on linearly oriented polysaccharide chains. The sign of birefringence is negative with respect to the radius *(negative spherulites)* indicating tangential orientation of the slow axis of retardation, i.e. the plane of the dye molecules and, in turn, radial arrangement of the polysaccharide chains within the cell wall (see Fig. 1c).

Acid or alkali hydrolysis is known to extract mannan and partly glucan, chains from the cell wall of yeasts [5, 11]. As a result of the extraction there was an intensive decrease in staining intensity of the cell walls, furthermore, a double-layered cell wall structure became visible (Figs 5a—b) probably corresponding to that seen by MUNDKUR [11] in the electron microscope after acid hydrolysis. At the same time the ABT-induced birefringence of the cell walls was also decreased (10—15 nm) and the optical character (negative spherulites) was changed into positive *(radially positive spherulites)*, indicating radially oriented dye molecules bound to a surface-parallel polysaccharide structure (see Fig. 1b).

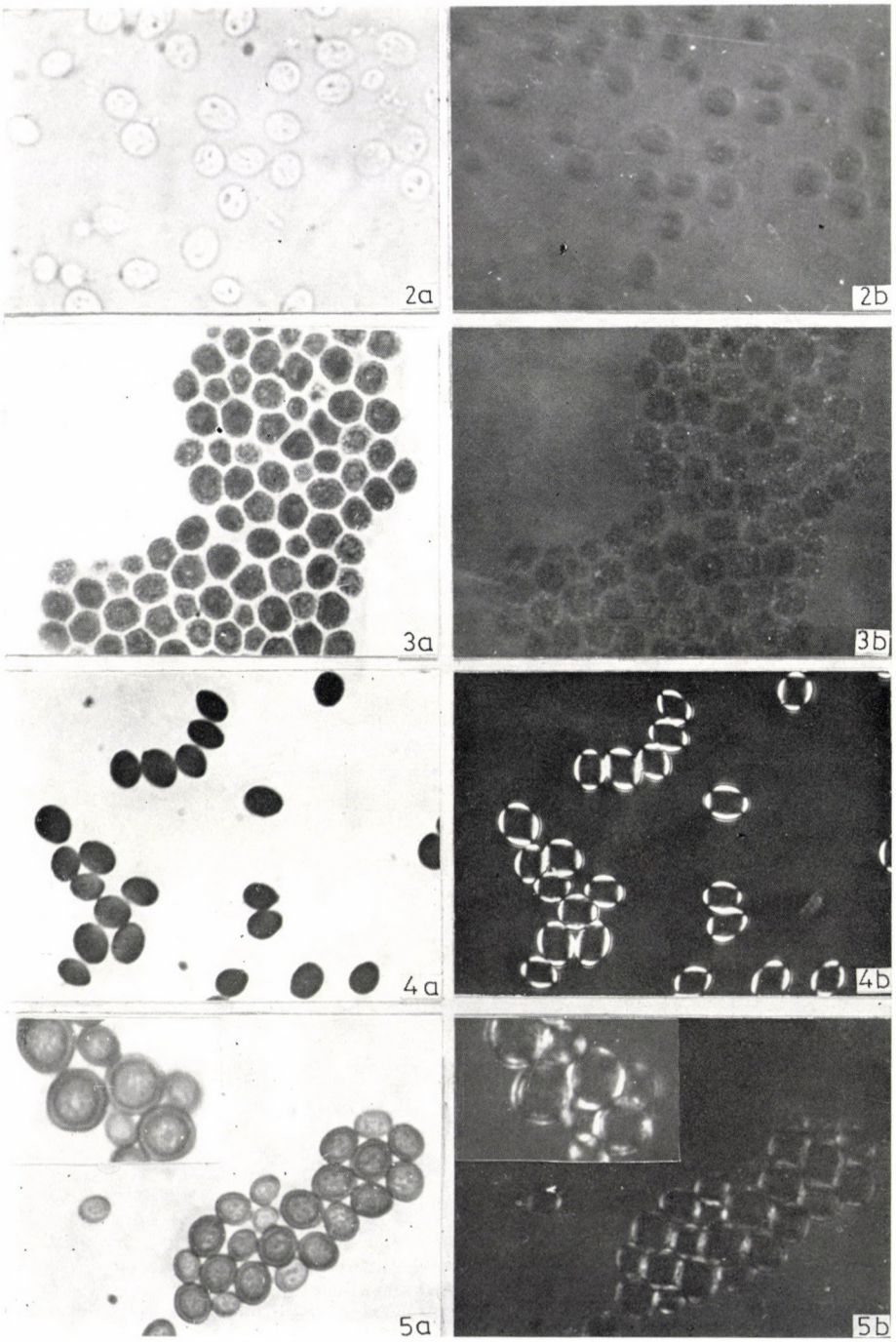
For investigation of the capsules and cell walls of *Cryptococcus neoformans*, we used various tissues from two human cases of generalized cryptococcosis. The capsules in the various tissues differed greatly in form and size. This is in

Fig. 2. Saccharomyces cerevisiae unstained in gum arabic *a* = in the light microscope, *b* = between crossed polars, lacking any intrinsic birefringence of the cell wall

Fig. 3. Saccharomyces cerevisiae stained with toluidine blue at pH 4.0. The cell walls are basophilic in *(a)* and isotropic between crossed polars *(b)*, indicating random association of the dye molecules

Fig. 4. Saccharomyces cerevisiae ABT reaction. The cell walls are strongly basophilic *(a)* and show an intensive birefringence (50—70 nm) *(b)* in the form of optically negative spherulites, indicating radially oriented polysaccharide chains

Fig. 5. Saccharomyces cerevisiae treated with 2N HCl at 56 °C for 3 h, followed by ABT reaction. The light microscope revelas double-layered cell walls *(a)* which show moderate birefringence in the form of optically positive spherulites (10—14 nm) indicating surface-parallel polysaccharide chains (opposite to the nonhydrolysed cell walls). The inserts show the same at higher magnification. The bilayered cell walls and their birefringence are clearly seen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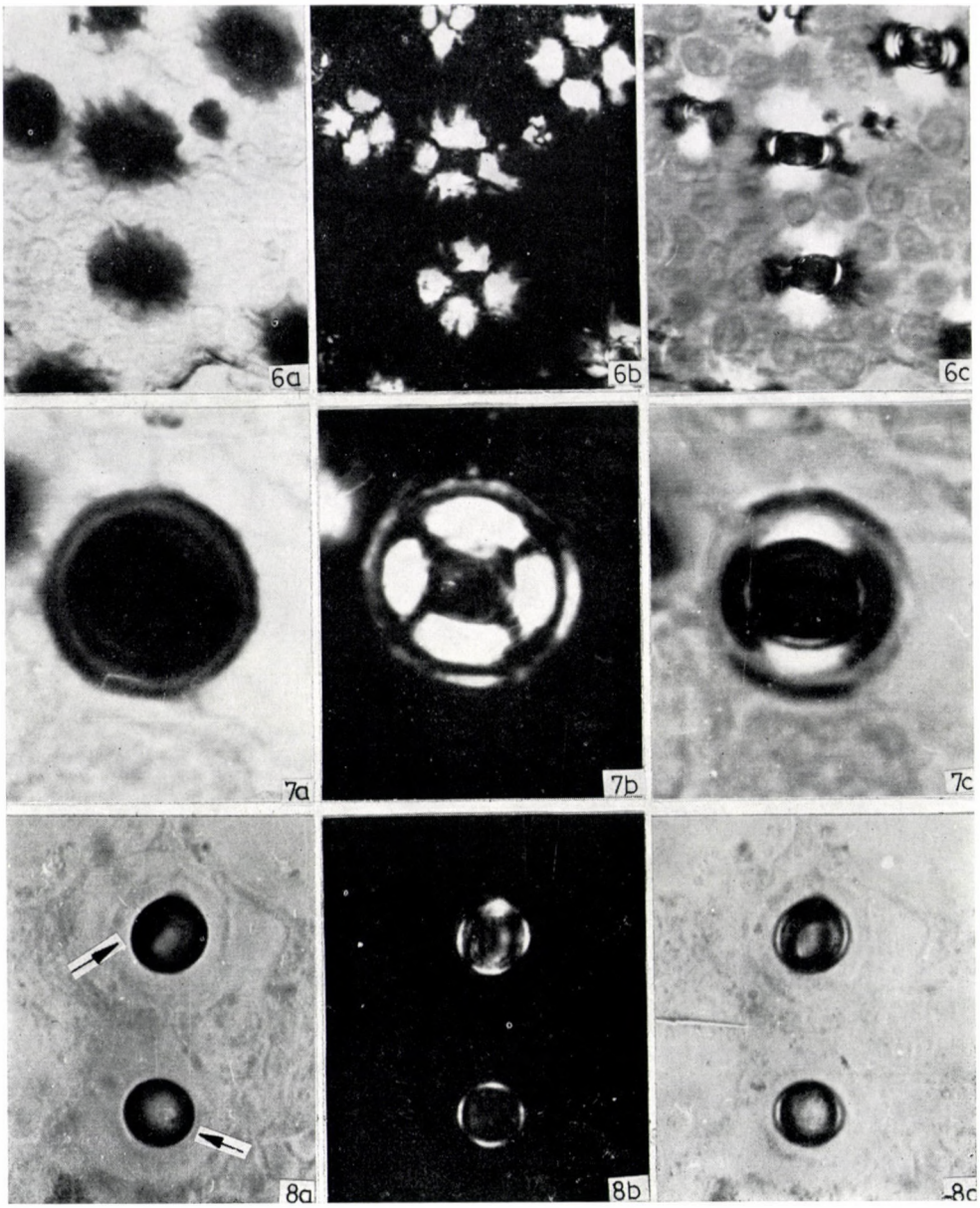
good agreement with the finding of TAKEO et al. [19], who pointed out that the largest capsules were seen in the lungs and spleen. We found that in an unstained state the cells walls and capsules of *Cryptococcus* did not show birefringence either in water (form birefringence), or in gum arabic or Canada balsam (intrinsic birefringence), that is, the yeasts in the tissues were invisible. The finding that they remained unstained with toluidine blue at pH 1.0 points to the absence of negatively charged (sulphated) polysaccharides. Toluidine blue staining of the capsules of *Cryptococci* began above pH 3.0 with a moderate birefringence, as reported earlier [6, 10], however, this gave no data on its intensity, optical character and pH dependence. On staining with toluidine blue at pH 3.5 we found birefringence in the form of optically negative spherulites with a retardation of 10—19 nm. These staining characteristics were suggestive of the presence of only weakly negatively charged (carboxylated) polysaccharides in an oriented pattern.

After the ABT reaction has developed the cell walls and capsules revealed very strong metachromatic, basophilic staining and an intensive birefringence with retardations of about 70—90 nm for the capsules and 100—110 nm for the cell walls (Figs 6a—c). The optical character of this birefringence was radially negative, similar to those cases in which the samples were stained only with toluidine blue at pH 3.5. In the tissue sections we found capsules varying in thickness. Some samples had capsules with uniform optical character in the form of optically negative spherulites and appeared as "hairy", radially ordered, filamentous structures, suggestive of an initial stage of capsule differentiation. In very large capsules one or two more basophilic ringlike zones were seen, the outer layer of which was opposite in optical character (positive spherulite) to the thick inner layer (negative spherulite). At the transition between the two layers, an isotropic layer is clearly seen (Figs 7a—c).

Fig. 6. Cryptococcus neoformans from the spleen in human systematic cryptococcosis — ABT reaction. The capsules are strongly basophilic and have a hairy, radially-ordered filamentous structure *(a)* and are strongly birefringent between crossed polars *(b)*. Note the radially-negative optical character at additive compensation with a retardation of about 80—90 nm, indicating radially-oriented polysaccharide chains in the capsule

Fig. 7. Cryptococcus neoformans from the spleen, with very large capsule — ABT reaction. In the capsule two stronger basophilic rings are seen in the light microscope *(a)*, which reveal different characters of birefringence between crossed polars *(b)*. There is an isotropic, ringlike zone at the transition between the outer and inner layers. Their opposite optical characters are clearly seen at compensation *(c)*; the horizontal segments of the outer layer are compensated dark and those of the inner layer are light. A similar character is observable on the lateral segments, however, with opposite signs. This optical behaviour indicates that the optical character of the outer layer is positive and that of the inner layer is negative with respect to the radius, indicating different molecular orientation of the polysaccharide chains in the different layers of the capsules

Fig. 8. Cryptococcus neoformans from the spleen, treated with 2N-HCl at 56 °C for 4 h, followed by ABT reaction. The capsules are completely removed (arrows) and do not show any staining or birefringence effect. However, the cell walls retain basophilic metachromatic staining *(a)* and show moderate birefringence *(b)* in the form of radially positive spherulites at compensation *(c)*, with a retardation of about 15—20 nm



Acid and alkali treatments removed the capsules completely (Fig. 8a—c), and no staining or birefringence effect could be detected after ABT. It is remarkable that at the same time, the cell walls retained a decreased basophilic staining and birefringence in the form of optically positive spherulites with about 15—20 nm retardation as compared with the 100—110 nm retardation of the radially negative birefringence seen before the extraction experiments.

Discussion

We have carried out polarization-optical investigations into the ultrastructure of the polysaccharides of the cell walls and capsules of yeasts, using a specific optical reaction demonstrating a linear order of the vicinal OH groups of polysaccharide chains [16].

So far it has been possible to use polarization-optical investigations only for plant cell walls which contain highly ordered micellar cellulose or chitin chains [2, 3, 17] and possess intensive intrinsic birefringence. Attempts to apply this method to the yeast cell wall have failed because the polysaccharides of this structure show neither form nor intrinsic birefringence.

As to the molecular mechanism of the ABT reaction, strong birefringence is induced by the dye molecules bound perpendicularly on the reacting polysaccharide chains if the vicinal OH groups are ordered in a linear pattern. Therefore, the ABT-induced birefringence is negative with respect to the chain lengths of polysaccharides [16]. We have found this method very useful in the study of the molecular order of polysaccharides in different biological structures [16]. It proved to be an excellent and very sensitive method for ultrastructural study of the molecular order of polysaccharide chains in the complex cell walls and capsules of yeasts, algae and bacteria [1]. As shown in our Figures, the ABT reaction induced very strong birefringence in the cell walls and capsules of yeasts.

Ultrastructure of the yeast cell wall

Figs 2—5 show the staining and optical character of the cell walls of *Saccharomyces cerevisiae* after different treatments. In Fig. 3, the basophilic cell walls stained with toluidine blue at pH 4.0 appear isotropic. After ABT reaction, there is a very strong basophilic staining, and the yeast cell walls appear as intensively birefringent spherulites (negative with respect to the radius). This indicates tangential orientation of the slow axis of transmission i.e. the plane of the dye molecules, suggesting radially-oriented polysaccharide chains (Fig. 1c). A similar anisotropy effect was found earlier on starch granules [16]. After acid or alkali hydrolysis, double-layered cell walls appeared (Fig. 5) with strongly decreased birefringence, and the optical character charged to positive with respect to the radius, suggesting radial orientation of the dye molecules associated on polysaccharide chains oriented parallel to the surface (Fig. 1b). This may be explained by assuming that a resistant, circularly oriented polysaccharide structure persisted after hydrolysis. However, the difference in intensity of the birefringence effect of the radially (50—70 nm) and circularly (10—19 nm) oriented components suggests quantitative prevalence of the former. This optical finding of the presence of a surface-parallel

polysaccharide structure after acid treatment seems to agree with electron microscopic observations demonstrating in the cell wall of yeasts circularly ordered fibrillar structures [5, 11], which were identified by X-ray diffraction studies as chitin [5, 8] or probably hydroglucan [8, 12]. Similar optical findings were obtained on the cell walls of *S. ellipsoideus*, *Candida albicans* and even on those of capsulated cryptococci. Especially remarkable was the very strong, ABT-induced birefringence of the cell walls of cryptococci (100—110 nm), which was often more pronounced than that of the birefringence of the thick capsules (see Fig. 6). The optical character of the cell walls was originally negative with respect to the radius but the staining intensity was decreased and the optical character was inverted into optically positive spherulites by acid or alkali hydrolysis. These findings may be explained by the assumption that the yeast cell wall is built up of two differently oriented micellar polysaccharide textures:

(1) polysaccharide chains with radially-oriented vicinal OH groups (radially or helically ordered polysaccharides) which are sensitive to alkali and acid hydrolysis and

(2) surface-parallel, circularly-ordered polysaccharide layers resistant to alkali and acid hydrolysis.

The opposite orientation of the polysaccharide chains in the cell walls of yeasts seems to explain the lack of birefringence of the cell wall in the unstained state.

Ultrastructure of the capsules of cryptococci

After ABT reaction has developed the capsules of cryptococci appeared as strongly basophilic and birefringent structures in the form of optically-negative spherulites, indicating tangentially-ordered dye molecules (surface-parallel orientation of the shlow axis of retardation i.e. the plane of the toluidine blue molecules) on a radially-oriented polysaccharide texture. This optical observation is in agreement with recent electron microscopic [4] and freeze-etching [19] findings which demonstrated radially-ordered fibrillar structures also in the capsule of cryptococci. Furthermore, it is of interest that the large capsules of cryptococci showed heterogeneous staining and an opposite optical behaviour. The outer layer of the capsules appeared more basophilic and opposite to the thicker inner layer in optical character.

At the transition zone between the two optically opposite layers, an isotropic layer was clearly seen. These findings indicate that similarly to the cell walls, both radially and surface-parallel oriented polysaccharide chains are involved in the building up of the capsules.

The partial resistance of the cell walls to hydrolysis suggests that the (not resistant) circular polysaccharide part of the capsule differed in its chem-

ical structure from that of the cell wall. This is in agreement with the findming that the capsules of cryptococci do not contain any chitionous component [12].

Our findings indicate that optical analysis by means of the ABT reaction is a very sensitive and useful method in the study of the molecular order of the polysaccharide chains of yeast cell walls and capsules. Therefore, this method seems to be able to give further information about the complex ultrastructure of the cell walls and capsules of microorganisms, a possibility which, in view of the molecular mechanism of the ABT reaction, is not shared by other ultrastructural methods.

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ELECTRONMICROSCOPICAL STUDIES ON THE ARGYROPHILIC STRUCTURES OF COLPIDIUM CAMPYLUM (CILIATA, TETRAHYMENIDAE)

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Abstract

Comparative light- and electron microscopical analysis of the argyrophilic structures in wet silvered *Colpidium campylum* has led to the following results. (1) The silverline meridians order 1 and 2 are located in the epiplasm, beneath the adjacent alveolar membranes of the pellicle. They are closely connected with the basal bodies, the protrichocysts and the excretion pore. Thus, the argyrophilic substance is continuous in the cortex of *C. campylum*. (2) The basal bodies are argyrophilic mainly on their proximal thirds and are surrounded by meridians order 1 in a ring-like fashion. However, the argyrophilic substance of the meridians order 1 breaks into the basal bodies and the distal parts of the cilia and surrounds the parasomal sacs. (3) The protrichocysts, except their apices, are surrounded by silver aggregates. These are localized on the outer side of the cytoplasmic membrane limiting the protrichocysts, and this membrane is connected with the endoplasmic reticulum. The disappearance of the silver aggregates soon after the protrichocysts have been discharged suggests that the argyrophilic substance is resorbed in the cytoplasm. The electron microscopical findings enable a better understanding of the light microscopical observations concerning the location of the argyrophilic substance in both resting and functioning protrichocysts. (4) The excretion pore in its whole extension is surrounded by silver aggregates which are located tightly beneath the pellicle. (5) Attempts were made to correlate the electron microscopical findings with the light microscopical ones, and a fibrillar nature of the silverline system of tetrahymenid ciliates is suggested.

Introduction*

Recently, having demonstrated by electron microscopy the subpellicular location of the SL* system (a formation discovered by KLEIN [12, 15]) in *Colpidium colpoda*, we [6] have refuted the hypothesis proposed by PITELKA [19] and other electron microscopists [for detailed literature, see 6] for the position and structure of this system. We have mainly studied the position and structure of SL meridians order 2. The arrangement and the location of the silver deposition at the basal bodies, protrichocysts, and SL meridians order 1 require further studies.

I had performed such investigations now in *Colpidium campylum*, a species closely related to *Colpidium colpoda*. In the present work, the findings

* In this paper the following abbreviations are used: SL = silverline, AS = argyrophilic substance.

in *C. colpoda* have been reproduced in *C. campylum*, and the site of silver deposition at the protrichocysts, basal bodies, parasomal sacs, SL meridians order 1 as well as those at the excretion pore have been investigated in detail.

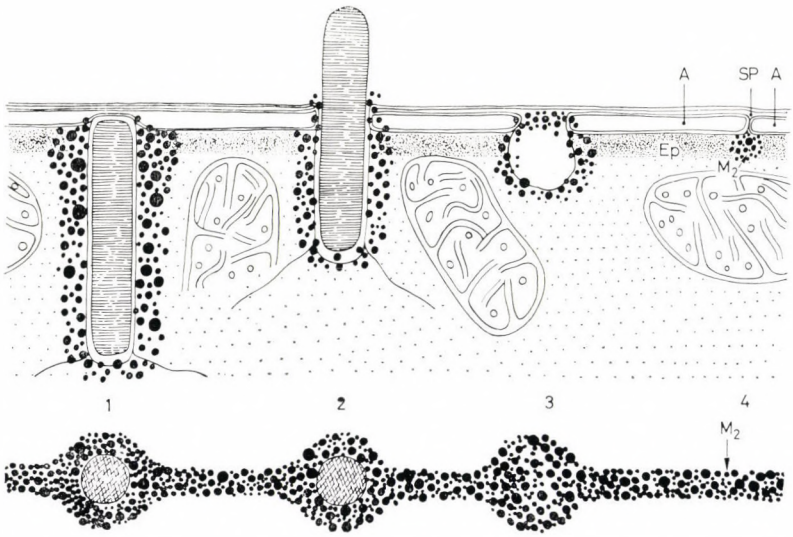


Fig. 1. The upper half is a schematic drawing of the silver deposition at resting and functioning (2—4) protrichocysts of *C. campylum* in longitudinal section. In the lower half the corresponding stages, recognizable even light microscopically, are shown in cross section. The SL meridians order 2 (M_2) are localized in the epiplasm (Ep), beneath the adjacent alveolar membranes of the pellicle (A) and enclose the protrichocysts. Further explanation in text

Material and method

Colpidium campylum, a ciliate of about 70 μm in size, very common in α-mesosaprobe and polysaprobe puddles, was cultivated in plant infusions. The species was determined according to the criteria recommended by KAHL [11], FOISSNER [5] and MAC COY [17].

For light and electron microscopy, the animals were sucked off from the surface of the infusion with a pipette, carefully centrifugated and, without any washing, poured over with the fixative.

Dry [8, 12, 15] and wet [3] silver preparations were comparatively examined under the light microscope. The procedure of wet silver impregnation was the same as described by CORLISS [3].

Preparation for electron microscopy. Fixation according to PALADE [18] at pH 7.5 for 15 min. The osmium tetroxide was washed off with the stock puffer solution, the preparation was dehydrated in an alcohol series, kept in propylene oxide for 30 min and embedded in Epon 812.

Dehydration of wet silvered specimens was interrupted at 50% alcohol concentration, then they were separated from the slide together with the gelatine layer with a razor blade. The dehydration in the alcohol series was continued subsequently (separation of completely dehydrated preparations fails!). The animals, embedded in the gelatine layer, were then placed in propylene oxide for 30 min and embedded in Epon 812.

Thin sections were made by means of a diamond knife with a Reichert OMU-2 ultramicrotome and spread on a carbon strengthened, pioloform filmed slide. Counter staining: 15 min in an alcoholic (50%) uranyl acid solution and 15 min in a lead citrate solution after REYNOLDS [20]. For electron microcopical investigations, the EM 9S of the firm Zeiss has been available for use.

Results

Light microscopical studies

The SL system in *C. campylum* as seen in dry silvered specimens (8)

Considering that numerous studies on the SL system of *Colpidium campylum* have been published [5, 10, 13, 14], we described this system very briefly, completed by several micrographs (Figs 2 – 5). Based on their connec-

Fig. 2. Right lateral aspect of the SL system of *C. campylum*. The SL meridians order 2 (M_2) originate near the apical pole from the SL meridians order 1 (M_1) and return into the same below the excretion pore (Ex). Dry preparation, × 1 200

Figs 3, 4. High power magnification of the SL system of *C. campylum*. The basal bodies (Bk) located in the SL meridians order 1 appear unevenly impregnated. The appearance of the relation bodies of the protrichocysts (P) depends on the stage of function of the organelles. Wet preparation, × 3 000, × 3 200

Fig. 5. A highly argyrophilic excretion pore (arrow) connected with the adjacent SL meridians order 1 and 2. Wet preparation × 3 200

Fig. 6. A slightly oblique cross section through *C. campylum*. Note the fibril system typical of tetrahymenid ciliates (kinetodesmal fibre = kf, postciliary tubuli = pt, transverse tubuli = tt), a pellicular alveole (A), and an epiplasm (E). Method "a", × 48 000

tions with certain organelles, SL meridians order 1 (Fig. 2, M_1) and order 2 (Fig. 2, M_2) are to be distinguished. The basal bodies of the cilia (Figs 3, 4, Bk) appear located in a regularly meridional row, viz., in meridians order 1, which often show an undulant course (Fig. 4). The basal bodies appear as heavy impregnated argyrophilic granules showing no special fine structure.

The meridians order 2, which continue in the relation bodies of the protrichocysts, are located just between the rows of the basal bodies. They arise near the apex from meridians order 1 and are emptied near the antapex into the same structure (Fig. 2). Unlike meridians order 2 in *C. colpoda*, those in *C. campylum* are usually not divided into two or three SLs [comp. 5]. In the rare cases when they are, there are well defined anastomoses between the separated parts of meridians order 2. Similar anastomoses may occur between SL meridians order 1 and 2 as well (Fig. 3). The relation bodies of the protrichocysts are arranged in the SL meridians order 2 at very irregular distances (Figs 2, 3, 4). They appear as homogenous AS (Fig. 2) accumulated in an area about 1 μm in diameter. Occasionally, heavily impregnated rings about 1.5 μm in diameter have also been seen.

The excretion pore (Fig. 2 Ex, 5 arrow) is an heavily stained argyrophilic ring with a much less argyrophilic centre. The neighbouring meridian order 1, sometimes also the neighbouring meridian order 2, runs into the argyrophilic ring of the excretion pore (Fig. 5).

The SL system in *C. campylum* as seen in wet silvered specimens (3)

Like in *C. colpoda* [comp. 6], there are no principal differences in *C. campylum* between dry- and wet silvered SL systems. The latter are sometimes impregnated so finely that they are practically invisible. Similar difficulties were met in the electron microscope technique, viz., one often could not decide if an intensely impregnated specimen was present in a semi-thin section. Too finely impregnated SL systems are of limited use in the electron microscopy because in such sections the SLs are scarcely elevated over the fine nonspecific precipitates.

The impregnation mode of the basal bodies and of the relation bodies of protrichocysts provides the only differences between pictures obtained with dry and wet preparation. As shown in Figs 3 and 4, the impregnation of the basal body apparatus is quite uneven. Some appear as a heavily argyrophilic grain, others as a ring carrying one or more argyrophilic grains of variable size. I could not differentiate between basal grain and accessory grain as described by KLEIN [13] and GELEI et al. [10]. It is a further difficulty that protrichocysts occasionally occurring adjacent to basal bodies and are then mistaken for an accessory body. In wet silvered specimens, ring-like silver deposits are

often seen at the site of the protrichocysts (Figs 3, 4, P). This finding, very important for an understanding of the function of protrichocysts, is dicussed in detail below.

Electron microscopical studies

Examination of *C. campylum* prepared by method "a"

The fine structure of *C. campylum* has been described in some detail by PITELKA [19]. I cannot add much to his data, which have been completed more recently by very detailed observations concerning the fine structure of related ciliates [1, 4, 21]. The organization of the cortex of *C. campylum* is very similar to that described in some detail for *C. colpoda* [6].

The pellicle consists of the cell membrane, which continuously surrounds the whole cell, and the membrane bounded alveoles (Figs 1, 6, A). The silver should be deposited at the joining sites of these alveoles [19]. The fibrogranular epiplasm (Figs 6, 8, EP), about 80 nm in thickness, is located beneath the inner alveolar membrane. It becomes very thin near the basal body and then passes through this body, in which it forms the basal plate. The arrangement of the cortical fibrillar systems (Fig. 6) corresponds to the situation described by ALLEN [1] and ELLIOT et al. [4] for *Tetrahymena pyriformis*, and the same is valid for the structure of the protrichocysts (mucocysts) and the excretion pore.

Examination of *C. campylum* prepared by method "b"

Silverline meridians order 1 and 2. As shown in all the Figures presented in this paper SL meridians order 1 and 2 are located in the epiplasm, beneath the joining site of the pellicular alveoles (Fig. 9, arrows). They are about 80 nm in diameter and built up of silver aggregates of variable size. The distances between these aggregates are sufficient to separate them from one another. The size of the silver aggregates and the diameter of the SLs depend on the quality of the preparation, viz., in heavily impregnated preparations the silver aggregates are large (some 40—80 nm) and are very near to each other (Fig. 7), whereas in finely impregnated specimens they are much smaller (some 10—40 nm) and are well separated from each other (Figs 8, 9, 11). Consequently, the SLs are larger in diameter in heavily impregnated animals than in finely impregnated ones. The SLs are indistinctly limited from the cytoplasm and the epiplasm (e.g. Figs 8, 9, 10, 11). They are packed most tightly at the border between epiplasm and cytoplasm (Figs 9, 11) and decrease in both size and number towards the pellicular membranes.

The basal bodies, cilia and parasomal sacs. It has been shown without any exception that, independently of the degree of impregnation, only the proximal thirds of the basal bodies are argyrophilic (Figs 12, 13, 16, 19). Their

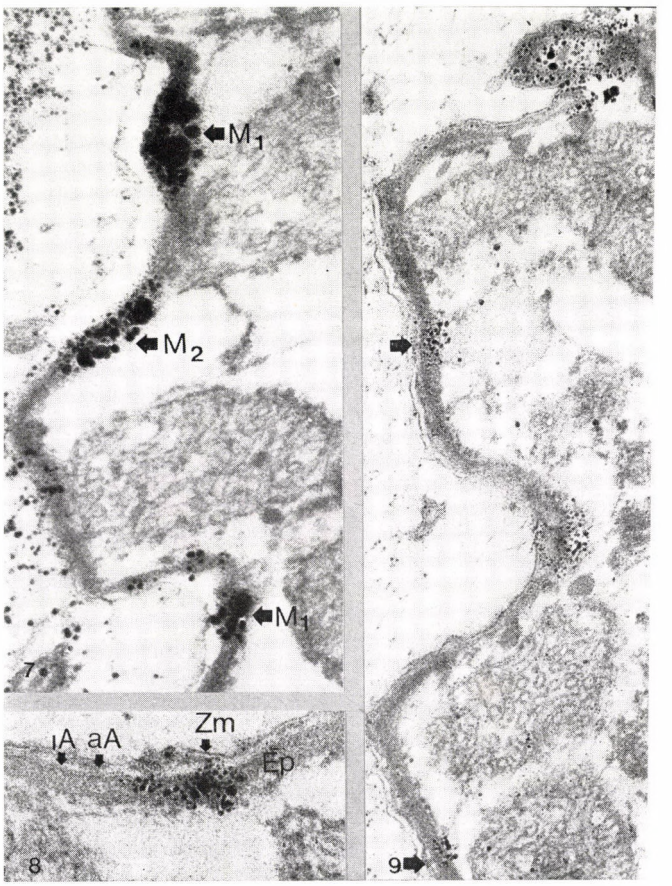


Fig. 7. A cross section showing two SL meridians order 1 (M_1) and a slightly obliquely sectioned SL meridian order 2 (M_2). SL meridians are built up of very distinctly large silver aggregates. Method "b", × 56 000

Fig. 8. High power magnification of SL meridians order 2. Silver aggregates are seen only in the epiplasm (Ep), beneath the cell membrane (Zm) and beneath the outer (aA) and inner (iA) alveolar membrane. Method "b", × 80 000

Fig. 9. A cross section showing two SL meridians order 1 with basal bodies, and two SL meridians order 2. It is clear that the latter are below the joining site of the pellicular alveoles. Method "b", × 80 000

middle and distal parts show very weak, if any, argyrophilia (Figs 12, 16). It is especially clear in cross sections (Figs 18, 20) that the majority of the silver aggregates was found around the basal bodies while much less aggregates

occurred in their centre. However, no sharp border could be demonstrated between the silver aggregates inside and those outside the basal bodies.

Beside the cilia, mainly the axonema part adjacent to the basal bodies shows argyrophilia (Figs 16, 19). Proximally to this, no specific silver deposi-

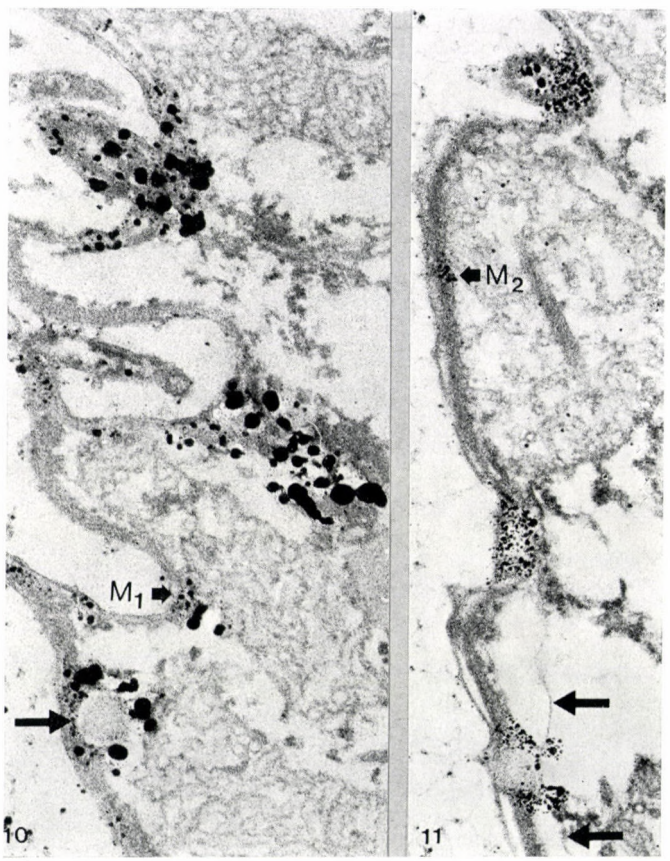


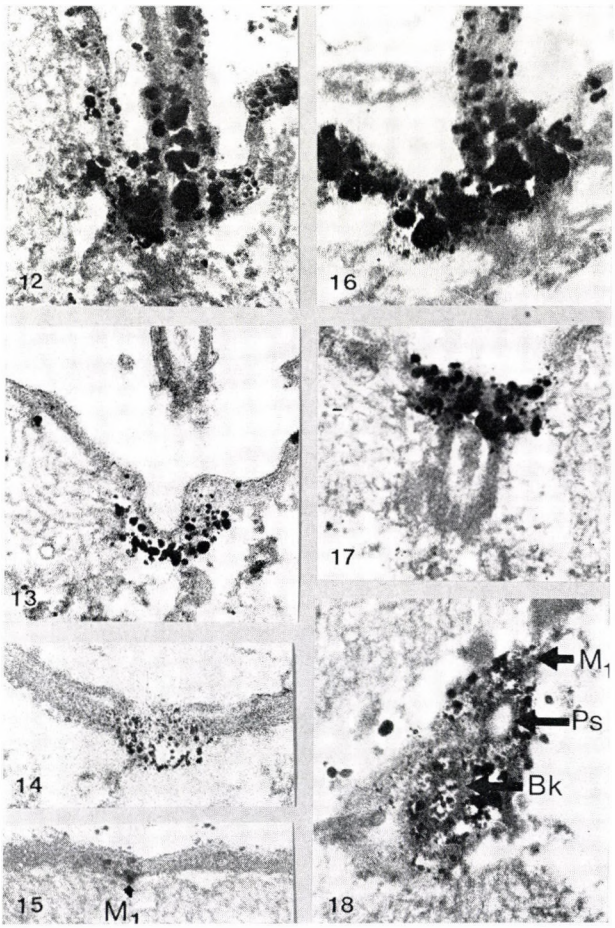
Fig. 10. An oblique section. Note the ring-like silver deposition around a protrichocyst (arrow) as well as around a basal body and a SL meridian order 1 (M_1). Method "b", × 64 000
Fig. 11. Cross section of a very finely impregnated *C. campylum* specimen. Note the SL meridian order 2 (M_2) in the epiplasm and the protrichocyst just before discharged. The membrane enclosing the protrichocyst (comp. Fig. 22) is connected with the endoplasmic reticulum (arrows). Method "b", × 56 000

tion could be demonstrated. Here, the occasionally observed aggregates were quite irregularly distributed.

The silver aggregates around the basal bodies continue without any interruption in aggregates around the parasomal sacs and build up the SL meridians order 1 (Figs 12—15, 18, 21). The parasomal sacs are surrounded

by silver aggregates in a bag-like manner (Figs 13, 18), but their central parts are free of them.

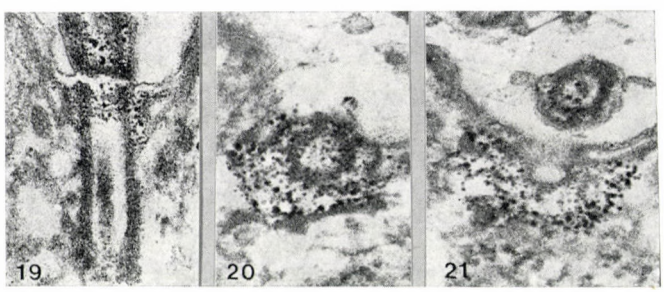
Protrichocysts. Examination of a great number of silver impregnated *C. campylum* enabled us to reconstruct, and control by light microscopy, the



Figs 12—15. This series of electron micrographs presents a good impression as regards the silver deposition at basal bodies and parasomal sacs. The silver aggregates in and around the basal bodies (Fig. 12) lead without any interruption to the parasomal sac and to the SL meridian order 1 (Fig. 15, M_1). Method "b", × 64 000, × 64 000, × 64 000, × 48 000
Figs 16, 17, 18. Irrespective of the degree of impregnation (comp. Fig. 16 with Figs 12, 19) it is clearly seen that only the proximal thirds of the basal bodies are argyrophilic. There are silver aggregates inside and around basal bodies. Method "b", × 64 000

exact location of the AS in relation to the resting and functioning protrichocysts (Fig. 1). Four phases could be distinguished, viz., (1) resting protrichocysts which are surrounded by a membrane originating in the endoplasmic

reticulum (Fig. 11, arrows); these are surrounded, in a bag-like manner, by a great number of silver aggregates (Figs 1 [1], 22). The aggregates, which continue without interruption in the SL meridians order 2 (Fig. 11), are carried tightly by the membrane surrounding the protrichocysts, but are not connected with the organelles themselves. The apex of the protrichocysts was always free of silver aggregates (Figs 22, 23). At the level of the epiplasm, however, these occur in a greater number, appearing in longitudinal sections as a wedge-like silver deposit (Fig. 22). The degree of silver accumulation as well as the size of the silver aggregates depends, just like in meridians order 1 and 2 and the basal bodies, on the preparation technique. (2) When the protrichocysts are pushed out, the pellicular membranes fuse and the organelles can leave the animal through the resulting circular gap (Fig. 1 [2]). Most of the AS remains in the animal (Fig. 23), but little amounts may be found tightly attached over the pellicle, suggesting that part of the AS is lost (Fig. 23, arrow) [compare 6]. (3) The bage-like vacuoles that contained the protrichocysts will diminish in size and soon disappear as the organelles have



Figs 19, 20, 21. Plane sections showing that silver aggregates of SL meridian order 1 (Fig. 18, M_1) continue without any interruption to the basal bodies (Fig. 18, Bk) and to the parasomal sacs (Fig. 17, 21, Ps). Method "b", × 64 000

been discharged (Figs 1 [3] 26). Meanwhile the silver aggregates are evenly distributed around the more or less spherical vacuole so that the silver aggregates appear diffusely distributed (Figs 1 [3], 25). (4) These aggregates diminish in number and eventually disappear at all as the gaps become smaller and smaller. The AS is obviously resorbed by the cytoplasm. Only SL meridian-order 2 remains visible; it runs without any interruption over the place where the organelles were released (Figs 1, 11).

The existence of four phases is consistent with the light microscopic findings (see discussion): phases 1 and 2 correspond to the ring-like silver deposit (Figs 4, P, 22–24), phase 3 correspond to the diffusely impregnated relation bodies (Figs 3, P, 25, 26) while phase 4 to meridians order 2 (Fig. 11).

The excretion pore. The light microscopical findings have been confirmed by electron microscopy also as regards the silver deposit at the excretion pore. In longitudinal sections of the excretion pores (Fig. 27) the very distinct, large silver aggregates were seen only under the pellicular membranes and under the alveolar membranes joining the pores. Although these should show light microscopically one or more uniformly impregnated grains, the silver deposit

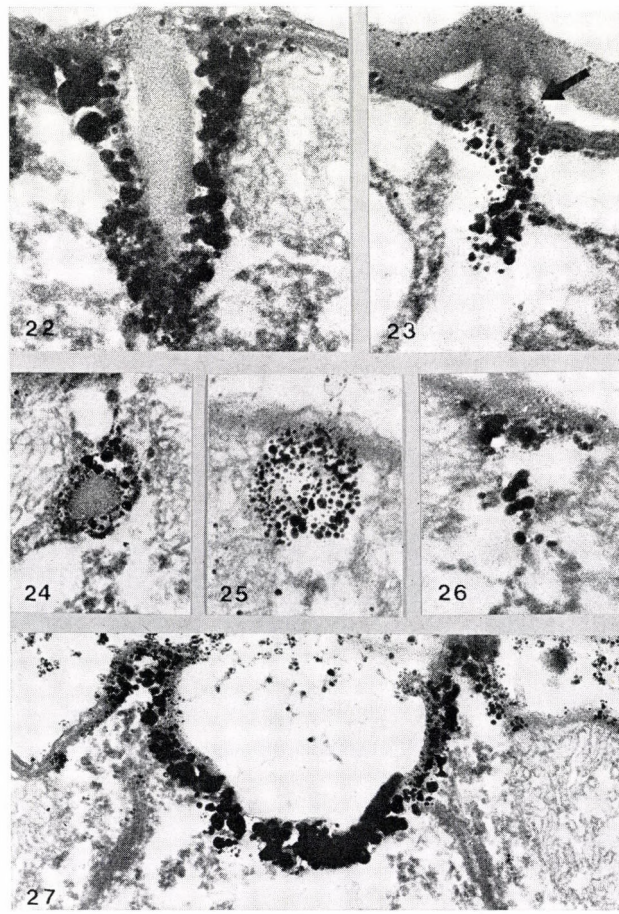


Fig. 22. Resting protrichocyst surrounded by bag-like arranged silver aggregates. These are located outside the membrane enclosing the organelle. Method "b", × 72 000
Fig. 23. When the protrichocysts are pushed out, a little AS (arrow) may escape. Method "b", × 64 000
Fig. 24. Resting protrichocyst. Cross section. Note the ring-like silver deposition. Method "b", × 64 000
Figs 25, 26. Protrichocyst "loop-holes" in plane section (Fig. 26). The silver aggregates are nearly evenly distributed on the surface of the loop-hole. Method "b", × 64 000
Fig. 27. Excretion pore, longitudinal section. Note the silver aggregates exclusively under the pellicular membranes. Method "b", × 48 000

often appears ring-like (Fig. 5). This discrepancy can easily be explained by the following considerations: (1) The ring-like formation may be an optical artefact which arises because the centres of the pores, being located considerably deeper, do not draw a sharp picture. In fact, weak argyrophilia can be seen in the centres of the pores as well if the specimens are examined correctly. (2) Furthermore, the silver-free centre may be an expression of a certain stage of function of the contractile vacuole. When the vacuole is emptied, the membranes limiting the pore are broken through [4]; consequently, the AS in the center of the pore must disappear. During the subsequent regeneration of these membranes AS must develop again beneath the membranes, making AS demonstrable by electron microscopy (Fig. 27). It is for the same reason, that homogeneously impregnated pores in variable number are seen in many light micrographs.

Discussion

SL meridians order 1 and 2

The present studies have shown that the SL meridians order 1 and 2 are located in the epiplasm of *C. campylum*. Accordingly, they can be regarded as subpellicular structures [6]. In *C. colpoda*, on the other hand, we [6] found the SL meridians order 2 in the epiplasm, whereas the meridians order 1 somewhat deeper. A revision of the *C. colpoda* preparations has recently shown that the SL meridians order 1 are located in the epiplasm too. The earlier erroneous conclusion can be explained by the fact that we did not take into consideration the silver deposit around the parasomal sacs which extends deep into the cytoplasm (comp. Figs 13, 14). Thus, we concluded that the SL meridians order 1 are located somewhat deeper, in a variable position.

The protrichocysts

KLEIN [15, 16] described the position of the AS at resting and functioning tricho- and protrichocysts as follows. (1) Tricho- and protrichocysts are connected by a relation grain, which is somewhat below the apex, with the SL system. (2) At the site where an organelle is being discharged, the SL forms a ring, i.e., a circular fibril through which the organelle leaves the animal. The relation grain, remaining on the relator, leaves the animal together with the secretion organelle. (3) The strong argyrophilia around the circular fibril is due to the activation of a finely meshed SL system at the site of discharge. (4) The gap (a "loop-hole") and the ring are closed soon after the secretion organelle has left the animal.

The present electron microscopical studies have led to a controversial interpretation of the light microscopical findings (see p. 000): (1) No relation grain as interpreted by KLEIN [15, 16] exists at the protrichocysts (Figs. 2, 23). The fact that KLEIN [16] found a relation grain in the lumen of a circular fibril might be explained as follows: as a protrichocyst has been discharged, part of the AS extends, more or less evenly, over the gap and thus a relation grain may be simulated (comp. Figs 25, 26). (2) There is no circular fibril around the protrichocysts, for the AS surrounding the protrichocysts along its full lenght leaves only its apex free (Fig. 22). KLEIN [16] himself emphasized the rarity of preparations showing such circular fibrils. This may therefore be interpreted so that resting protrichocysts around which the light microscope shows a ring-like silver deposition caused by the bag-like silver accumulation around these organelles (see Fig. 22) occur very seldom in dry prepared specimens because the protrichocysts, owing to the stimulus due to dehydration, are soon discharged and, consequently, mainly the resorption stages of "loopholes" are seen (Figs 25, 26). This interpretation is supported by the observation that such circular fibrils have been seen much more frequently in wet prepared specimens (Figs 3, 4), which had been killed by a procedure of much shorter duration. (3) The intensive argyrophilia of the "circular fibril" described by KLEIN [15, 16] arises from the bag-like AS surrounding the protrichocysts (Fig. 22). The concentration of AS here causes a light microscopically demonstrable argyrophilia, more intensive than in SL meridians order 1 and 2. (4) The AS around the protrichocysts is resorbed as the organelles have been discharged. This is supported first of all by the light microscopic findings in dry prepared specimens, viz., the majority of SL meridians order 2 show neither argyrophilic grains nor rings [13].

Based on these findings we recommend the term "relation body" (Relationskörper) instead of the terms "Zirkularfibrille" and "Relationskorn" proposed by KLEIN [16].

The basal body

The electron microscopic findings concerning the silver deposition at the basal bodies and parasomal sacs do not correlate with the light microscopic observations unequivocally. This is because the structural units are at the threshold of light microscopic visibility and thus can be misinterpreted. These difficulties point to the fact that the structure of the basal body apparatus of *C. campylum* as described by KLEIN [14] and GELEI et al. [10] is somewhat different. The circular fibrils of these authors may correspond to the silver deposits around the basal bodies (Figs 18, 20). The argyrophilic granules (basal grain and accessory grains) on and in these rings should mostly correspond

to the basal bodies with their parasomal sacs. Since these grains may occur in great number and are not always round (Figs 3, 4), we attribute some role to preparation artefacts and/or optical deformations.

Structure and function of the SL system

It was found shortly before our electron microscopical studies on *C. campylum* that, in peritrichous and hypotrichous ciliates, the silver lines are identical with certain cortical fibrils [7, 9]. The same could be not demonstrated in tetrahymenid ciliates. In conventionally prepared specimens of *C. colpoda* [compare 6] and *C. campylum* no specific material was found at the sites of silver deposits. I am nevertheless convinced that the SLs are fibrils even in tetrahymenid ciliates, though, these fibrils cannot be demonstrated with the preparation techniques used so far [compare 6]. The SL systems of ciliata show light microscopically such a very similar structure that it would be highly unlikely that they were build up of fibrils only in peritrichous and hypotrichous ciliates and not in tetrahymenid ciliates. It is however possible that the SL systems of different ciliates show some structural dissimilarities.

The present investigations emphatically suggest that the SL system is connected with several cortical structures, viz., basal bodies, protrichocysts and excretion pore. These and many other observations [see 6, 7, 9] support the view that the SL system plays part in conveying locomotoric impulses and in building up new formations [15].

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UTILIZATION OF KEROSENE BY A HYDROCARBON-ASSIMILATING STRAIN OF FUSARIUM OXISPORUM

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Abstract

Fusarium oxysporum IFO 6384 was grown in three different media, one containing glucose and two containing kerosene as carbon source. One of the kerosene media contained also Tween 60. The harvested dry mats were weighed at various stages of growth. Substitution of glucose with kerosene led to a sharp drop in the production of fungal mycelia. Analysis of the mat samples representing maximum yields indicated that the mycelia grown in the kerosene media were richer in free amino acid and poorer in protein as well as in polymeric and low-molecular weight carbohydrates than those grown in the glucose medium. Treatment of the culture filtrates with three volumes of acetone led to the separation of two fractions. In the first, acetone soluble fraction the free sugars and amino acids were determined, whereas the second, acetone precipitable one, was examined for its proteolytic activity. The gelatinase activity of the acetone precipitable material (APM) obtained from the kerosene media was lower than that from the glucose medium. The former activity, in contrast to the latter reached its maximum and minimum values at pHs 4 and 9, respectively.

Introduction

The ability of microorganisms to assimilate hydrocarbons was recognized many years ago and since then much attention has paid on the production of microbial cells and metabolites, such as organic acids and vitamins, from n-paraffins. However, little information is yet available on the production of enzymes from hydrocarbons. In recent years, there has been a growing interest in the extracellular protease produced by hydrocarbon assimilating fungi. Nakao et al. [7] and Suzuki et al. [9], investigating fungi producing alkaline protease from n-paraffins, selected *Fusarium* sp. S-19—5 as an excellent strain and successfully obtained the kabicidine resistant mutant No. 5-128B. This mutant was found to be capable of accumulating large amounts of alkaline protease in the culture broth, and excreted larger quantities of enzymes, such as amylase and nuclease, than the parent. Furthermore, Suzuki et al. [8] have investigated the culture conditions for industrial production of alkaline protease from n-paraffins by the mutant No. 5—128B. The effects of oxygen supply, initial pH and temperature, nitrogen source, phosphate concentration, and surface active agent were studied.

The present work aimed at studying the utilization of kerosene (instead of glucose) by *Fusarium oxysporum* IFO 6384 and production of fungal mycelia as well as some metabolites, such as amino acids, proteins, carbohydrates, and protease.

Material and method

Organism. The microorganism used in this work was *Fusarium oxysporum* IFO 6384, kindly supplied by the "Institute for Fermentation, Osaka (IFO), Japan".

Culture media and conditions. The composition of the media is given in Table 1. The fungal strain grown on slants was used to inoculate 10 ml of medium (I) (see Table 1) in large test tubes and the tubes were incubated at 28 °C for 48 h in a shaker. One millilitre volumes of the culture broth thus obtained were transferred into 250-ml Erlenmeyer flasks each containing 25 ml medium and the flasks were shaken for 144 h at 28 °C on a rotatory shaker. Fungal mycelia were harvested at different incubation periods by filtration followed by quick washing with 0.85% saline. The collected mats were dried over P_2O_5 *in vacuo* to constant weight.

Table 1

Composition of the media used for growing of Fusarium oxysporum IFO 6384

Component	Amount present in 1000 ml		
	Medium I	Medium II	Medium III
Kerosene	—	50 ml	50 ml
Glucose	15.00 g	—	—
KH_2PO_4	1.00 g	1.00 g	1.00 g
$NaNO_3$	2.00 g	2.00 g	2.00 g
$MgSO_4 . 7H_2O$	0.50 g	0.50 g	0.50 g
KCl	0.50 g	0.50 g	0.50 g
$FeSO_4 . 7H_2O$	0.01 g	0.01 g	0.01 g
Yeast extract	1.00 g	100 g	1.00 g
Tween 60	—	—	0.80 g
pH	5.00	5.00	5.00

Analysis of the fungal mycelia. The isolated mycelia were extracted several times with hot ethanol (85%) and the alcoholic extract was analyzed for its amino acid and low-molecular weight carbohydrate contents. On the other hand, the alcohol extracted mycelia were then subjected to two acid hydrolysis procedures, one specific for hydrolysis of polysaccharides [4], and the other for proteolysis [2]. Thus in the first hydrolysate the carbohydrates, whereas in the second the amino acid contents were determined.

Determination of amino acids. The method of MUTING and KAISER [6] was used for estimation of amino acids. A standard curve for glycine was used for calculation.

Preparation of the acetone precipitable material (APM). The culture filtrate was added to 3 volumes of ice-cold acetone and the APM was separated by centrifugation, then dried over P_2O_5 under reduced pressure. The supernatant was shaken three times with equal volumes of ether, and the aqueous layer was submitted to amino acid and carbohydrate analysis.

Determination of proteins. This was done according to the method of LOWRY et al. [5].

Determination of gelatinase activity. The APM was dissolved in 0.2 mol/l glycine buffers (pHs 4, 7, and 9) to obtain the final concentration of 1 mg/ml (in each case). In the resulting solutions the gelatinase activity was determined at the afore-mentioned pH-values, according to the method of BERGKVIST [1]. Briefly, a 5% gelatin solution containing the AMP was incubated at 37 °C, and the reduction in viscosity was determined.

Results and discussion

The growth rates for *Fusarium oxysporum* IFO 6384, grown in three culture media, are given in Table 2. The results clearly show that the substitution of glucose with kerosene as carbon source led to a sharp drop in the production of mycelia. This observation seems to be in accordance with that of SUZUKI et al. [8], who found that the growth of the mutant No. 5—128B of *Fusarium* sp. decreased to a quarter of its value when kerosene was used instead of glucose.

Table 2

Production of fungal mycelia in three media containing different carbon sources*

Incubation period (h)	Weight (in g) of mats harvested from different media		
	Medium I	Medium II	Medium III
48	5.00	0.72	0.32
72	5.12	1.52	0.68
96	4.40	2.00	1.16
120	4.09	2.48	1.64
144	3.92	2.12	1.40

* See Table 1

Addition of Tween 60, a surface active agent, to the kerosene medium resulted in a further decrease in mycelial yield. However, the maximum weight of mycelia was reached as late as after 5 days of incubation in contrast to the 3 days required is the glucose medium. The results, collectively, indicate that kerosene was utilized more slowly and had a lower efficiency for production of *F. oxysporum* mycelia, as compared to glucose. It is likely that glucose can easily be utilized without being transformed into an intermediary metabolite, whereas kerosene requires some conversions to be ready for absorption and assimilation. The mycelial samples representing maximal yiedls were analyzed for low-molecular weight and polymeric carbohydrates as well as free and bound amino acids. The results recorded in Table 3 indicate that the fungal mycelia grown in the kerosene media were characterized by their higher free amino acid content and lower carbohydrate and protein contents as compared to those of mycelia grown on the glucose medium. Furthermore, it is evident that there is no significant difference in composition between mycelia grown in either of the two kerosene media. These analytical data may be valuable for any further investigation of economical production of fungal amino acids, proteins, and carbohydrates using a cheap carbon source.

Table 3

Composition of the mat samples representing the maximum yields

Culture media**	Components(%)*			
	Low-MW carbohydrates	Polymeric carbohydrates	Free amino acids (as glycine)	Bound amino acids (as glycine)
Medium I	4.80	24.54	1.60	15.82
Medium II	2.33	17.29	3.00	12.81
Medium III	2.25	16.53	2.25	12.52

* On dry basis
** See Table 1

Treatment of the culture filtrate of *F. oxysporum* with 3 volumes of acetone led to the separation of two fractions, viz., an acetone soluble fraction and an aceton precipitable one. In the former, the free amino acids and sugars, whereas in the letter the proteolytic activity were determined. Table 4 shows the concentration of extracellular free sugars present in culture filtrates of *F. oxysporum* obtained after different incubation periods. The results revealed that in the first 48 h more than 99% of the initial amount of the free sugars present in glucose medium was consumed and 5 g/l mycelium was pro-

Table 4

Concentration of free sugars present in culture filtrates of F. oxysporum IFO 6384 grown in three media containing different carbon sources*

Incubation period (h)	Free sugars (mg/l) determined as glucose		
	Medium I	Medium II	Medium III
0.00	15,138.00	138.00	138.00
48	52.00	96.60	41.08
72	53.64	88.51	42.15
96	52.91	79.00	45.62
120	60.16	60.32	50.05
144	65.40	62.16	52.84

* See Table 1

duced. On the other hand, 56.29% of the initial amount (0.138 g/l) of the free sugars present in the kerosene containing medium was consumed in the first 120 h of incubation, and 2.48 g/l mycelium was produced. Furthermore, when Tween 60 was added to the kerosene medium, 64% of the initial amount

(0.138 g/l) of free sugars was consumed in the 120 h of incubation and 1.64 g/l mycelium was obtained. These results indicate that in the presence of high concentration of glucose stimulates the utilization of this source of carbon and the production of relatively high amounts of mycelia. On the contrary, in the presence of kerosene as main source of carbon, the very little amount of sugars was utilized at a slower rate and, also, the mycelium yield was lower.

The extracellular free amino acids present in the different culture broths (Table 5), showed a drop in concentration by the 48th of incubation. The data in Table 5 indicate that 93% and 96% of the initial amounts of free amino

Table 5

Concentration of free amino acids present in culture filtrates of F. oxysporum IFO 6384 grown in three media containing different carbon sources*

Incubation period (h)	Free amino acids (mg/l) determined as glycine		
	Medium I	Medium II	Medium III
0.00	180.00	180.00	180.00
48	7.00	13.00	13.00
72	6.85	13.12	13.20
96	6.07	13.12	13.20
120	5.14	13.15	13.25
144	4.80	13.50	14.00

* See Table 1

acids, present in the kerosene media, and the glucose medium, were consumed within 48 h. The rapid consumption of extracellular amino acids can be attributed to the high amino acid requirement of *F. oxysporum*, especially in the early stages of growth (48 h).

Production of APM was also influenced by the carbon source. Table 6 shows that the maximum production of APM was attained after 120 h of incubation in the glucose medium and after 144 h in the kerosene media. Furthermore the maximum yield (1080 mg/l) of APM obtained from the kerosene containing medium II was slightly higher than that (936 mg/l) produced from the glucose medium. Addition of Tween 60 to the kerosene medium reduced the APM yield (800 mg/l).

The effect of carbon source and incubation period on the composition of the separated APM are shown in Table 7. Both of carbohydrate and protein contents of the APM decreased when the incubation period was prolonged

up to 120 h. A sudden increase in these components was then observed on the sixth day of incubation. These results indicate that the first decrease in carbohydrate content was due to its consumption by the growing fungus. To avoid confusion, it should be noted that the apparent slight decrease in protein content was accompanied by an appreciable increase (Table 6) in the yield of APM. Thus, the production of extracellular protein was actually increased with the increase of incubation period. The next sudden increase in both of carbohydrate and protein contents was supposedly due to an autolysis of the fungal mycelia and liberation of their cellular components. In determination of proteolytic activity of APM the presence of three types of protein hydrolyzing enzymes [1] with different optimum pHs was taken into consideration. Therefore, the gelatinase activities of the APM samples was assayed at three

Table 6

Production of the acetone precipitable material (APM) by F. oxysporum IFO 6384 grown in three media containing different carbon sources*

Incubation period (h)	Acetone precipitable material (mg/l)		
	Medium I	Medium II	Medium III
48	648	648	480
72	723	695	560
96	870	781	641
120	936	896	723
144	860	1 080	800

* See Table 1

Table 7

Composition of the APM obtained from filtrates of different cultures of F. oxysporum IFO 6384*

Incubation period (h)	Component (per cent)					
	Total carbohydrates			Proteins		
	Medium (I)	Medium (II)	Medium (III)	Medium (I)	Medium (II)	Medium (III)
48	15.00	13.25	16.16	39.57	39.57	35.41
72	12.14	11.68	14.71	37.15	38.26	35.00
96	10.08	10.92	11.97	37.60	37.15	34.82
120	8.75	7.35	9.05	37.49	37.49	34.82
144	17.88	12.06	16.89	45.83	39.89	38.92

* See Table 1

pHs, i.e., 4, 7 and 9. Table 8 shows the effect of the carbon source on the gelatinase activity of the produced APM. The maximal gelatinase activities (at pHs 4, 7 and 9) were attained after 120 h incubation irrespective of the medium. Generally, the APM obtained from the glucose medium had higher gelatinase activities than the kerosene media. This result is, to some extent, in agreement with that obtained by SUZUKI et al. [8], who studied the effect of carbon sources on the production of alkaline protease by the mutant No.

Table 8

Gelatinase activity of APM obtained from the filtrates of different cultures of F. oxysporum IFO 6384*

Incubation period (h)	Gelatinase activity determined at pH 7			Gelatinase activity determined at pH 4			Gelatinase activity determined at pH 9		
	Medium (I)	Medium (II)	Medium (III)	Medium (I)	Medium (II)	Medium (III)	Medium (I)	Medium (II)	Medium (III)
48	21.5	15.0	12.2	12.2	26.1	22.5	29.9	3.7	5.1
72	30.9	20.6	22.1	23.3	38.3	34.2	36.5	15.1	19.5
96	41.2	31.5	31.1	36.7	45.7	42.8	47.6	28.0	26.9
120	53.2	42.0	38.2	49.0	48.1	43.1	55.9	41.2	39.9
144	46.7	37.4	31.4	35.1	29.9	35.9	49.8	36.0	35.5

* See Table 1

5—128B derived from *Fusarium* sp. S-19—5. Furthermore, in contrary to the results of SUZUKI et al. [8], the addition of Tween 60 to the kerosene medium showed an inhibitory effect on the protease production. It is worthy to note that the APM obtained from the kerosene media showed its maximum and minimum activities at pH values 4 and 9, respectively. On the other hand, the maximum and minimum gelatinase activities of APM obtained from the glucose medium were found at pH's 9 and 4, respectively.

It may be concluded that *F. oxysporum* IFO 6384 can utilize different carbon sources and produce more than one types of extracellular proteases. However, it seems likely that the glucose medium stimulated the production of alkaline protease, whereas kerosene media enhanced the synthesis of the acidic type of this proteolytic enzyme.

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COMPARISON OF VEGETATIVE ANATOMY OF PIPERALES

I. JUVENILE XYLEM OF TWIGS

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Abstract

Medullary bundles of *Piperaceae* resemble those of *Ranunculaceae*. The nature of tracheary elements of primary xylem suggests that *Houttuynia cordata (Saururaceae)*, *Piper cubeba (Piperaceae)* and *Chloranthus officinalis (Chloranthaceae)* are of lower evolutionary status than others. Among these three, *P. cubeba* shows stratification of secondary xylem, a specialized character. Lateral wall of metaxylem tracheary elements and distribution of bundles of *Peperomia*, suggest their primitive status and distinctness, supporting separation of "Peperomiaceae" (of NOVAK). *Piper cubeba*, *Houttuynia* and *Chloranthus* bear one important Ranunculaceous character: scalariform perforation in primary vessels. Primitive species of *Peperomia* carry probably another Ranunculaceous character, i.e., many circles of medullary bundles. Shape and pattern of vascular bundles of *Piper cubeba*, *Houttuynia* and *Chloranthus* are similar. Other species of *Piper* show modifications. *Peperomia* represents another distinct pattern.

Introduction

Of the three families of the orders, *Piperaceae*, *Saururaceae* and *Chloranthaceae*, the first two are placed in the same family and the third as the related next family by BENTHAM and HOOKER [4]. They are included as separate families in the same order by ENGLER and DIELS [20], GUNDERSEN [22], LAWRENCE [33], RENDLE [42], CORE [15], CAMPBELL [9] and HUTCHINSON [27]. ROUSSEAU [44] affirms that these three families should be amalgamated. Thus, authors are unanimous about the close relation of the three families. Regarding relations with other orders there are controversies, (1) some placing the order in *Amentiferae* after *Casuarinales (Verticillatae)* and followed by woody orders like *Salicales* or *Hydrostachyales* [9, 15, 20, 33], (2) others placing in *Amentiferae* but near herbaceous families like *Polygonaceae* [14, 42] or (3) between *Aristolochiales* and *Myristicales* [4] and (4) still others regarding the order as an offshoot of the Ranalian ancestry [5, 22, 23, 27].

The sequences of advancement of the three families are: (1) *Piperaceae*—*Saururaceae*—*Chloranthaceae* [9, 15, 27, 35, 42], (2) *Saururaceae*—*Piperaceae*—*Chloranthaceae* [4, 19, 33], (3) *Lactoridaceae*—*Saururaceae*—*Piperaceae*—*Chloranthaceae* [22]. Of the two genera of *Piperaceae (Piper* and *Peperomia)*,

the former may be more primitive than the latter [4, 19, 27]. According to ROUSSEAU [44], *Piper* is the basic group, from which *Chloranthus* and *Saururus*, (commonly placed in distinct families) are derived by minor modification, while *Peperomia* has evolved by major changes. METCALFE and CHALK [35], on the contrary, have shown that the wood of *Piperaceae* is more specialized than that of *Chloranthaceae* and the vessels of the genus *Piper* are "outstanding" in this group.

A number of species were examined discretely by anatomists [1, 7, 8, 13, 16, 17, 18, 24, 25, 26, 30, 34, 37, 38, 39, 40, 41, 44, 45, 46, 47, 48, 51, 54, 56, 57, 59]. The conclusive taxonomic suggestion should wait critical comparative study of more species. Structures of secondary xylem are often utilized for tracing phylogeny [2, 3, 10, 11, 12, 21, 32, 35, 43, 49, 52, 53, 55, 58]. Possibility of utilizing primary xylem has been particularly emphasized in the exhaustive work of BIERHORST and ZAMORA [6], which is an essential field for tracing phylogeny in herbaceous plant groups, like *Piperales*.

Material and method

Stem with leaf and cuttings representing *Piper*, *Peperomia*, *Houttuynia* and *Chloranthus* were studied (Table 1). The collected materials were compared with the specimens of the herbaria of Calcutta University and Indian Botanic Garden, Sibpur, Calcutta.

For studying the xylem, pieces of twig apices just near the seventh plastochron were macerated by modified Jeffrey's method [6] and hand sections (stained with safranin and fast green [31]), were used. Free hand or paraffin-embedded microtomic sections (16—18 μm) were stained with safranin and fast green [31]. Mean values of measurements mentioned in the text were based on 50 readings from 10 sections in each case. Measurements of every structure in the text are represented in three numerical values, the first is minimum, the middle mean (bold) and the last maximum.

Results

Saururaceae

[Lindl. Nat. Syst. ed. 2. (1836) 184]

Genus — *Houttuynia*
(Thunb., Fl. japon. (1784) 12].

Topography: 22—24 vascular bundles are arranged in a ring each showing 6—8 parenchymatous cells, 6—12 tracheary elements in T. S. Xylem in each bundle includes a U-shaped group of tracheary elements, either solitary or in cluster, external sclerenchyma and internal parenchyma (Fig. 1).

Shape and measurements of tracheary elements: Proportion of tracheids and vessels is 50 : 50 in protoxylem and 40 : 60 in metaxylem. Length of tracheids and vessel members in protoxylem are 408.3—**450**—541.6 μm and 300—**416.6**—500 μm respectively, in metaxylem 550—**664.1**—758.3 μm and

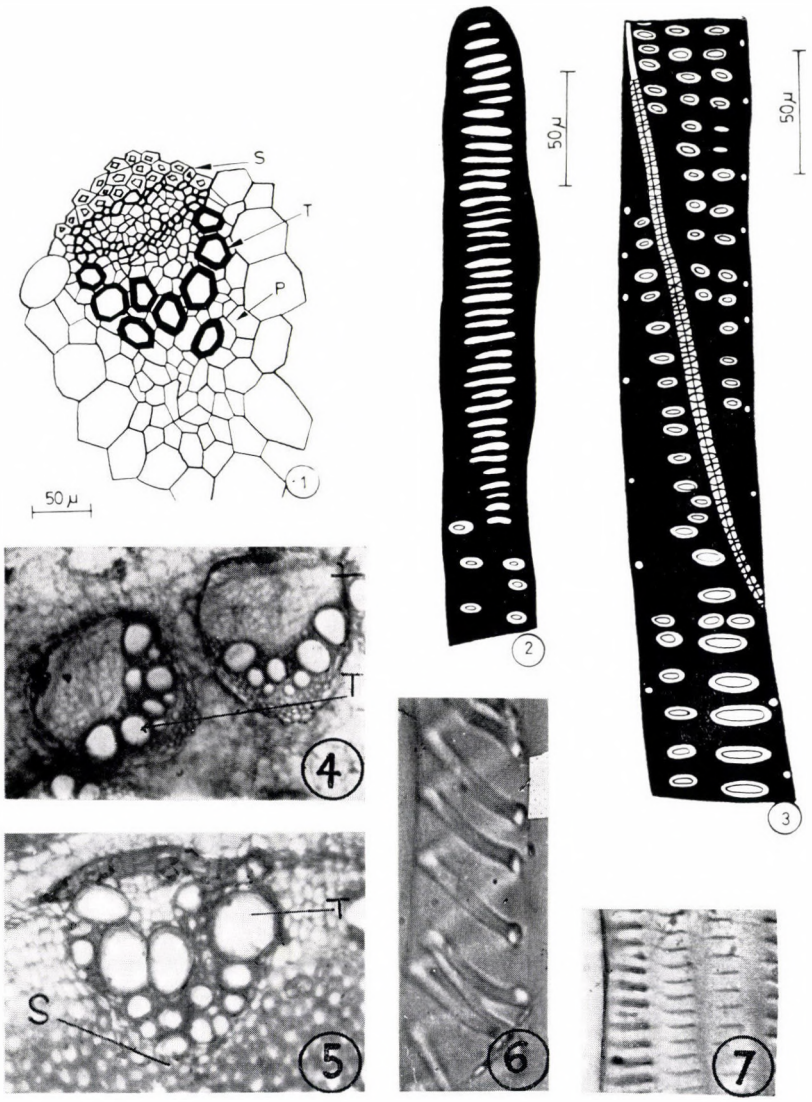
Table 1

*Specimens used in the investigation**

Name	Collector, source	Material
Family — Saururaceae		
Houttuynia cordata THUNB.	Nair 35 775, DD	Dry twig, Stem
Family — Piperaceae		
Genus — *Piper*		
Piper betle L. (Cultivar. *jhal pan*)	Sen 1 (Pi), CU Imperial Nursery	Cutting
P. betle L. (Cultivar. *mitha pan*)	Sen 10 (Pi), CU	Cutting
P. betle L. (Cultivar. *chhachi pan*)	Sen 11 (Pi), CU	Cutting
P. brachystachyum WALL.	IBS 9499	Dry twig
P. cubeba LINN. f.	Sen 2 (Pi), CU	Cutting
P. longum L.	Datta 3 (Pi), CU	Cutting
P. nepalense Miq.	IBS 89	Dry twig
P. nigrum L.	Sen 5 (Pi), CU	Cutting
P. nigrum L.	Datta 6 (Pi), CU	Cutting
P. pedicellosum WALL.	IBS 93	Dry twig
Genus — *Peperomia*		
Peperomia argyreia E. MORR.	Sen 12 (Pep), CU	Cutting
Pep. metallica LINDL. RODIG	Sen 8 (Pep), CU	Cutting
Pep. obtusifolia A. DIERTR.	Sen 7 (Pep), CU	Cutting
Pep. pellucida H.B.K.	Sen 9 (Pep), CU	Cutting
Family — Chloranthaceae		
Genus — *Chloranthus*		
Chloranthus officinalis BL.	IBS 184	Dry twig

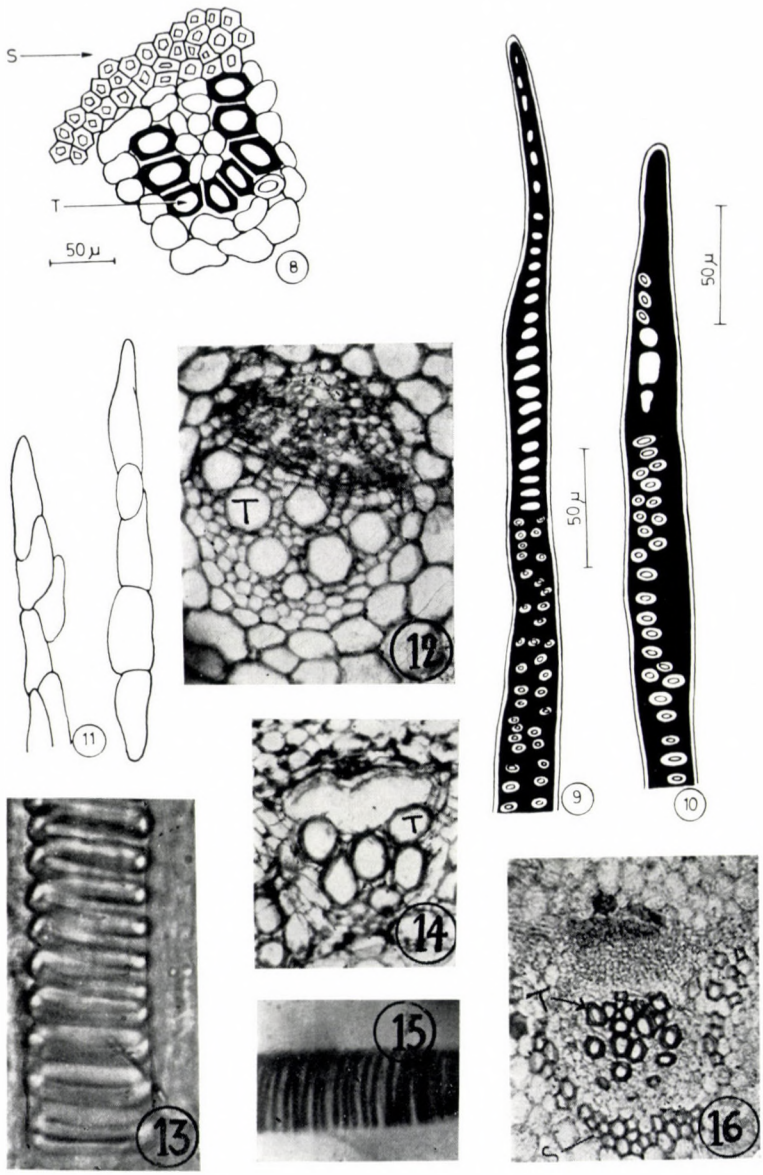
* Specimens cited according to procedure recommended by STERN and CHAMBERS [50]
DD = Botanical Survey of India, Dehradun, India
CU = Botany Department, Calcutta University, Calcutta, India
IBS = Botanical Survey of India, Indian Botanic Garden, Sibpur, Calcutta

500—**666.6**—708.3 μm, respectively. Diameters of tracheids and vessels are respectively, 44—**53.8**—60 μm and 56—**64.4**—72 μm in protoxylem, 68—**80**—88 μm and 60.2—**68**—74.1 μm in metaxylem. Length of the slopes of tracheid ends in protoxylem and metaxylem are 100—**140**—160 μm and 200—**220**—250 μm, respectively. Angles of end walls of vessel members of protoxylem and metaxylem are 110—**120**—130° and 120—**125**—130°, respectively. Angles of the slopes of tracheids of protoxylem and metaxylem are 110—**120**—130 and 115—**120**—125, respectively. Position of perforation plate is generally terminal on the inclined walls.



Figs 1—3. Houttuynia cordata; 1 = T.S. of a single bundle showing a more or less semilunar group of 12 tracheary elements and internal parenchyma; *2* = perforation plate of a protoxylem vessel member; *3* = thickening and bordered pits of metaxylem vessel members
Fig. 4. Piper betle; T. S. through two bundles showing 7—9 tracheary elements arranged in a semilunar pattern
Figs 5—7. P. brachystachyum; 5 = T.S. through a bundle showing 15—16 tracheray elements in a semilunar group and internal sclerenchyma; *6* = thickening of protoxylem tracheary elements; *7* = thickening on lateral wall of metaxylem tracheary elements

Perforation of lateral wall: End of vessel members are uniperforate. Percentage of vessel members having perforation at both ends and one end, are, respectively, 70 and 30 in protoxylem and 75.6 and 24.4 in metaxylem.



Figs 8—11. P. cubeba; 8 = T.S. through a bundle showing U-shaped tracheary group of eight, and external sclerenchyma; *9* = perforation plate of the metaxylem vessel member; *10* = perforation and long tale of vessel members of secondary xylem; *11* = ray in tangential section
Figs 12—13. Piper longum; 12 = T.S. through a bundle showing a semilunar patch of 8 tracheary elements; *13* = thickening of metaxylem tracheary element
Figs 14—15. P. nepalense; 14 = T.S. through a bundle showing about five tracheary elements arranged in a semilunar bundle; *15* = thickening on the lateral wall of a protoxylem tracheary element
P = parenchyma, T = tracheary elements, S = sclerenchyma
Fig. 16. T.S. through a bundle of *P. nigrum* showing about 18 tracheary elements arranged in a semilunar pattern

In addition to simple perforation, scalariform type occurs with about forty bars (Fig. 2). Simple pits and spiral annular transitional forms of thickening occur in protoxylem vessel members. Distance between the pits is 3—5 μ in metaxylem vessel members and that between the gyres in protoxylem are 3—5 μm and 2—4 μm, respectively. Pits are as broad as faces or intraface-opposite, trans-edge opposite and alternate. Metaxylem vessel members have scalariform bordered pits. Fibres are absent (Fig. 3).

Piperaceae
[L. C. Rich. in: Humboldt (1815) 46E]

Genus — *Piper*
[L. Gen. ed. 1. (1737) L. Spec. pl. ed. 1 (1753) 28. — Endl. G. n. 1820.]

Topography: Number of peripheral bundles — 7—8 in *P. betle* and *P. cubeba;* 24—26 in *P. brachystachyum;* 15—18 in *P. longum;* 20—24 in *P. nepalense, P. nigrum* and *P. pedicellosum;* medullary bundles — 5—7 in *P. betle, P. cubeba, P. longum, P. nigrum;* 9—12 in *P. brachystachyum;* 12—14 in *P. nepalense;* 8—10 in *P. pedicellosum.* Each xylem has mostly 7—10 tracheary elements, 3—9 in *P. pedicellosum,* and *P. nigrum* enclosing a group of parenchymatous cells, mostly 8—12 (8—18 in *P. longum, P. nigrum, P. brachystachyum* and *P. pedicellosum*) forming a semilunar patch (U-shaped in *P. cubeba*). Sclerenchyma is mainly internal in *P. betle, P. brachystachyum* (also external), *P. longum, P. nepalense* and *P. pedicellosum,* external in *P. cubeba* and *P.* nigrum (Figs 4, 5, 8, 12, 14, 16, 17).

Table 2

Proportion of tracheids and vessels in Piper

	Protoxylem	Metaxylem
P. betle	33:67	25:75
P. brachystachyum	0:100	0:100
P. cubeba	85:15	18:82
P. longum	4:96	0:100
P. nepalense	0:100	0:100
P. nigrum	0:100	0:100
P. pedicellosum	0:100	0:100

Shape and measurement of tracheary elements (Figs 6, 7, 9, 10, 13, 15, 18). Proportion of tracheids and vessels are stated in Table 2.
Lengths of tracheids (T) and vessel members (V) are given in Table 3.

Table 3

Lengths of tracheids and vessel members (μm) in Piper

	Protoxylem	Metaxylem
P. betle	T 320—**416**-483	T 166—**208.3**—258
	V 160—**175**—189	V 160—**182.2**—195
P. brachystachyum	V 400—**554.1**—785	V 250—**314.1**—358
P. cubeba	T 550—**652.1**—790	T 270—**500.2**—782
	V 550—**639.1**—785	V 280—**435**—490
P. longum	T 250—**300**—590	
	V 250—**300**—350	V 180—**215.8**—260
P. nepalense	V 150—**250.8**—315	V 150—**190.2**—215
P. nigrum	V 475—**617.5**—750	V 200—**310.8**—425
P. pedicellosum	V 285—**316.6**—350	V 140—**189.2**—230

Diameters of tracheary elements in proto- and metaxylem are given in Table 4.

Mean length of the slopes of tracheids varied from 26—50 μm; mean angle of the slopes are 30—50°. Mean angle of vessel perforation plates are 130—145° in *P. cubeba*, 100—120° in the others.

Distance between the gyres of tracheary thickening of protoxylem are 2—5 μm in *P. nepalense*, 4—8 μm in others; of metaxylem are 1.5—3 μm in *P. longum* and *P. nepalense*, 2—5 μm in others.

Perforation plates are simple an are generally at a terminal position on slopes. Vessel member ends are mostly uniperforate, both uniperforate and multiperforate in *P. cubeba*. Percentages of vessel members having perforation at one end only are 20 in *P. longum*, 32 in *P. nepalense*, 18 in *P. cubeba*, 17 in *P. brachystachyum*, 45 in *P. betle*, 3 in *P. nigrum* and 30 in *P. pedicellosum*.

Fibres are short, mostly 500—700 μm, 15—27 μm broad, absent in *P. betle*. Pits simple.

Secondary xylem of P. cubeba: Ring is present; pores very numerous [12], 46 pores per mm^2. Pores usually solitary, some in chains and clusters, pores are angular in shape (pentagonal to hexagonal); radial dimension of pores very small to moderately small [29]. Vessel members are medium-sized [28] and moderately inclined; vessel members with simple perforation and bordered pits. Intervascular pits are bordered and vessel-parenchyma pits are simple. Length, diameter and angle of the vessel members are 400—**458.3**—512 μm, 36—**49.8**—70 μm, 120°—**130**°—140°, respectively. Shape of the perforation plate is elliptical. Perforation is terminal, and at the base of the slope. Tail is present.

Table 4

Diameters of tracheary elements (μm) in Piper

	Protoxylem	Metaxylem
P. betle	T 44—**48**—50	T 44—**50**—58
	V 44—**53.8**—60	V 60—**70**—80
P. brachystachyum	V 42—**51.6**—60	V 68—**76.2**—90
P. cubeba	T 30—**32**—34	T 36—**40**—44
	V 30—**34.4**—48	V 36—**49.8**—70
P. longum	T 30—**32**—34	V 36—**42**—44
	V 36—**42**—44	
P. nepalense	V 20—**40**—60	V 30—**35**—40
P. nigrum	V 30—**33.1**—36	V 38—**45**—50
P. pedicellosum	V 68—**74.8**—78	V 74.2—**76.3**—78

Axial parenchyma is terminal to initial marginal banded (wide). Symbol formula Mt—Mi b.

The radial section shows ray cells with simple pitting. Crystals are present in the ray cells. The ray is of heterogeneous type I (Fig. 11) with a few procumbent cells. The height of ray is 126—**183**—240 μm, breadth being 16—**14**—18 μm. Ray abundance is very numerous [12], 10—12 rays per mm. Fibres are short to medium-sized [28]. Length and breadth of the fibres 475—**571.6**—916.6 μm and 16.6—**26.6**—33.3 μm, respectively. Fibres have simple pits.

Genus — *Peperomia*

[Ruiz. et Pav., Fl. peruv. et chil. prodr. (1794) 8]

Topography: Number of peripheral bundles 8—10 in *Pe. argyreia*, 9—12 in *Pe. metallica*, 10—14 in *Pe. obtusifolia*, 4—5 in *Pe. pellucida;* medullary bundles 5—6 in *Pe. argyreia*, 15—18 in *Pe. metallica*, 18—20 in *Pe. obtusifolia*, 1—2 in *Pe. pellucida*. Each xylem strand contains a variable number of tracheary elements, 5—8 in *Pe. argyreia*, 20—26 in *Pe. metallica*, 8—12 in *Pe. obtusifolia*, 6—8 in *Pe. pellucida;* surrounding a group of parenchyma 6 - 8 in *Pe. argyreia*, 25—30 in *Pe. metallica*, 11—25 in *Pe. obtusifolia*, 38—40 in *Pe. pellucida*. Tracheary elements are arranged commonly in a semilunar pattern, semicircular or in a straight tangential row in *Pe. pellucida*. Sclerenchyma patch is absent (Figs 19, 22, 25, 27).

Shape and measurement of tracheary elements (Figs 20, 21, 23, 24, 26, 28, 29, 30).

Tracheids absent commonly, present in protoxylem of *Pe. pellucida* 4.4%. Length of tracheids (T) and vessel members (V) are in Table 5.

Diameters of tracheary elements in protoxylem and metaxylem are in Table 6.

Length of the sloped part of tracheids is 38—**41.6**—49 μm in *Pe. pellucida*, mean angle of the slope being 30°. Mean angles of perforation plate commonly vary from 95°—100° in protoxylem and 90°—95° in metaxylem; 120° in proto- and metaxylem of *Pe. argyreia*.

Table 5

Length of tracheids and vessel members (μm) in Peperomia

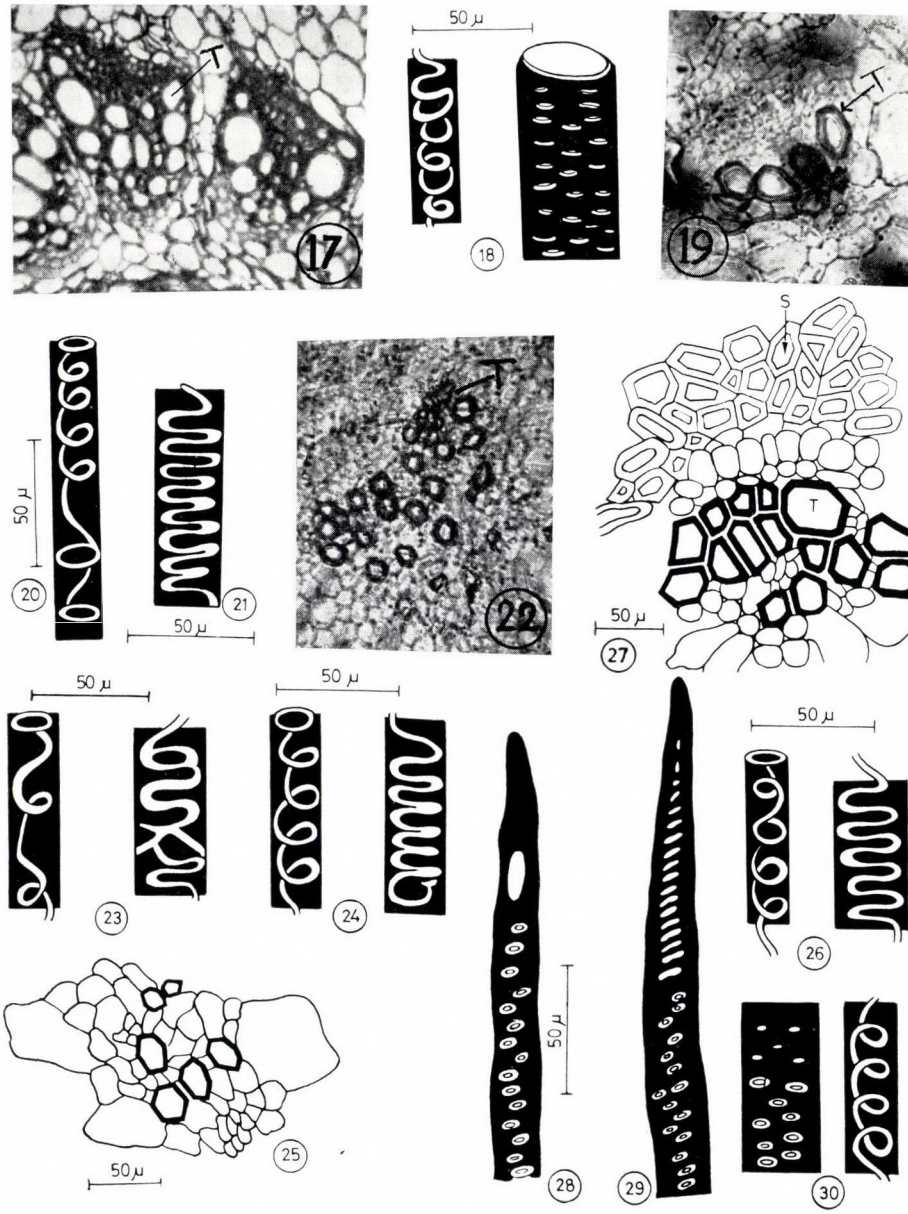
	Protoxylem	Metaxylem
Pe. argyreia	V 358—**416.6**—480	V 166—**208.3**—250
Pe. metallica	V 333—**413.3**—458	V 188—**234.1**—258
Pe. obtusifolia	V 268—**411.2**—450	V 188—**235.2**—260
Pe. pellucida	T 390—**454.2**—508	
	V 375—**434.1**—508	V 166—**291.6**—366

Table 6

Diameter of tracheary elements (μm) of Peperomia

	Protoxylem	Metaxylem
Pe. argyreia	V 26—**35**—38	V 30—**40.5**—52
Pe. metallica	V 20—**28**—32	V 36—**40**—44
Pe. obtusifolia	V 20—**28.3**—34	V 36—**42**—48
Pe. pellucida	T 26—**34.2**—39	
	V 26—**35**—38	V 40—**45.6**—58

Figs 17—18. P. pedicellosum; 17 = T.S. through a bundle showing 12—16 tracheary elements in semilunar patterns; *18* = lateral thickening of vessels in proto- and metaxylem
Figs 19—21. Peperomia argyreia; 19 = T.S. through a bundle showing about 10 tracheary elements; *20—21* = lateral wall thickening on proto- and metaxylem tracheary elements
Figs 22—23. Pe. metallica; 22 = T.S. through a bundle showing about 26 tracheary elements in a semilunar patch; *23* = lateral wall thickening of proto- and metaxylem of tracheary elements
Fig. 24. Lateral wall thickening of proto- and metaxylem tracheary elements of *Pe. obtusifolia*
Figs 25—26. Pe. pellucida; 25 = T.S. through a bundle showing about 6 tracheary elements; *26* = lateral wall thickening of tracheary elements of proto- and metaxylem
Figs 27—30. Chloranthus officinalis; 27 = T.S. through a bundle showing oval tracheary patch and a sclerenchyma patch external to phloem; *28* = simple perforation at the end of vessel member; *29* = scalariform perforation at the end of a vessel member; *30* = lateral wall thickening of proto- and metaxylem tracheary elements



Distance between the gyres of tracheary thickening in protoxylem is commonly 4—8 μm, 3—5 μm in *Pe. metallica;* in metaxylem commonly 3—5 μm, 2—3.5 μm in *Pe. metallica.*

Perforation is commonly terminal on the slopes of tracheary elements, occasionally proximal in *Pe. pellucida;* member ends are commonly uniper-

forate; percentages of vessel members having perforation at one end only are 5 in *Pe. argyreia* (proto & meta), 17.9 in *Pe. pellucida* (protoxylem), absent in others. Thickening is partly annular and partly spiral. Fibre is absent.

Chloranthaceae
[Blume, Enum, pl. Javae I. (183) 78]

Genus — *Chloranthus*
[Swartz. in: Phil. Trans. LXXVI (1787) 359]

Topography: Number of bundles 4—5; pattern of distribution of tracheary elements in T. S. semilunar to oval in shape (Fig. 27). A sclerenchyma patch occurs external to phloem.

Shape and measurement of tracheary elements: Proportion of tracheids and vessels are 83 : 17 in protoxylem and 30 : 70 in metaxylem. Lengths of the tracheids and vessel members are respectively 300.1—**490**—520.1 μm, 266.6—**450**—466.6 μm in protoxilem and 300.1—**470**—500 μm, 166.6—**225**—241.6 μm in metaxylem. Diameters of tracheids and vessels 30—**32**—34 μm and 30—**40**—50 μm in protoxylem and 36—**48**—52 μm and 54—**66.6**—70 μm in metaxylem. Lengths of the slopes of tracheid ends are 33.3—**41.6**—50 μm in protoxylem and 32—**34**—36 μm in metaxylem. Angles of the end walls of vessels in protoxylem and metaxylem are 110°—**120**°—130° and 112°—**116**°—125°, respectively. Angles of the slope of the tracheids of protoxylem and metaxylem are 20°—**30**°—40° and 20°—**30**°—40°, respectively.

Perforation and lateral wall. Position of the perforation plate is generally terminal on the slope of the inclined end. Vessel member ends are often uniperforate. Percentages of vessel members having perforation at both ends and at one end, are 62.9 and 37.1 in protoxylem and 36 and 64 in metaxylem. Simple and scalariform type of perforation at the end of the cylindrical vessel members or on slopes (Figs 28—29). Number of bars is about 30—35 in a scalariform perforation plate (Fig. 29). Spiral thickening occurs in protoxylem tracheary elements. Metaxylem tracheary elements have scalariform simple or bordered pits on faces or trans-edge opposite (Fig. 30). Distances between gyres on lateral walls of tracheary elements of protoxylem and metaxylem are 3—5 μm and 2—3.5 μm, respectively.

Secondary xylem. Ring is absent; pore abundance is "very numerous", 53 per mm^2; pores are in chains, angular in shape (pentagonal to hexagonal); diameter of pores is very small to moderately large, with scalariform bordered pits. Vessel members are medium-sized. Length, diameter and angle of the vessel members are 439.2—**583.3**—600 μm, 30—**38**—64 μm, 50°—**60**°—70°, respectively. Perforation terminalor at the base of the slope. Axial parenchyma is diffuse. The rays are 15—18-seriate.

The radial sections show ray cells with simple pitting. The ray is of heterogeneous type III, "extremely low" and "extremely fine to very fine". The height and breadth of rays are 158 —**169.9** —200 μm and 10 —**14**—16 μm. Fibres are "extremely short" and show bordered pits. Length and breadth of the fibre are 185 —**214.4** —300 μm and 16.6 —**25** — 29.1 μm, respectively.

Discussion

On the basis of the fundamental ideas of advancement of characters, the following classes for each character have been assumed:

For angle of perforation plates (Class 1) 90°—110°, (Class 2) 110°—130°, (Class 3) 130°—150°, (Class 4) 150°—170°.

For perforation of protoxylem and metaxylem vessel members (Class 1) simple, (Class 2) transitional or multiperforate, (Class 3) scalariform about 20 bars, (Class 4) scalariform, about 25—40 bars.

For length of protoxylem vessel members (mean values) (Class 1) 300—400 μm, (Class 2) 400—500 μm, (Class 3) 500—600 μm, (Class 4) 600—700 μm.

For length of metaxylem vessel members (mean values) (Class 1) 100—200 μm, (Class 2) 200—300 μm, (Class 3) 300—400 μm, (Class 4) 400—500 μm, (Class 5) 500—600 μm, (Class 6) 600—700 μm.

For diameter of vessel members (Class 1) 70—80 μm, (Class 2) 60—70 μm, (Class 3) 50—60 μm, (Class 4) 40—50 μm, (Class 5) 30—40 μm. Fig. 31 represents these class values and their total values for each species.

Vasculature of related families

Piperales are related to different families by different taxonomists, e.g. *Casuarinaceae, Salicaceae, Hydrocharitaceae, Polygonaceae, Aristolochiaceae, Myristicaceae, Ranunculaceae,* etc. Occurrence of cauline and cortical vascular bundles is the only character suggesting similarity of *Piperales* with *Casuarinaceae* and *Hydrostachyaceae.* Primary xylem of *Salicaceae* and *Myristicaceae* [35], suggest no similarity with *Piperales.*

Simple perforation in primary vessel members of *Polygonaceae* is of a more advanced character than those of the primitive species of *Piperales,* e.g. *Houttuynia cordata, Piper cubeba* and *Chloranthus officinalis.* It is impossible to relate *Piperales* with *Casuarinaceae* or *Hydrostachyaceae* unless detailed information on the primary xylem of those families are obtained. According to HUTCHINSON [27], the vascular bundles in the stem of *Piperaceae* are sometimes scattered like monocotyledons instead of being arranged in concentric rings. This, according to HUTCHINSON, is an indication of the Ranalian origin.

Ranunculaceae are most closely related to monocotyledons, like *Alismataceae*, and has both simple and scalariform perforations *(Paeonia)*. Concave nature of xylem strands, several circles of vascular bundles, often showing scattered irregular arrangement (as in *Actaea*, *Cimicifuga*, *Thalictrum* of *Ranunculaceae*), suggest similarity with *Peperomia obtusifolia* and *Peperomia metallica*. Medullary bundles, common in *Piperales*, occur in many species of *Ranunculaceae* also [*vide* 35]. All these characters suggest similarity of *Piperales* with *Ranunculaceae* and supports the concept of HUTCHINSON [27].

Tracheary elements of the families and genera (compared)

Two major sequences of advancement of the families of this order are suggested by taxonomists:

(1) *Piperaceae* — *Saururaceae* — *Chloranthaceae*,
(2) *Saururaceae* — *Piperaceae* — *Chloranthaceae*.

In addition, METCALFE and CHALK suggest *Chloranthaceae* and *Saururaceae* as more primitive than *Piperaceae*. Angle of end wall perforation and length of vessel member slopes (Fig. 31) suggest lower evolutionary status of *Houttuynia cordata (Saururaceae)*, *Piper cubeba* and *Chloranthus officinalis*, than other species of *Piperales*. The first column drawn by adding the class values of different characters also clearly suggests that these three species are more primitive than the others studied. The thickening of the lateral walls of metaxylem tracheary elements of *Houttuynia cordata*, *Piper cubeba* and *Chloranthus officinalis* are very similar, having scalariform bordered pits, in addition to spiral ones and generally trans-edge opposite pits, located on the faces. *Chloranthus officinalis* possesses scalariform simple pits, also. Some of the primary vessels of *Houttuynia cordata* represent trans-edge alternate bordered pits. Thus, on the basis of the primary xylem character, it is difficult to state which of these three species is more advanced and which is primitive. The characters of secondary xylem are different in *Chloranthus officinalis* and *Piper cubeba*. Vessels are nonstratified in the former and stratified in the latter. This may suggest a less specialized status of *Chloranthus officinalis*. The progress of evolution of different parts may not be concomitant always. Thus, in comparison to *Piper cubeba*, the total class value of *Chloranthus officinalis*, suggests more evolved primary xylem and less specialized secondary xylem. But, it is quite logical to suggest that *Piper cubeba*, *Chloranthus officinalis* and *Houttuynia cordata* are basic in pattern of primary xylem.

In comparison to the species of *Piper*, the class values of the primary xylem of *Peperomia* appears primitive (Fig. 31). Primitiveness of *Peperomia* is also suggested by the occurrence of reticulate and spiral thickening and sometimes annular thickening on lateral walls of metaxylem tracheary elements (mostly scalariform in *Piper*) and several circles of vascular bundles,

often scattered (similar to *Ranunculaceae*). Vascular pattern of *Peperomia* diverging widely from that of *Piper*, supports separation of *Peperomia* by NOVAK [36] as *Peperomiaceae*. Thus specialization of primary xylem in *Piperales* show two major lines, one showing persistence of scalariform perforation of the Ranunculaceous type (primitive *Piper* species, *Chloranthus officinalis* and *Houttuynia cordata*) and the other line showing absence of scalariform perforation but persistence of several circles of concave vascular bundles of the Ranunculaceous pattern.

The graph (Fig. 31) shows that the species arranged according to the total class values of primary xylem are roughly correlated with the class values of vessel length, angles of end walls, perforation of metaxylem vessels and diameter of tracheary elements.

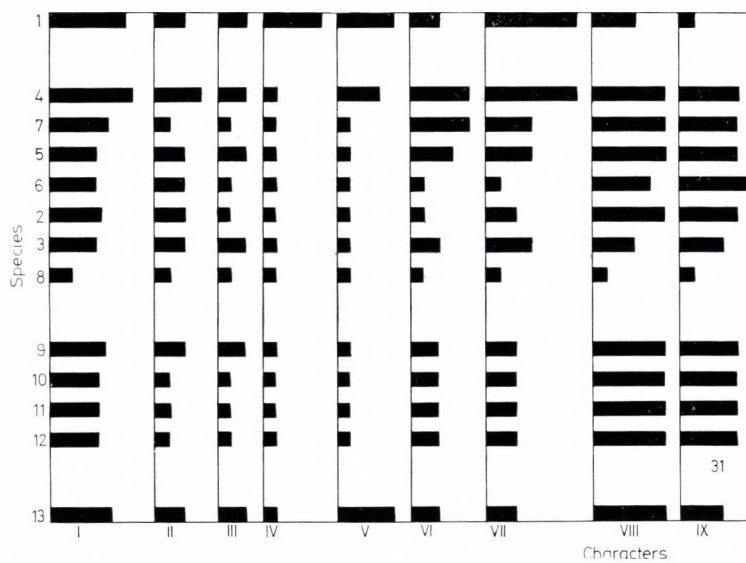


Fig. 31. Class values of I, plate angle (protoxylem); II, plate angle (metaxylem); III, perforation nature (protoxylem); IV, perforation nature (metaxylem); V, length of protoxylem vessel members; VI, length of metaxylem vessel members; VII, diameter of protoxylem vessel; VIII, diameter of metaxylem vessel; IX, total of all class values. Species are (1) *Houttuynia cordata*, (2) *Piper betle*, (3) *P. brachystachyum*, (4) *P. cubeba*, (5) *P. longum*, (6) *P. nepalense*, (7) *P. nigrum*, (8) *P. pedicellosum*, (9) *Peperomia argyreia*, (10) *P. metallica*, (11) *P. obtusifolia*, (12) *P. pellucida*, (13) *Chloranthus officinalis*

Vascular bundles of genera (compared)

The pattern of vascular bundles as revealed in transverse sections are distinctly uniform in *Piper cubeba*, *Houttuynia cordata* and *Chloranthus officinalis*. The tracheary elements form U-shaped, semilunar or kidney shaped structures. The protoxylem is situated at the inner periphery of the middle part of the arches. In these species, sclerenchymatous patches occur external

to the phloem. *Piper nigrum* represents slight modifications of this type, by the occurrence of both internal and external (mainly external) sclerenchymatous patches. Further modification is noticed in other species of *Piper*, where the sclerenchymatous patches are mainly or solely internal. *Peperomia* species are distinct for the absence of such sclerenchyma patches accompanying the bundles. Thus the patterns of individual vascular bundles, also correspond to the trends of evolution, drawn from the structure of primary tracheary elements.

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COMPARISON OF VEGETATIVE ANATOMY OF PIPERALES

II. LEAF

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Abstract

Many characters of leaf (hair, hypodermal cells, palisade layers, intercellular space, distinction between spongy and palisade parenchyma, "palisade ratio", distribution of collenchyma and sclerenchyma, presence or absence of starch grains, calcium oxalate crystals, number, shape and arrangement of bundles of petiole) are useful distinguishing characters. Reduction of palisade layers seems to be the trend of evolution in *Piper* and *Peperomia*.

Introduction

Many authors of the last decades of the 19th century, the first quarter of the 20th century and of recent years stress on the taxonomic importance of leaf anatomy [3, 4, 5, 9, 10, 11, 13, 17, 19]. Discrete records on some leaf tissue of a few species of *Piper* and *Peperomia* are in literature [1, 7, 12, 15, 16, 18, 20]. A comprehensive comparative study is the main aim of the present paper.

Material and method

Collected specimens are enlisted in Table 1.

For studying the leaf structure, about five leaves, usually 6th to 10th leaves from the tip, were cleared by boiling in water, 95% ethanol and saturated aqueous chloral hydrate solution [2]. Sections (16—18 μm) obtained from living specimens were stained with safranin and fast green [6] and those from dry oxidized materials were examined unstained. Quantitative data were calculated from the five sections and 50 readings. All measurements in the text have been indicated in the sequence of minimum, mean and maximum.

Results

Genus—*Piper*

Leaf dorsoventral. *Hairs* sparsely distributed on the lower surface, unicellular in *P. betle* var. *chhachi pan*, both unicellular and multicellular in *P. cubeba*, multicellular in *P. longum*, *P. betle* var. *jhal pan*, absent in other

Table 1

Collected specimens

Name	Collector, source	Material
Family Piperaceae		
Genus — *Piper*		
Piper betle L. (Cultivar. *jhal pan*)	Sen 1 (Pi), CU Imperial Nursery	Cutting
P. betle L. (Cultivar. *mitha pan*)	Sen 10 (Pi), CU	Cutting
P. betle L. (Cultivar. *chhachi pan*)	Sen 11 (Pi), CU	Cutting
P. cubeba LINN. f.	Sen 2 (Pi), CU	Cutting
P. longum L.	Datta 3 (Pi), CU	Cutting
P. nigrum L.	Sen 5 (Pi), CU	Cutting
P. nigrum L.	Datta 6 (Pi), CU	Cutting
P. nigrescence C. DC.	IBS 190	Dry twig
P. pedicellosum WALL	IBS 93	Dry twig
Genus — *Peperomia*		
Peperomia argyreia E. MORR.	Sen 12 (Pep), CU	Cutting
Pep. metallica LINDL. RODIG	Sen 8 (Pep), CU	Cutting
Pep. obtusifolia A. DIERTR.	Sen 7 (Pep), CU	Cutting
Pep. pellucida H.B.K.	Sen 9 (Pep), CU	Cutting
Family Chloranthaceae		
Genus — *Chloranthus*		
Chloranthus officinalis BL.	IBS 184	Dry twig

CU = Botany Department, Calcutta University, Calcutta, India
IBS = Botanical Survey of India, Indian Botanic Garden, Sibpur, Calcutta
All species (except the last one) were grown in our University garden in the usual sunlight

species; crystalline pearl glands absent. *Epidermis* unilayered, papillose, particularly on the abaxial surface of major veins of *P. betle* var. *jhal pan*, var. *chhachi pan*, *P. pedicellosum* and *P. longum* and normal in other species; epidermal cells near the major veins smaller than the cortical and hypodermal cells in *P. cubeba*, *P. betle* var. *mitha pan* and larger than hypodermal cells in *P. longum*, *P. nigrum*, *P. nigrescence* and *P. betle* var. *jhal pan* and almost equal in *P. longum*, *P. pedicellosum* and *P. betle* var. *chhachi pan* (as recorded by CHIBBER [1]); cells elliptical in T. S. and having longer dimension vertical to the surface in *P. longum*, *P. betle* var. *jhal pan;* cells ovate in *P. betle* var. *mitha pan* and rectangular and with longer dimension parallel to surface in *P. cubeba*, *P. pedicellosum;* cells roundish to rectangular having longer axis parallel to surface in *P. nigrescence*, *P. betle* var. *chhachi pan*.

Stomata always confined to the lower surface; surrounded by a rosette of cells smaller than other epidermal ones in *P. nigrum*, *P. betle* var. *jhal pan;*

surrounding cells similar to other epidermal cell in *P. pedicellosum*, *P. betle* var. *mitha pan* and *P. cubeba;* intermediate condition in *P. longum*, *P. nigrescence* and *P. betle* var. *chhachi pan;* clear cruciferous type reported in *Piaeraceae* [8] absent; stomatal index for different species are: *P. betle* var. *jhal pan* — 6.2—**10.84**—12.5; *P. betle* var. *mitha pan* — 3.3—**5.58**—7.9; *P. betle* var. *chhachi pan* — 3.2—**5.51**—5.8; *P. cubeba* — 2.0—**3.22**—3.8; *P. longum* — 5.8—**7.51**—7.6; *P. nigrum* — 2.1—**3.44**—7.3; *P. nigrescence* — 5.8—**6.58**—7.9; *P. pedicellosum* — 4.1—**8.33**—12.5.

Hypodermis absent on the adaxial side in *P. cubeba;* adaxial hypodermis unilayered in *P. nigrescence*, and *P. longum*, 1—2-layered in *P. pedicellosum* and *P. nigrum*, and clearly 2—3-layered in all varieties of *P. betle;* abaxial hypodermis unilayered in *P. cubeba* and *P. longum*, 1—2-layered in *P. betle* (all vars.), *P. nigrescence* and *P. pedicellosum* and 2—3-layered in *P. nigrum*. Except *P. cubeba*, hypodermal cells of laminar region larger than the epidermal ones. Cells of adaxial hypodermis generally larger than those of the abaxial one, very distinctly in *P. nigrum*, but smaller in *P. longum*, *P. betle* var. *chhachi pan*. "*Cross bands*" (reported in *Piperaceae* by SKOTTSBERG [15, 16]) absent. *Hydathodes* absent.

Mesophyll including unilayered palisade, sometimes two-layered near the major veins of *P. cubeba;* palisade and spongy parenchyma indistinguishable in *P. nigrescence* and *P. pedicellosum;* shape of palisade cells more or less funnel-like in *P. betle* var. *jhal pan* and cylindrical with rounded ends in the rest of the species. Spongy parenchyma compact and almost without intercellular spaces in *P. cubeba*, *P. betle* vars. *chhachi pan* and *mitha pan*, with small intercellular spaces in *P. longum*, *P. nigrum*, *P. betle* var. *jhal pan;* more or less compact, 2—3-layered mesophyll, indistinguishable into palisade and spongy parenchyma in *P. nigrescence* and *P. pedicellosum*. Chloroplasts in mesophyll common in most of the species uncommon and irregular in *P. cubeba*. Palisade: epidermal cell ratios for different species are: *P. betle* var. *jhal pan* — 1—**3.1**—4; *P. betle* var *mitha pan* — 2—**3.2**—5; *P. betle* var. *chhachi pan* — 3—**4.6**—7; *P. cubeba* — 6—**7.57**—8.2; *P. longum* — 5.2—**6.15**—9.2; *P. magnificum* — 2.5—**3.18**—4; *P. nigrum* — 8—**9.89**—13; *P. nigrescence* — 4.2—**4.35**—6.5; *P. pedicellosum* — 1.5—**2.57**—4 (Figs 1—6).

Cortical cells of major veins generally hexagonal parenchymatous on the abaxial surface and circular to slightly angular on the adaxial surface in *P. betle* var. *chhachi pan*, *P. cubeba* and *P. pediqellosum* more or less roundish on both sides in other species; cells on the abaxial side larger. A multilayered chlorenchymatous tissue continuous to the palisade below the adaxial ridge against the vascular bundle of the major veins, found in *P. betle* vars. *jhal pan* and *mitha pan*, *P. longum*, *P. pedicellosum*, *P. nigrescence* and *P. nigrum*, absent in *P. cubeba* and *P. betle* var. *chhachi pan*. An adaxial sclerenchymatous cap in *P. betle* var. *chhachi pan* and *P. longum*, small patches of sclerenchyma

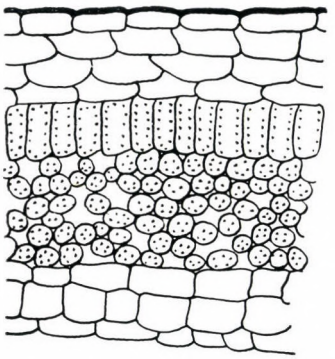

Fig. 1

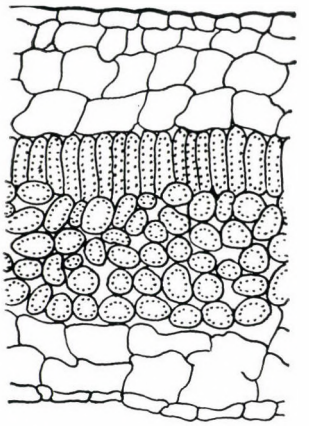

Fig. 2

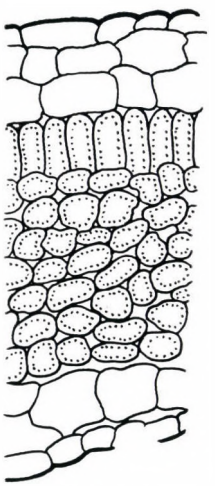

Fig. 3

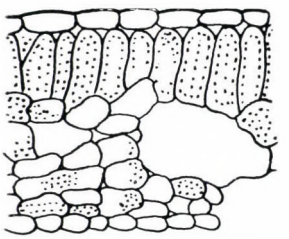

Fig. 4

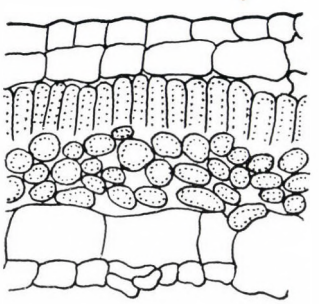

Fig. 5

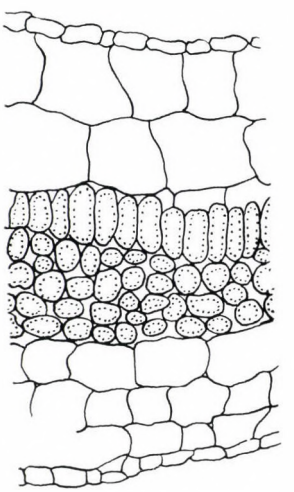

Fig. 6

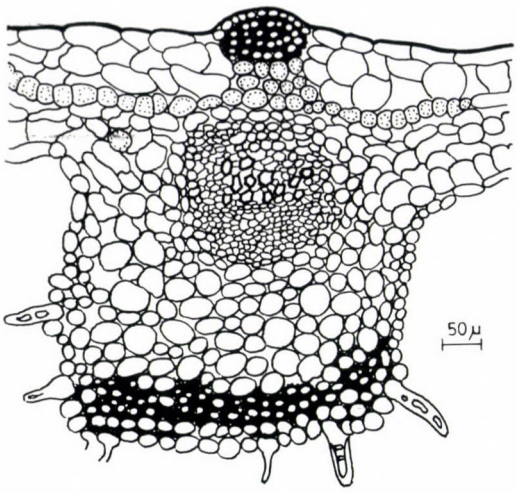

Fig. 7

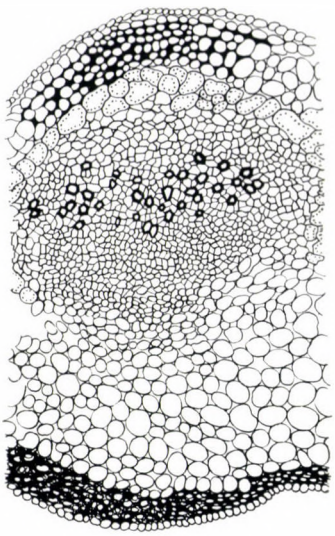

Fig. 8

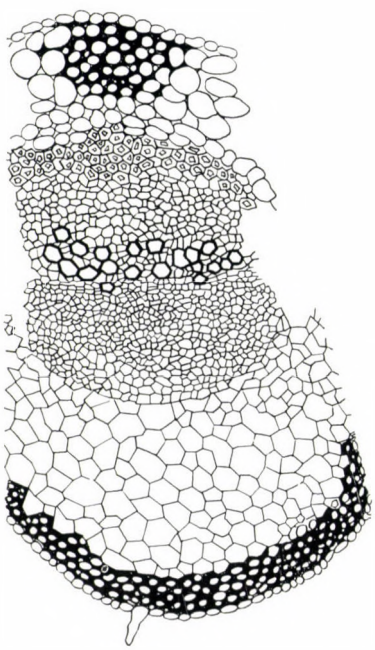

Fig. 9

Figs 1—9. Camera-lucida drawings of transverse sections of lamina (1—6) and midrib (7—9) of *Piper*. *1 = Piper betle (jhal pan); 2 = P. betle (mitha pan); 3 = P. betle (chhachi pan); 4 = P. cubeba; 5 = P. longum; 6 = P. nigrum; 7 = P. betle (jhal pan); 8 = P. betle (mitha pan); 9 = P. betle (chhachi pan)*

in the same position in *P. cubeba*, *P. betle* vars. *mitha pan* and *jhal pan*, absent in others. Collenchymatous adaxial hypodermis forming a more or less rectangular patch in *P. betle* (all vars.), *P. longum* and *P. nigrum*, covering the whole adaxial ground tissue in *P. nigrescence*, absent in *P. cubeba* and *P. pedicellosum;* collenchyma in abaxial hypodermis a 4—6 layered sheath in *P. betle* (all vars.), *P. nigrum* and *P. nigrescence*, 1—2 layered in *P. longum* and absent in *P. cubeba* and *P. pedicellosum;* type of collenchyma lacunar in *P. nigrum*, *P. cubeba*, *P. betle* (all vars.), *P. nigrescence* and angular in *P. longum* (Figs 7—14).

Vascular bundle open, collateral. Cambium zone 1—2-layered in *P. betle* var. *chhachi pan*, 2—3-layered in *P. cubeba*, 3—4-layered in *P. longum*, *P. nigrum*, 3—5-layered in *P. betle* var. *jhal pan*, not distinct in *P. betle* var. *mitha pan*, *P. nigrescence* and *P. pedicellosum*, spheroidal but dorsoventrally flattened in *P. betle* var. *chhachi pan*, *P. cubeba*, almost circular in *P. betle* var. *jhal pan*, *P. nigrum*, *P. longum*, *P. pedicellosum*, *P. nigrescence*. Number of tracheary elements in transverse section $\pm$ 15 in *P. pedicellosum*, $\pm$ 40 in *P. betle* var. *mitha pan*, $\pm$ 20 in *P. betle* var. *chhachi pan* and *P. nigrum*, $\pm$ 25 in *P. betle* var. *jhal pan*, $\pm$ 30 in *P. longum*, *P. nigrescence*, $\pm$ 120 in *P. cubeba;* tracheary elements hexagonal to pentagonal in shape. Proportion of phloem in relation to xylem almost equal in all species except *P. cubeba*, *P. pedicellosum*, where the phloem portion smaller; fibres in phloem and xylem very rare; venation reticulate; vein islet numbers per mm^2 are: *P. betle* var. *jhal pan* — 2—**6.72**—7; *P. betle* var. *mitha pan* — 2—**2.3**—6; *P. betle* var. *chhachi pan* — 3.7—**4.44**—5; *P. cubeba* — 2—**3.94**—6; *P. longum* — 1—**1.34**—4; *P. nigrum* — 2—**3.35**—5; *P. nigrescence* — 1—**3.5**—4; *P. pedicellosum* — 1—**2.08**—4. Petiole — epidermis unilayered in *P. nigrescence*, *P. cubeba* and *P. pedicellosum*, two-layered in *P. nigrum* and *P. longum*, 2—3-layered in *P. betle* (all vars.), cells barrel-shaped in *P. longum*, *P. nigrum*, *P. betle* (all vars.), *P. nigrescence*, *P. pedicellosum* and *P. longum*. Collenchyma on the adaxial side of petiole angular in *P. nigrum*, *P. longum* and *P. betle* (all vars.), absent in *P. cubeba*, *P. nigrescence* and *P. pedicellosum*. Cortical parenchymatous cells of petiole angular in T.S., more or less compact in *P. betle* vars. *chhachi pan* and *mitha pan*, isodiametric in *P. pedicellosum*, *P. betle* var. *jhal pan*, *P. longum*, *P. nigrescence* and *P. nigrum*, angular with intercellular spaces in *P. cubeba;* layers varying as 4—5 in *P. nigrum*, *P. betle* vars. *chhachi pan* and *mitha pan*, *P. longum*, 5—7 in *P. nigrescence* and *P. betle* var. *jhal pan*, 8—10 in *P. cubeba* and *P. pedicellosum*. Starch grains scattered in the ground tissue of *P. longum* and *P. nigrum*. Calcium oxalate crystals present on the ground tissue of *P. betle* var. *jhal pan*, absent in rest of the species. Open collateral vascular bundles varying in number, about seven main and nine branches in *P. betle* (all vars.), six branches in *P. nigrum*, four branches in *P. longum*, five to seven vascular bundles in *P. pedicellosum* and *P. nigrescence*, single in *P. cubeba*. Mucilage

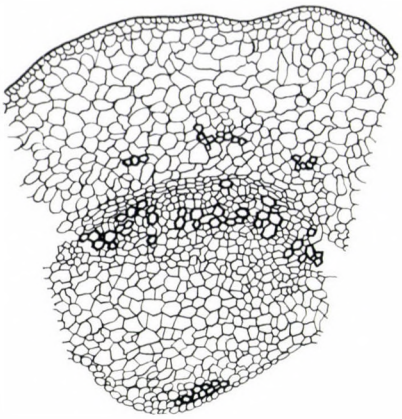

Fig. 10

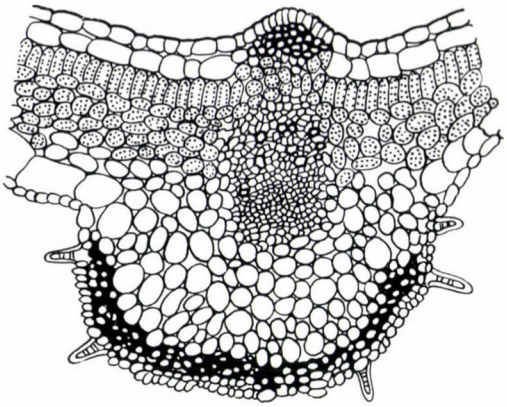

Fig. 11

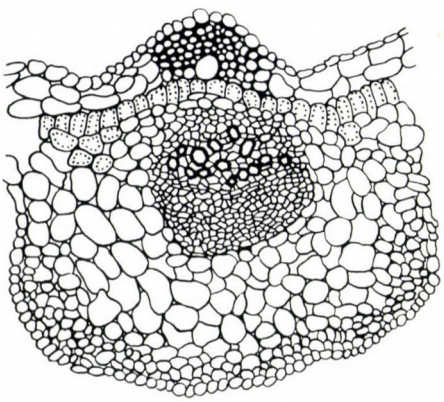

Fig. 12

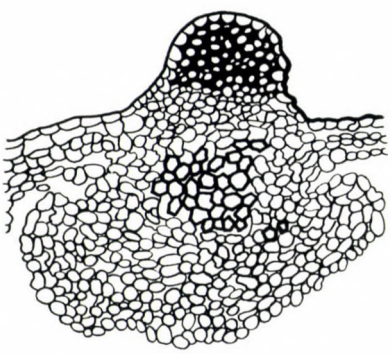

Fig. 13

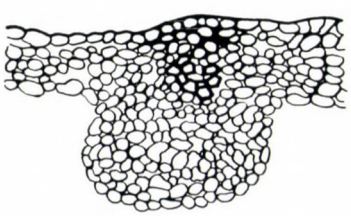

Fig. 14

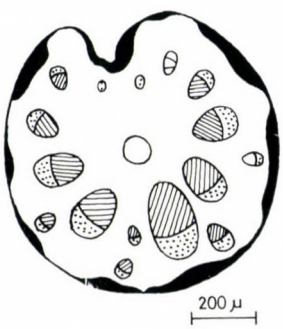

Fig. 15

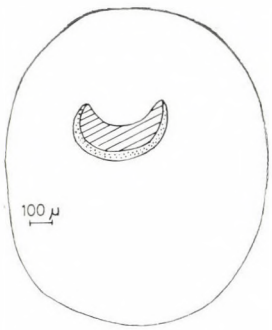

Fig. 16

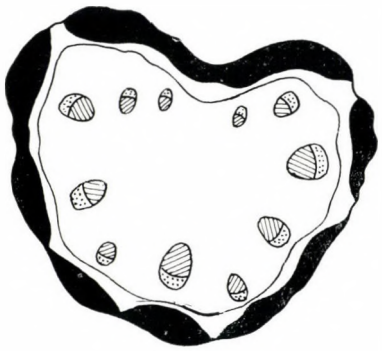

Fig. 17

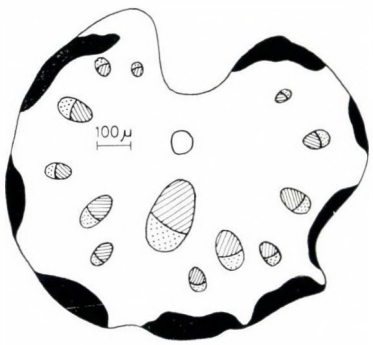

Fig. 18

Figs 10—18. Camera-lucida drawings of transverse sections of midrib (10—14) and petioles (15—18) diagrammatic of *Piper. 10 = P. cubeba; 11 = P. longum; 12 = P. nigrum; 13 = P. nepalense; 14 = P. pedicellosum; 15 = P. betle (jhal pan); 16 = P. cubeba; 17 = P. longum; 18 = P. nigrum*

canal present, almost at the centre of petiole of *P. nigrum*, absent in rest of the species. Cambium 3—5-layered in *P. longum*, *P. nigrum*, *P. betle* (all vars.), 1—2-layered in *P. pedicellosum*, not distinct in *P. cubeba* and *P. nigrescence*. Vascular bundle of petiole ovoid in *P. nigrum*, P. *betle* (all vars.) and *P. longum*, crescent shaped in *P. cubeba*, spheroidal and dorsoventrally flattened in *P. pedicellosum* and *P. nigrescence*. Vascular system generally forms a crescent in *P. cubeba*, a complete circle in *P. longum*, *P. nigrescence* and *P. pedicellosum* and few of adaxial bundles tending to be medullary in *P. nigrum*, *P. betle* var. *jhal pan*, *mitha pan* and *chhachi pan* (as in *P. excelsum*, CHIBBER[1]), (Figs 15—21).

Number of tracheary elements per main bundle $\pm$ 8 in *P. nigrescence* and *P. betle* vars. *mitha pan* and *chhachi pan*, $\pm$ 14 in *P. pedicellosum*, *P. nigrum* and *P. longum*, $\pm$ 16 in *P. betle* var. *jhal pan*; $\pm$ 74 in *P. cubeba*; tracheary elements hexagonal or pentagonal in transections. Proportion of phloem to xylem vary little in almost all species. *Fibres* very rare in phloem and xylem.

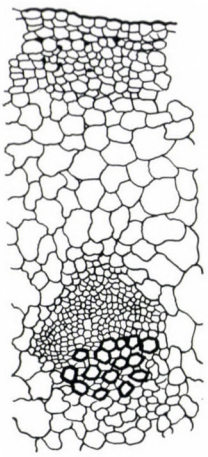

Fig. 19

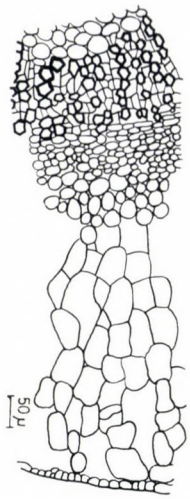

Fig. 20

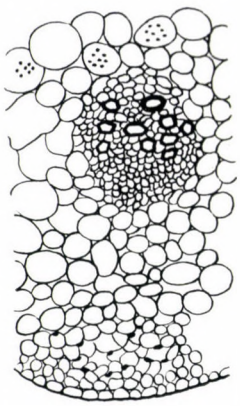

Fig. 21

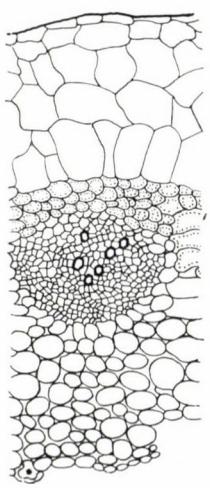

Fig. 22

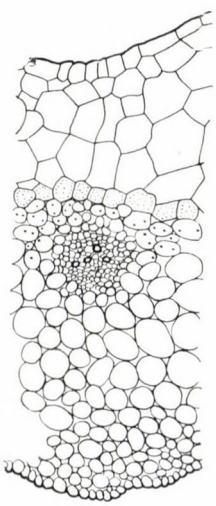

Fig. 23

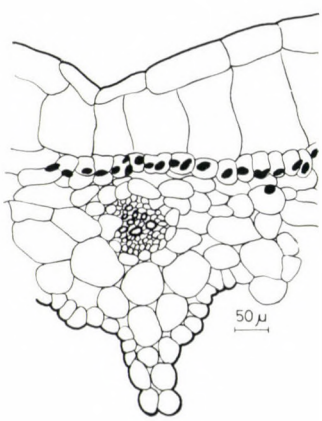

Fig. 24

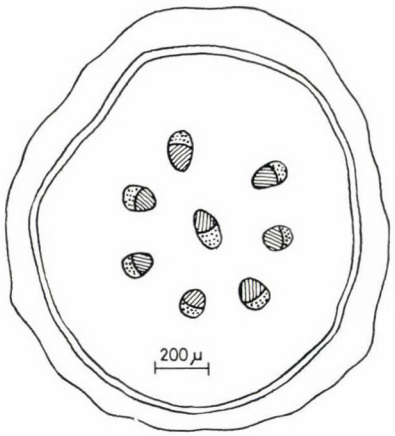

Fig. 25

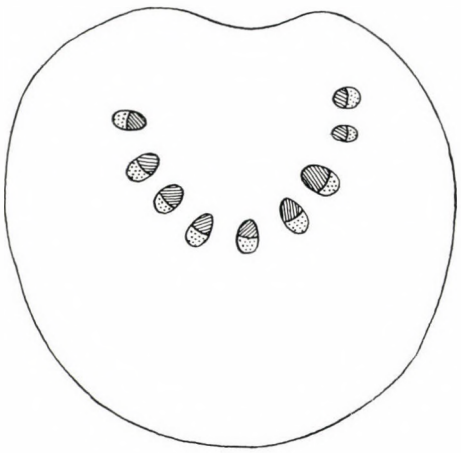

Fig. 26

Fig. 27

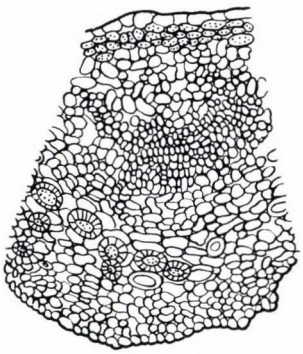

Fig. 28

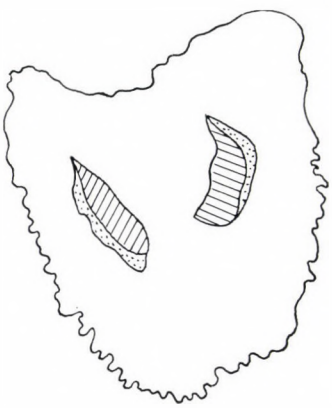

Fig. 29

Figs 19—29. Camera lucida drawings (diagrammatic and cellular) of transections through petiole and lamina of a few species of *Piper*, *Peperomia* and *Chloranthus*. *19* = *Piper betle (jhal pan)*; *20* = *P. cubeba*; *21* = *P. longum*; *22* = *Peperomia argyreia*; *23* = *Pe. obtusifolia*; *24* = *Pe. pellucida*; *25* = *Pe. argyreia*; *26* = *Pe. obtusifolia*; *27* = *Pe. pellucida*; *28* and *29* = *Chloranthus officinalis*

Genus — *Peperomia*

Leaf dorsoventral. *Hairs* sparsely distributed on the lower surface; glandular hair uniseriate on the lower surface in *P. pellucida*, *Pe. argyreia*, *Pe. obtusifolia* and *Pe. metallica;* glands consisting of basal short stalk and an enlarged head in all species. Epidermis unilayered; papillose in *Pe. obtusifolia* and *Pe. metallica* on the adaxial side and normal in *Pe. argyreia* and *Pe. pellucida;* cells near the major veins smaller than the cortical cells in all the species; cells with larger dimension perpendicular to the surface in *Pe. pellucida*, ovate in *Pe. obtusifolia* and *Pe. argyreia;* rectangular with longer dimension parallel to the surface in *Pe. metallica.*

Stomata always confined to the lower surface, surrounding cells smaller than other epidermal cells in *Pe. obtusifolia* and *Pe. metallica*, similar to other epidermal cells in *Pe. pellucida* and *Pe. argyreia*, clear cruciferous type absent in the studied species. Stomatal indices are: *Pe. argyreia* — 2.1—**8.1**—10.5, *Pe. metallica* — 2.2—**7.37**—8.8; *P. obtusifolia* — 2—**4.25**—7.6; *Pe. pellucida* — 9.1—**12.09**—17.4.

Hypodermis (adaxial) consisting of angular cells in *Pe. argyreia*, *Pe. metallica* and *Pe. obtusifolia*, single-layered with longer axis perpendicular to the surface in *Pe. pellucida*, 4—5-layered in *Pe. argyreia*, *Pe. obtusifolia*, *Pe. metallica;* cells compact without intercellular spaces. Abaxial hypodermis absent.

Mesophyll consisting of 4—5-layered palisade in *Pe. argyreia*, 1—2-layered in *Pe. metallica* and *Pe. obtusifolia* and one-layered in *Pe. pellucida;* deposits of calcium oxalate crystals present in palisade cells of *Pe. argyreia* and absent in other species; shape of the palisade cells angular in *Pe. argyreia*, *Pe. obtusifolia*, ovoid in *Pe. pellucida* and finger-like with blunt heads in *Pe. metallica;* chloroplasts present in palisade cells of all species; very distinct and larger in size in *Pe. pellucida*. Spongy parenchyma compact and with small intercellular spaces in *P. obtusifolia* and *Pe. metallica* and without intercellular spaces in *Pe. argyreia* and *Pe. pellucida*. Palisade ratio — *Pe. argyreia* — 7—**9.2**—12, *Pe. metallica* — 8—**11.03**—14, *Pe. obtusifolia* — 9.2—**11.53**—15.5, *Pe. pellucida* — 6.5—**8.90**—11.5 (Figs 22—24).

Vascular bundle spheroidal, dorsoventrally flattened in *Pe. argyreia* and ovoid in rest of the species; number of tracheary elements ± 10 in *Pe. argyreia*, *Pe. obtusifolia* and *Pe. metallica*, ± 6 in *Pe. pellucida;* shape hexagonal to pentagonal in T. S.; proportion of phloem in relation to xylem is equal in *Pe. argyreia* and smaller in rest of the species; fibres in phloem and xylem very rare (Figs 22—24). Vein islet number per mm^2 — *Pe. argyreia* — 1—**2.3**—4 *Pe. metallica* — 1—**2.34**—4, *Pe. obtusifolia* — 1—**1.16**—2, *Pe. pellucida* — 1—**2.04**—4.

Petiole contains epidermis, unilayered in all the species. Collenchyma of hypodermis angular, 8—10-layered in *Pe. argyreia*, absent in other species.

Deposits of calcium oxalate crystals present in the ground tissue of *Pe. argyreia*, *Pe. obtusifolia*, absent in *Pe. pellucida* and *Pe. metallica*. *Vascular bundle* open-collateral, almost ovoid; number of bundles, 9 in *P. obtusifolia*, 7—9 in *Pe. metallica*, 8 in *Pe. argyreia*, (5 in *Pe. berteroana*, SKOTTSBERG [15]), and 3 in *Pe. pellucida*, 3 also in *Pe. hispidula*, JOHNSON [7] and *Pe. langsdorfii*, METCALFE and CHALK [8]). Bundles arranged as a half-moon in *Pe. pellucida*, *Pe. obtusifolia*, *Pe. metallica*, or a complete circle *(Pe. argyreia)*. Cambium 2—3-layered in *Pe. obtusifolia*, *Pe. metallica* and *Pe. argyreia*, 1—2-layered in *Pe. pellucida*. Number of tracheary elements ±8 in *Pe. metallica*, ±5 in *Pe. pellucida*, ±14 in *Pe. obtusifolia* and ±20 in *Pe. argyreia;* tracheary cells hexagonal in T.S.; fibres of phloem and xylem very rare (Figs 25—27).

Genus — *Chloranthus*

Leaf dorsoventral; hairs absent; *epidermis* unilayered, both on the abaxial and adaxial surfaces; cells papillose on the abaxial side; size of the abaxial epidermal cells smaller than the adaxial epidermal cells; depth of cells, **50**—**58.3**—66.6 μm and 43.3—**48.3**—58.3 μm in upper and lower surface, respectively; dimension of cells in surface view 43.3—**48.3**—58.3 μm and 58.3—**100**—133.3 μm in upper and lower surfaces, respectively (Fig. 28). *Stomata* always confined to the lower surface, surrounded by ordinary epidermal cells. Stomatal index 11.1—**14.08**—16.6. *Hypodermis* absent. *Cortical* cells of major veins 8—10-layered, situated below the abaxial epidermis, cortical cells interrupted by stone cells.

Mesophyll not differentiated into palisade and spongy parenchyma, next to adaxial epidermis; chloroplasts evident (Fig. 28), palisade ratio — 23.5—**32.88**—38.7.

Vascular bundle crescentic in shape, cambium not distinct, ±120 vessels, tracheids angular in T.S.; proportion of phloem less than xylem; fibres rare in xylem and phloem (Fig. 28). Vein islet number per mm^2 — 1.

Petiole. Epidermis unilayered; collenchyma of hypodermis compact, 5—6-layered, more or less ovoid in T.S. Large stone cells in groups scattered throughout the parenchymatous cells of the ground tissue, star-shaped secretory cells present, the vascular system formed in two lateral crescentic patches; cambium 4—5-layered, ±120 vessels, proportion of phloem less than xylem; fibres rare in xylem and phloem (Fig. 29).

Discussion

Epidermal hairs form a clear criterion of taxonomic distinctions, which are non-glandular on the lower surface of *Piper*, glandular on the lower surface of *Peperomia* and absent in *Chloranthus*. Hairs provide no evolutionary significance.

Stomata of both *Piper* and *Peperomia* are of the same nature. The rosette of cells surrounding stomata may be similar to, or smaller than, epidermal cells. The character may differ in different varieties of the same species. Stomatal index differ distinctly in different species and varieties, and therefore, has taxonomic significance.

The hypodermis provides a good taxonomic character. Abaxial hypodermis is absent in *Peperomia*, present in *Piper*. The number of layers of hypodermis is specific. In the genus *Piper*, it is lowest in *P. cubeba* (adaxial absent and abaxial unilayered), higher in other species. The size of hypodermal cells is also characteristic of species. Among *Peperomia* species, the adaxial hypodermis is unilayered with highly specialized cells in *Peperomia pellucida*, but multilayered in other species.

The number of palisade layers, shape of palisade cells, nature of spongy parenchyma cells, intercellular spaces and presence or absence of difference between spongy parenchyma and palisade cells, provide good taxonomic characters of *Piper*. Two-layered palisade is found in *P. cubeba* and gradual reduction or loss of distinction seems to be a trend of evolution. Among *Peperomia* species also *P. argyreia* has the highest number of layers of palisade (4—5) and single layer in *P. pellucida*. The trend therefore seems to be of gradual reduction, in both the genera.

Palisade—epidermal cell ratio also varies markedly according to species. From 2.5 *(Piper pedicellosum)* to 9.8 *(Piper nigrum)* in *Piper* and from 8.9 *(Peperomia pellucida)* to 11.5 *(Peperomia obtusifolia)* in *Peperomia*. Similar taxonomic significance is found in vein islet number.

Distribution of collenchyma and sclerenchyma on the adaxial or abaxial hypodermis, against the major veins, is also a distinctive character for a species. Presence or absence of starch grains and calcium oxalate crystals in the ground tissue of petiole, number and shape of bundles in the petiole, also provide good identifying characters of species. The arrangement of vascular bundle in petiole, is also distinct in a species, circular in *Piper longum*, *P. nigrum*, *P. pedicellosum*, *P. nigrescence*, crescent shaped in *P. cubeba* and few tending to form medullary bundles in *P. betle* (all varieties). Similarly in *Peperomia* it may be crescent shaped *(Pe. pellucida*, *Pe. obtusifolia* and *Pe. metallica)* and circular with a medullary bundle in *Pe. argyreia*.

In *Chloranthus* cortical cells of petiole are interrupted by stone cells. Differentiation of mesophyll into palisade and spongy parenchyma is absent. Vascular system consists of two lateral crescentic patches. Palisade ratio is very high (23.5—38.7). Vein islet number is very low (1 per mm^2). All these characters distinguish *Chloranthus* from other studied species of *Piperales*.

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EFFECT OF VITAMIN C ADMINISTRATION ON BLOOD CHOLESTEROL LEVEL IN MAN

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Abstract

Vitamin C (1.0 g/day) was administered orally to 20 healthy males for 1 month under controlled conditions. The blood ascorbic acid level rose from 0.76 ± 0.21 mg% to 1.24 ± 0.19 mg% in young subjects (20—30 years), and from 0.74 ± 0.29 mg% to 1.22 ± 0.22 mg% in middle-aged ones (31—50 years). Simultaneously, the serum cholesterol levels decreased from 204 ± 16 mg% to 177 ± 21 mg% in the young and from 256 ± 11 mg% to 225 ± 36 mg% in the middle-aged, a statistically significant fall of 10—15%, on the average ($P < 0.01$). The effect in normo-cholesteraemic subjects, in particular, supports the cholesterol-lowering action of vitamin C.

Introduction

The possible relationship between vitamin C and cholesterol metabolism has attracted considerable attention during the last few years since it has an important bearing on the wider problem of atherosclerosis. Some workers have reported that vitamin C can lower the blood cholesterol [3, 10], while others have refuted this [1, 2]. On the other hand, NAMBISAN and KURUP [7] have recently suggested that vitamin C deficiency could promote atherosclerosis even without appreciably affecting the blood cholesterol level. In view of these controversial findings, we have investigated the effect of vitamin C administration on the blood cholesterol level of two groups of normal adults, one young and the other elderly, under controlled conditions.

Material and method

This study is based on 40 healthy adult males. All of them were chosen from amongst doctors and medical residents so that their dietary and environmental conditions were essentially similar. All were symptomless and without any abnormality on routine clinical examination. Fasting blood sugar was estimated in all subjects to exclude any potential diabetics. The 40 selected subjects were divided into two equal groups, by age (20 to 30 years and 31 to 50 years of age). Each group was further subdivided randomly into two: one set of 10 receiving vitamin C and the other 10 serving as a control. All the subjects were given to understand that the purpose of the study was only to see if added vitamin C effectively raised the blood ascorbic acid level or was merely washed out; the cholesterol part of the study was not disclosed.

All estimations were done by one of the authors (K.J.) unbiased by the identity of the samples. Blood was collected after an overnight fast. Serum ascorbic acid was estimated colorimetrically using 2,4 dinitrophenyl hydrazine [8]. Serum cholesterol was estimated by the Liebermann—Burchard reaction as described by KIM and GOLDBERG [5]. The tests were performed before starting vitamin C and at the end of 30 days treatment. The control groups were simultaneously tested.

Vitamin C (ascorbic acid, Glaxo) was given orally in a single dose of 1.0 g daily for exactly 30 days. The subjects did not take any other medicine during this period and made no change in their usual dietary and working schedules.

Results

The serum cholesterol and ascorbic acid levels, before and after the vitamin C administration for 30 days, are summarized in Table 1.

It is obvious that both the control groups are evenly matched with their respective experimental groups and the serum cholesterol and ascorbic acid

Table 1

Serum cholesterol and ascorbic acid levels before and after vitamin C administration for one month

Group	No. of subjects	Cholesterol, mg/100 ml		Ascorbic acid, mg/100 ml	
		Before	After	Before	After
Young:					
Vitamin C	10	204 ± 16*	177 ± 21	0.76 ± 0.21	1.24 ± 0.19
Control	10	191 ± 26	205 ± 20	0.77 ± 0.25	0.77 ± 0.21
Middle-aged					
Vitamin C	10	256 ± 11	225 ± 36	0.74 ± 0.29	1.22 ± 0.22
Control	10	262 ± 24	257 ± 18	0.70 ± 20	0.68 ± 0.18

* Mean ± 1 standard deviation

levels are closely similar. The initial values show that ascorbic acid in the middle-aged subjects was somewhat lower than in the younger ones, but the difference is not statistically significant ($P > 0.05$). On the other hand, cholesterol has significantly increased with age ($P < 0.01$).

Vitamin C administration for one month has considerably raised its own level in the blood, both in young and middle-aged subjects. The increase is statistically significant ($P < 0.01$). As a matter of fact, only 4 out of 20 subjects had an initial ascorbic acid level of 1.0 mg/100 ml or more while, after one month of vitamin C administration, as many as 17 out of these 20 moved above this level. Along with this, serum cholesterol levels have consistently decreased in all the 20 subjects. In the young group, who had a lower initial serum cholesterol, the average fall was by 27 mg/100 ml and the change is highly significant ($t = 4.38$ and $P < 0.01$). In the case of the middle-aged subjects, who had a higher initial cholesterol level, the average fall was by 31 mg/100 ml and this again is highly significant statistically ($t = 3.78$, $P < 0.01$).

The control groups did not show any appreciable change during the one month, indicating that all conditions remained essentially identical.

Discussion

The present investigation has brought out a consistent fall in the serum cholesterol level of all the subjects on a daily supplement of 1.0 g vitamin C while keeping all other conditions essentially constant. In 30 days time the decrease had been of the order of 10—15% below the initial value (Table 1) and the change is statistically significant ($P < 0.01$). Young adults, below 30 years of age, have shown the change just as much as the more aged subjects who had higher initial values. The contention that vitamin C can lower blood cholesterol is thus strengthened by the fact that it can do so, in the accepted therapeutic dose of 1.0 g/day, even in young, perfectly normo-cholesteraemic subjects, i.e. the effect seems not to be confined against abnormally high cholesterol levels.

The serum ascorbic acid levels have in the meantime risen appreciably, indicating that enough scope existed for further saturation of the blood with this water-soluble vitamin, at least amongst the population under study. At what blood level would vitamin C exert its optimum effect on the blood cholesterol remains to be seen.

SPITTLE [10] has reported that vitamin C, in the dose of 1.0 g per day, produces not only a marked fall in the blood cholesterol but might even mobilize any cholesterol already deposited in the vessel walls. For the same reason, several Russian workers led by Miasnikova have been regularly using vitamin C, orally or by intravenous injection, as a therapeutic measure in patients with atherosclerosis [9]. Recently, however, the pendulum has swung to the opposite side and it has been claimed that too much vitamin C might actually raise the circulating cholesterol level [2], possibly by changing the copper zinc ratio [6]. NAMBISAN and KURUP [7] have shown that ascorbic acid deficiency results in increased cholesterol in the liver and increased triglycerides in the liver and aorta of guinea pigs, although there is little change in the blood cholesterol level. However, they have affirmed that ascorbic acid deficiency would contribute to the development of atherosclerosis because it decreases the level of sulphated glycosaminoglycans.

It can be concluded that, within any given set of conditions, vitamin C intake can be an important determinant of the serum cholesterol level, but no doubt not the only one. Whether vitamin C primarily influences the intestinal absorption of cholesterol, promotes its conversion into bile acids and secretion by the liver [4] or controls its *in vivo* synthesis would require further elucidation.

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RADIATION-GENETIC STUDIES IN GARDEN PEA

V. PERFORMANCE OF SOME PROMISING MUTANTS AND RECOMBINATS

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Abstract

Genetic upgrading of seed protein production of garden pea was undertaken by developing 8 genotypes of having seed protein content leguminous plants through mutation breeding. Also 8 high yielding mutants and recombinants obtained from Germany were examined for their performance under Indian conditions by growing them in conjunction with commonly grown pea variety of Northern India. The seed protein content of high protein genotypes ranged from 130 to 135% of the control local line. Besides, the majority of the high protein genotypes were dwarf and higher yielding than the control line, and all of these flowered, fruited and matured later than the control local line. All the mutants and recombinants obtained from Germany were shorter, earlier and higher yielding than the control line. Mutant 68C is characterized by an increased ovule number per pod resulting in an enhanced seed number per pod. While, in Germany, its pod number gets reduced drastically, in India, it increased significantly. Thus, the positive and negative association in Germany changed to positive function in N. India. Mutant 1201A is characterized by the formation of shoot dichotomy but the penetrance of the gene in Germany varied from 22 to 83%. In India (Kurukshetra and Varanasi), the penetrance of the gene was nearly 1%. Therefore in the northern or central parts of India, the isolation of this mutant would have been impossible. A similar behaviour of the mutant gene was found in R98B, which is characterized by stem fasciation and diminutive vegetative and reproductive structures. The penetrance of the gene causing shoot dichotomy in 1201A could further be reduced by incorporating the gene for earliness and the gene for increased ovule number per carpel into the genome of 1201A mutant. On the contrary, its penetrance could firmly be established by an incorporation of the gene inducing reduced seed size. Thus, the expression of a mutant gene is considerably modified by the presence of other mutant genes in the genome; the penetrance and expressivity can therefore be enhanced or reduced by the incorporation of other mutant genes in the genome. Mutant 489C exibits a pronounced stem fasciation, and numerous aggregated flowers and represents one of the highest yielding genotypes under West-European conditions. In India (Bombay, Kurukshetra, Udaipur and Varanasi), unlike in Germany, it does not bear seeds at all. Thus, this mutant is without any breeding value in tropical and subtropical regions of India, in its present form. However, after incorporating the Cochleata gene into the genome of this mutant, an enormously increased floral and seed production was obtained at Kurukshetra in these recombinants.

Introduction

Recent FAO report reveals that the people of developing countries exhibit numerous symptoms associated directly with protein calory malnutrition. In fact, the high extent of malnutrition represents merely the top of an ice-

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berg and beneath its surface there is a great mass of undiagnosed marginal protein malnutrition. In India, high child mortality and retarded physical and mental growth of children represent the clear manifestation of protein deficiency [2]. SUKHATME [12] has found that since calories are basically deficient in the low income groups, meagre quantity of the available protein gets used as fuel in them. Thus, the protein situation assumes still worse dimensions with their reduced availability. Also in the lower income groups, protein absorption is limited due to infection and parasitism and, thereby, protein requirement increases further. Furthermore, of all the food ingedrients, protein foods are the most income-dependent and are therefore subject to uneven distribution even within a household, thus seriously threatening the proper nutrition of infants; pregnant women and lactating mothers need specially, high amounts of protein, which they rarely get.

Legumes form the cheapest source economical protein and contain three times more protein than that of the cereals. Besides, diverse ranges of species, varieties and variants exhibiting high adaptability and wide ecological amplitude are available in them [1]. Hence, more and more protein should come from legumes and an increased production of conventional plant protein should be initiated briskly, especially in India, where majority of the population is vegetarian. Garden pea, *Pisum sativum*, is one of the most widely consumed legume, utilized fresh, in dried and in canned state. Therefore, an attempt was made to improve seed protein yield of pea by mutation breeding. Also many mutants having an increased seed production but moderateley low seed protein yield were obtained from W. Germany and grown at Kurukshetra (N. India). The present paper gives a preliminary account of the performance and prospects of these improved genotypes under Indian conditions. This was done by growing these in conjunction with the local variety (probably a selection of Boneville variety), which is popularly grown throughout the northern, pea-growing, belt of India.

Material and method

The following genotypes, mutants and recombinants (R) were used.

Group A. (a) *HP1—HP2:* these genotypes were obtained as high protein (HP) mutants in a 5KR gammaray-treated Boneville pea population in m_3 generation and then stabilized after three back-crossing cycles with the initial line; the selection criteria were enhanced seed protein content and better grain size and yield.

(b) *HP5—HP10:* these were obtained as above but using Kashmir local as the initial line.

Group B. (a) *68C:* a single-gene (recessive) mutant characterized by an increased number of ovules per carpel.

(b) *1 201 A:* another single-gene (recessive) mutant having dichotomous stem bifurcation but the penetrance of this gene is most unstable.

(c) 489C: pronounced stem fasciation.

(d): 176: diminitive vegetative and reproductive structures.

(e) *R98:* this was obtained from the cross 489×176A., the recombinant has shortened fasciated stem and bears narrow leaves.

(f) *R177:* this was obtained from the cross 489C × 1201A; the recombinant has non-fasciated but bifurcated stem, the penetrance of the gene 1201A causing stem bifurcation is completely stabilized in it.

(g) *R350:* this was derived from the cross 68C × 1201A, and is homozygous for these two mutant genes, but the penetrance of the gene 1201 is highly unstable in this recombinant as well.

(h) *R657*, *R701*, and *R710:* these were produced from the cross 489C Cochleata (modified and reduced stipules). The recombinants exhibit a relaxed fasciation; R657 is earlier than R710 by 7—10 days.

The above material was grown in Kurushetra University Botanical Garden in a randomized block design using 4 replications of 100 plants per genotype. Further details about group A genotype are given by KAUL and MATTA [10] and about group B by GOTTSCHALK [3, 4] and GOTTSCHALK and KAUL [8]. The plants of group B were obtained from Prof. GOTTSCHALK's collection (Bonn, Germany), in which an increased seed yield served as the bais of selection; group-A plants were developed by the present author and in these an increased seed protein content was the selection criterion. Therefore, group-A and -B plants represent distinct genetic entities and, hence, the data obtained for these are classed separately in the present paper. The seed protein content of HP lines was analyzed with the help of the micro-Kjeldahl method. The value of protein content has been calculated for the dry weight of the seed.

Results and discussion

Pea unusually tolerates environmental stress probably because of its hidden genetic variability which is released even when low mutagen doses are applied to it. This is apparent from Table 1, a perusal of which reveals the existence of a wide variation even between the genotypes of group-A plants, though a rigorous selection was followed to isolate only protein rich lines. The reasons for the maintenance of this large variability are not clearly known but evidences suggest the existence of opposing selective forces within a genetic type [11]. While HP1, HP2 and HP10 are distincly dwarf mutants, the shoot height of HP7—9 is also significantly less than that of the local variety. But this reduced height is not distinctly associated with the decreased node number or internode length. Though the HP lines flower, fruit and mature later than the local variety, they exhibit a significant increase in pod number per plant. Furthermore, except for HP2, seed production per plant is significantly increased (Fig. 2). The lower seed production of HP2 line is due to a poor seed-bearing capacity within its pod.

All the mutants and recombinants of group B are significantly shorter in height than the local variety (Table 1). Like group-A plants, this reduced shoot height is not associated with the node number. Though flowering in the mutants and recombinants of group-B plants is initiated earlier, fruit maturity of these does not differ significantly from the control line. Even though seed number per pod of these (except in 68C) is considerably reduced, their seed production is significantly higher than that of the control line (Fig. 1).

Mutant 68C is characterized by an increased ovule number resulting in an increased seed number per pod. This expression of an increased ovule number which is controlled by a recessive gene is maintaned fully at Kurukshetra,

Table 1

Genotype	Shoot height (cm)	Node number	Internode length (cm)	Days required for		Pods per plant	Seeds per pod	Seeds per plant	Protein value.
				50% flowering	50% fruit maturity				
Group A									
Local Line	192.00	25.0	7.08	64	119	24	4.40	105.60	19.40
HP 1	106.75	20.00	5.34	74	128	49.20	3.89	191.39	28.14
HP 2	112.62	24.86	4.53	75	126	42.00	2.32	97.44	28.42
HP 5	180.66	24.44	7.39	80	129	58.22	5.62	327.20	26.91
HP 6	178.20	25.33	7.06	89	138	75.06	4.07	305.49	26.10
HP 7	148 25	20.50	7.23	92	130	45.00	4.92	221.40	28.40
HP 8	176.75	27.00	6.52	81	129	135.62	3.09	419.06	25.80
HP 9	170.80	23.83	7.16	90	130	87.17	2.95	257.15	25.40
HP 10	115.70	21.14	5.47	94	132	51.57	3.47	178.95	28.20
CD at 5% P level	15.35	3.91	2.88	9.77	13.20	16.07	1.20	29.55	4.70
Group B									
Local Line	192.00	25.00	7.08	64	119	24	4.40	105.60	19.40
68 C	74.73	14.75	5.06	48	112	48.41	4.36	248.76	
120 IA	121.05	20.01	6.50	54	112	66.2	2.91	193.0	
R 98	97.02	23.60	4.11	49	108	42.2	2.75	116.09	
R 117	107.21	21.51	4.98	49	111	79.0	2.40	190.0	
R 350	98.04	19.30	5.07	49	114	58.4	3.79	221.1	
R 657	108.15	17.07	6.33	52	118	41.9	3.57	149.8	
R 701	114.54	20.10	7.23	44	116	63.2	2.91	184.4	
R 710	77.40	19.21	4.02	51	106	73.5	3.66	269.5	
CD at 5% P level	24.82	03.25	2.61	8.19	9.68	13.56	1.04	22.82	

too, however, the pod number per plant in Germany gets significantly reduced [8]. Thus in Germany, a positive and a negative character expression are conjointly operative in this mutant. Both these expressions are caused by the single recessive gene [3]. Surprisingly, this mutant showed a different behaviour at Kurukshetra. Not only the seed number per pod but also the pod number per plant was increased, due to an extraordinarily vigorous and profuse branching of the plant. Therefore, this mutant at Kurukshetra produced an exceedingly high number of seeds, due to the additive effect of these positive yielding components (Fig. 2). Thus, one of the reactions of this mutant gene to a different ecological condition is indicative by a positive alteration of the pleiotrop-

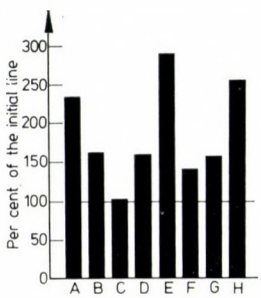

Fig. 1. Seed production of mutant and recombinants of pea

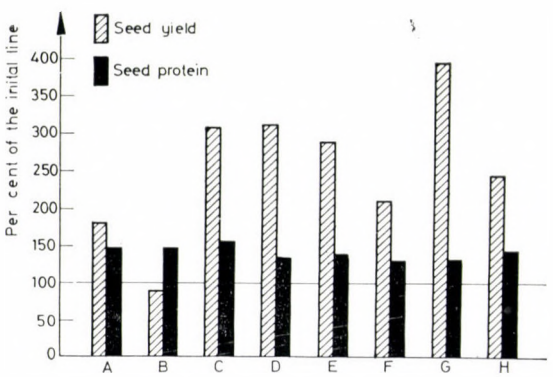

Fig. 2. Seed yield and seed protein contents of high-protein genotypes of pea. (All the values recorded are in per cent of their respective initial lines)

ic spectrum resulting in an increaxed seed production. Hence, this genotype has an improved breeding value in India, and therefore can profitably be incorporated in a cross-breeding programme with other promising varieties.

The characteristic feature of mutant 1201A is an alteration of monopodial branching system of the shoot into a dichotomous one, and the mutant has

a bifurcated apical shoot system due to which the pod number per plant gets increased significantly. This stem bifurcation is caused by a single recessive gene [3]. Thus, this gene would be of a profound practical value in pea breeding and yield improvement if it would have a stable manifestation. But this is not the case. In each generation, the stem bifurcation appears only in a certain proportion of the plants, whereas the remaning plants, even though carrying the same genotype, are non-bifurcated and do not exhibit an increased seed production. At Bonn, Germany, the penetrance of this gene varied from 22 to 83% [5]. In Belgrade (Yugoslavia), its penetrance was lower [9]. Both at Kurukshetra and Varanasi (India), the penetrance of the gene was found to be nearly 1% when 200 plants of this genotype were analyzed at each place. Therefore, this gene is unable to express its action under the environmental conditions of Northern and Central India and it would have been impossible to select this mutant, in India, at least.

Theoretically, the stabilization of the penetrance of this gene should result in a considerably increased seed production of the mutant. In the frame of a comprehensive crossing programme, gene 1201A was combined with many other mutant genes, and recombinants of different genetic constitutions were selected. These experiments have revealed that the penetrance behaviour of gene 1201A does not only depend on specific environmental factors, but to a considerable extent on the presence of other mutated genes in the genome. Its penetrance causing stem bifurcation is dramatically reduced in association with the gene for earliness (got from mutant 468C). On the other hand, a complete stabilization of the unstable penetrance of gene 1201A occurs if it is combined with a gene causing a reduced seed size (obtained from mutant 489C), all the plants (recombinant R177) are bifurcated and have an increased seed number per plant, both at Kurukshetra and at Bonn.

In 89% of R350 plants, a recombinant homozygous for the genes increased ovule number per pod (obtained from 68C) and stem bifurcation (obtained from 1201A), the stem bifurcation did not appear at all under Indian climatic conditions. Thus, R350 at Kurukshetra behaves in this respect like its parental mutant 1201A. The remaining 11% exhibited stem bifurcation and produced 68% more seed than the non-bifurcated ones even though both had the same genotypic constitution.

R98B was selected at Bonn in the F2 generation of the cross 489C (stem fasciation) × 176A (diminutive vegetative and reproductive structures) and exhibited the above-mentioned specific parental traits. But at Kurukshetra and Varanasi, the stem fasciation did not appear in 93% of the plants of this population; in the remaining ones the anomaly was very weakly developed and just hardly discernible. Hence this gene combination, which expresses its full potential in Central-European conditions in every plant, shows an extremely low expressivity and penetrance in India. Consequently, it would not

have been possible to select this genotype as a recombinant in Northern and Central India.

It is known that multiple mutations may be induced by mutagenic treatments of the cells of the growing point of an embryo. Each of such mutations present in M1 plants can be separated from one another in the subsequent generations and gradually pure lines can be developed. We tried this by crossing genetically simple mutants like 176A, 1201A, afila and cochleata with 489C, which genetically is a very complicated mutant characterized by fasciated stem and the aggregation of an extremely large number of flowers in the apical region, leading to an increased pod and seed production. We made this crossing to show how many genes mutated in the X-irradiated embryo of 489C, and how many distinct traits they influence. In the F3 and F4 generations of all the above crosses, five different degrees of internode length, divergent from that of the initial line, resulted in 5 different classes of plant height. Since 489C was the common partner in all the above crosses, this distinct internode length was transmitted from it alone, other partners being homozygous for their height genes. Therefore, at least six different genes seem to have mutated in the embryo that produced mutant 489C and at least five of them influence the internode length [3].

As mentioned above, 489C exhibits a pronounced stem fasciation and is one of the highest yielding genotypes under West-European conditions [3, 4, 6]. In Germany, it flowers very profusely but 10 days later than the initial line. On the other hand, in India (Bombay, Kurukshetra, Udaipur and Varanasi) and in Egypt, it develops vigorously, exhibits a luxuriant vegetative growth, a marked stem fasciation but not flower production at all [8]. Nevertheless, 6—7 weeks after the initial line had flowered, a few plants at Kurukshetra developed minute flowers. But these were never able to develop the seeds. Thus, this mutant, unlike in Germany, is without any breeding value in tropical and subtropical regions in its present form. However, it was crossed with cochleata mutants and the recombinants R701 and R710 exhibiting stem fasciation and modified stipules were selected and made homozygous. They were then grown at Kurukshetra in 1974. Both these recombinants flowered and fruited earlier than the initial line and had a tremendously high seed production; R710 being shorter with an increased seed production than R701 (Fig. 1).

Conclusion

The improvement of the grain yield in garden pea by means of the action of mutated genes could theoretically be achieved by an increase in the ovule number, number of carpels per flower, the number of flowers and pods per plant or kernel size [1]. Furthermore, improvement in seed protein can be realized by producing the genotypes rich in seed protein and by upgrading

the protein quality of the seeds. All the above-mentioned desirable traits have been realized in pea and are available in different genotypes of group-A and- B plants. Hybridization among group-A and -B plants should lead to the production of improved pea genotypes as envisaged by BLIXT and GOTTSCHALK [1].

The selection value of pea mutants and recombinants studied presently lies in their having early flowering and ripening, increased flower and pod number per plant and an overall improvement of yield. Also the HP genotypes, though higher yielding than the local variety, may prove inferior in seed production in other regions of India or elsewhere. Therefore, these lines should be grown along with the respective local lines in various regions of India, as well as in other pea-growing countries and then evaluated for their yielding ability. The same is valid for the group-B plants. However, such a high yield per plant and high protein content in the seeds have not been reported so far from India. Hence, these lines are of much significance to this country.

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ON THE POSSIBLE ROLE OF UNSATURATED FATTY ACIDS IN THE ANAEROBIOSIS OF ANODONTA CYGNEA L. (MOLLUSCA, PELECYPODA)

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Abstract

The total lipid content, the fatty acid composition and the amounts of saponifiable as well as non-saponifiable fractions of total lipids were measured in the ganglia on the 6th day, and in the total body on the 8th and 9th days of prolonged anoxia. In the ganglia the total gas chromatographic peak area of the saturated fatty acids obtained from the same amount of total lipids increased about 55%, whereas that of the unsaturated ones increased only 17% by the 6th day of anoxia; the ratio of saturated to unsaturated fatty acids increased from 0.43 to 0.57. At the same time the absolute quantities of total lipids present in the ganglia somewhat decreased. At this time of anaerobiosis the anoxic energy producing mechanisms are still functioning well. In the whole body the saponifiable fraction of total lipids showed a drastic decrease by the 8th and 9th days of anoxia (from 55—60 to 15—30 weight%), whereas the non-saponifiable fraction increased correspondingly. By this time the anoxic tolerance of the animals was nearly exhausted. The unsaturated fatty acids may play a role as terminal electron and H-acceptors in the anoxic metabolism of molluscs. The possibility is discussed that this electron transfer may be coupled to phosphorylating mechanisms whithin the yellow pigment granules (cytosomes) producing a high amount of ATP (two-thirds of the aerobic production) from the same quantities of carbohydrates even in complete anoxia.

Introduction

The known basic biochemical mechanisms of energy production during facultative anaerobiosis of invertebrates have recently been reviewed [6]. Although the production of succinate, propionate, etc. may yield somewhat more energy (up to 11 moles ATP/mole glucose-6-phosphate + 2 moles aspartate + 2 moles glutamate) than the classical glyco- or glycogenolytic pathways (2 or 3 moles ATP/mole glucose, resp.), the whole energetic balance remains still unexplained. Experimental data has shown that in certain molluscs, anaerobiosis causes very little or no Pasteur effect [see for ref.: 13, 24] and at the same time a surprisingly high ATP level is maintained: e.g. in *Anodonta cygnea* about two-thirds of the normal value was found till the 6th day of complete anoxia [24]. A possible explanation for these phenomena may be the existence of a special mechanism of energy production called "anoxic endog-

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enous oxidation" [24, 26, 29]. In this mechanism an intrinsic electron acceptor substance was thought to substitute for molecular oxygen.

Experimental evidence shows a correlation between the presence of yellow pigment granules (cytosomes) in the tissues and their anoxic tolerance [21, 22, 27]. On the other hand, the cytosomal lipochrome pigment consisting of neutral and phospholipids [28] as well as carotenoids [12], proved to be a good electron acceptor under anoxic conditions [23]. These results suggested that the assumed electron acceptor substance of the "anoxic endogenous oxidation" should be sought among the components of the cytosomal lipochrome pigment, first of all among the lipids [26, 31].

Recent biochemical findings have thrown some more light onto the possible role of unsaturated fatty acids in the energy production of facultative anaerobic molluscs. Namely, it has been revealed that the active incorporation of radiolabel from ^{14}C-acetate into fatty acids in the land snail *Cepaeae nemoralis* L. which is of high anoxic tolerance, persists even during anoxia, and the incorporation into saturated fatty acids becomes significantly stimulated. This probably involves a saturation process which may act as an electron acceptor mechanism for the reoxidation of NADH or NADPH [15, 18]. Furthermore, the production of some volatile fatty acids has also been demonstrated as a possible contributor to the anoxic ATP synthesis of facultative anaerobes [1, 3, 20].

The present paper provides further data regarding the possible role of unsaturated fatty acids in the anaerobiosis of *Anodonta cygnea* by studying the composition of fatty acids in the ganglia and the whole body after prolonged anoxic periods.

Material and method

Two series of experiments were performed:

Experiment 1. Adults of the fresh-water mussel *Anodonta cygnea* L. of 10—12 cm body length were collected from the Lake Trasimeno (Italy) in the summer (July). After collection the animals were kept for 5 days in a well aerated aquarium containing water of the same lake, then 10 randomly selected animals were exposed to anoxia (see below), at 20—21 °C for 6 days. Another group of 10 animals remained constantly in well oxygenated water during the same period (controls). The cerebral, visceral and pedal ganglia of the animals were dissected and pooled together within the control and anoxic groups, respectively. The fresh weights were measured and the total lipid concentration as well as the fatty acid composition were analyzed. The choice fell on the ganglia because among all the tissues the nervous tissue is the richest in cytosomes (25).

Experiment 2. This experiment was performed during the period October—January. Adult samples of *Anodonta cygnea* of 16—18 cm body length were taken from the aquaria of the Tihany Institute (Hungary). (These animals had been collected from fish-ponds and subsequently were kept in aquaria supplied with the water of Lake Balaton for several months. During the transportation to Italy, the animals were exposed to anoxia for 2 days; therefore, in order to eliminate an eventual effect of this anoxic period, we kept them in a well aerated aquarium of fresh-water for 20 days before the experiments. Commercially available fish food was given time to time to the water of the animals.) In these experiments animals were exposed to anoxia for 8 or 9 days at 15—16 °C and subsequently the total lipid concentration, the fatty acid composition and the amounts of saponifiable as well as non-saponifiable fractions

were analyzed in the total lipids of the whole body. The controls were kept in aerated water during the anoxic period of the experimental group, at the same temperature. We decided to study whole animals starting from the prediction that if the theory of "anoxic endogenous oxidation" involves the unsaturated fatty acids as electron acceptors, one should get some essential changes in the fatty acid pattern during the terminal phase of the anoxic tolerance.

Exposition to anoxia. Each animal was placed in a volume of water amounting to 8 times its total weight, then the water surface was covered with a paraffin oil layer of about 2 cm thickness. Under these conditions the water had become anoxic (no oxygen measurable by polarography) by the 17th h at 15 °C. About two-thirds of the normal ATP level of the whole body was maintained for 6 days. On the 7th day the ATP content had dropped to 11% of the normal, and the animals could survive maximally until the 10th day [24]. It should be noted that this mode of creating anoxic conditions had apparently no drawbacks for the anoxic survival of *Anodonta cygnea*, since no accumlation of acidic metabolites was observed in the water (for more details see: Ref. 31).

Extraction of lipids. Lipids were extracted by 2:1 chloroform: methanol (containing 1 mg BHT/100 ml) using a weight to volume ratio of 1:20. The samples were homogenized in the extracting solvent, then filtered. A volume of 0.88% KCl was added corresponding to 20% of the total extract volume, then the chloroform phase was separated from the aqueous one. The chloroform extract was brought to a determined volume by evaporation with N_2 gas stream.

Determination of total lipid concentration. A known volume (50 μl) of the chloroform extract was evaporated and the weight of lipids was measured by using a microbalance with a precision of $\pm$ 0.1 μg.

Methylation of fatty acids. A volume of the chloroform extract containing 2 or 4 mg total lipids of the ganglia or the whole body, respectively, was evaporated to dryness in N_2 stream, then 2.5 ml 6% H_2SO_4 in methanol was added and this mixture was kept at 70 °C for 2 h. After cooling, 5 ml distilled water was added and the fatty acid methylesters were extracted with 2$\times$5 ml n-hexane. The methyl ester yield was measured in numerous experiments in our laboratory giving always a value above 90%.

Purification of methyl esters. The hexane extract was evaporated to about 0.1 ml in N_2 stream and the methyl esters were purified by thin-layer chromatography (5). This method assures a 98% recovery of methyl esters as confirmed by experiments with radiolabelled fatty acids. The purified methyl esters were kept in n-hexane and immediately before the gas chromatography the solvent was changed for CS_2 (30, 50 or 100 μl) from which 1 or 2 μl were injected into the gas chromatograph. In order to avoid an error originating in the inequal evaporation of the solvent, this procedure was carried out as quickly as possible. Different runs of the same sample differed maximally 20% in total peak area which represents obviously the total reproducibility of the gas chromatograms.

Gas chromatography. A Carlo Erba Model 2300 equipped with a glasscolumn (2 m$\times$3 mm i.d.) packed with 5% DEGS was used. Automatic temperature programming was applied from 130 °C to 210 °C with an increase of 2.5 °C/min. Special care was taken to assure constant conditions for GLC. Peak areas were directly measured by means of an automatic computer (Autolab System). The peak areas are expressed in arbitrary units identical for all the analyses. The identification of fatty acids was made partly by adding authentic standards to the sample (16:0 and 18:0) and comparing the relative amounts before and after these additions. The other fatty acids were identified on the basis of relative retention times obtained in our laboratory during other experiments and of those published by others [17].

Saponification experiments. Saponification of total lipids was performed according to VAN DER HORST [17]. Non-saponifiable lipids were extracted with petroleum ether from the water-diluted saponification mixture (the great majority of the yellow pigments came out with this fraction). After acidification to pH 1.0, the residue was extracted again with petroleum ether or ether. This second fraction contained very little or no visible pigments. After having evaporated the solvent under nitrogen stream, the weights of the saponifiable and non-saponifiable fractions were measured.

Results

The total fresh weight of the ganglia taken from 10 control animals amounted to 152 mg, whereas that of the 10 anoxic clams was 98 mg (Experiment 1). The total lipid concentration in the control and anoxic groups was

24.3 and 31.9 mg/g fresh weight, respectively, i.e. it increased about 31% during the 6 days of anoxia. However, this increase is due to the decrease of the total fresh weight of the ganglia, namely, the absolute quantity of lipids present in the ganglia of 10 animals decreased 15.4% as compared to the control value.

Before describing the gas chromatographic results concerning the composition of fatty acids in the total lipids, one has to make some considerations. Namely, since the extraction of lipids was carried out from the pool of ganglia of 10 animals, the results obtained should be regarded as average values. Unfortunately, the pooling of ganglia does not permit to calculate the statistical scatter of individual values; on the other hand, individual analyses are impossible because of the very small weights of the ganglia. The statistical scatter obtainable from different gas chromatographic runs of the same sample reflects the reproducibility of the chromatograms; the standard errors were around 5—6% of mean total peak areas for 3 runs, and about 1—2% for the percentual representation of the fatty acids. Since this statistics has nothing to do with the scatter of lipid composition of individual ganglia, the indication of these standard errors in the table values might have been misleading. Therefore, Table 1 shows the average values of 3 runs without indicating the statistical scatter. In spite of the missing individual statistics, the data obtained may indicate some general tendencies.

The anoxic group showed an increase of 29% in the total peak area (Table 1). Considering what was said above on the reproducibility of the total peak areas, this increase could even be interpreted as marginal. This increase, however, is not homogenously distributed among the fatty acids: the saturated ones increased 55% while the unsaturated ones showed only +17%. Another important fact is that the ratio of saturated to unsaturated fatty acids also increased during the anoxia. This increase is statistically significant, if the standard error of mean does not exceed 8—9%. Taking into consideration that this ratio is regulated rather strictly in poikilothermic animals [4, 8, 10, 11], one can assume that the real standard error of mean obtainable from 10 individual measurements would fall within the limits of 8—9%. Thus one can accept that the anoxia caused a real change in the fatty acid composition of the ganglia.

Another aspect of the chromatograms obtained in the anoxic ganglia is that certain unsaturated fatty acids maintained an almost constant peak area as compared to the controls. The total peak area of fatty acids with retention times longer than that of 20:3 showed an increase of only 5.5% which may well be the error of the method used. Since these fatty acids are most probably components only of the structural phospholipids, one can assume that this group has not been altered by the anoxia. On the other hand, the fatty acids having retention times shorter than 20:4 fatty acid has, undergo a rather

Table 1

Composition of fatty acids in the total lipids of ganglia. The figures represent average values of 3 chromatographic runs obtained from identical amounts of total lipids of the pooled ganglia of 10 normal and 10 anoxic animals. For statistics see the text

Fatty acid (Tentative identity)	Normal		6 Days of anoxia	
	peak area	per cent	peak area	per cent
14 : 0	6 100	1.03	7 942	1.04
15 : 0	4 736	0.80	6 861	0.90
15 : 1	2 090	0.35	2 922	0.38
15 : 0	116 201	19.63	192 155	25.16
16 : 1	35 274	5.96	51 758	6.78
17 : 0	7 912	1.34	11 714	1.53
17 : 1	8 950	1.51	14 639	1.92
18 : 0	39 517	6.68	53 220	6.97
18 : 1	51 239	8.66	56 514	7.40
18 : 2	20 977	3.54	25 579	3.35
18 : 3	4 706	0.80	6 835	0.90
19 : 0	2 993	0.51	4 117	0.54
20 : 1	51 406	8.69	71 084	9.31
20 : 2	6 879	1.16	9.963	1.30
20 : 3	7 232	1.22	10 247	1.34
20 : 4 ω 6	41 201	6.96	48 344	6.33
20 : 4 ω 3	24 186	4.09	24 255	3.18
20 : 5	22 801	3.85	22 648	2.97
22 : 3	23 381	3.95	18 938	2.48
22 : 4	37 371	6.31	42 000	5.50
22 : 5 ω 6	31 682	5.35	25 236	3.30
22 : 5 ω 3	31 260	5.28	36 443	4.77
22 : 6	11 161	1.89	17 537	2.30
24 : 4	2 570	0.43	2 728	0.36
Total	591 825	100.0	763 677	100.0
(Increase in % of normal)			(29.0%)	
Saturated	177 459	29.98	276 009	36.14
			(55.5%)	
Unsaturated	414 366	70.02	487 668	63.86
			(17.7%)	
Satur./Unsatur.	0.4283		0.5660	
Total above 20 : 3	225 613	38.12	238 127	31.19
			(5.5%)	
Total below 20 : 4	366 212	61.88	525 550	68.81
			(43.5%)	

significant quantitative change during anoxia. Their total area increased 43% suggesting an intense synthesis of these fatty acids in anaerobiosis. Especially the fatty acids 16:0 and 16:1 increased, their peak areas were 65 and 47% higher, respectively, after 6 days of anoxia.

In the total body (Experiment 2) the lipid content was found in the range 12.6—14.0 mg/g fresh weight (without the shells) in the control animals. After being exposed to anoxia for 8 or 9 days (at 15—16 °C), the total lipid content varied between 11.8—13.3 mg/g fresh weight. These data were obtained from 3 normal and 6 anoxic animals. The control values are identical with the total lipid concentration found in *Cepaea nemoralis* (1.36% of the fresh weight [17]).

Gas chromatographic analysis of the fatty acid composition in the total lipids of the whole body was carried out in one experiment involving 1 normal and 2 anoxic (8th and 9th days) animals. A very strong decrease of the total peak areas was observed in the anoxic animals (to 13—23% of the control) when starting from the same amounts of total lipids. Such high differences can hardly be attributed to methodical errors. Nevertheless, the gas chromatographic peak area in itself cannot be regarded as a safe measure of the fatty acid content. For this reason, we measured the saponifiable fraction of the total lipids in further experiments.

The saponifiable portion of the total lipids amounted to 55—60% in the control animals (6 measurements in 3 animals). After 8 or 9 days of anoxia the values were in the range of 15—30% (12 measurements in 6 animals), whereas the non-saponifiable fraction increased correspondingly. These results confirm that the decrease of total peak areas of gas chromatograms were not due to methodical errors and permit the conclusion that the anoxia of 8 or 9 days causes a strong decrease in the fatty acid content of the total lipids.

Discussion

Interpreting the results obtained in the ganglia on the 6th day of anoxia (Experiment 1), one has to take into consideration the fact that at this time the anoxic energy production mechanism of *Anodonta cygnea* still functions well [24]. The results indicate that this level of anoxia induces an increase of fatty acid synthesis (or transport of fatty acids to the ganglia) in such a way that the saturated fatty acids increase to a higher extent than the unsaturated ones. This interpretation is coincident with the observations of others [15, 18], obtained by radioisotope techniques in *Cepaea nemoralis*.

The question arises what can be the physiological significance of the increased fatty acid synthesis during anaerobiosis. Since the beta-oxidation of fatty acids is very improbable in anoxia [3], the sense of an increased fatty acid synthesis remains obscure. On the other hand this process requires some ATP, therefore, it represents an energy demand which would be uneconomic

for the tissues, unless this energy expense could be repaid later on with a higher energy yield. We assume that this "repayment" of the energy may be realized by hydrogenation of the unsaturated chains. The following data show that this hypothesis may be much more than a mere speculation. An enzyme has recently been found in *Candida* cells and in rat liver (NADPH-Enoyl-CoA-Reductase) performing the biohydrogenation of unsaturated chains [7, 14]. This enzyme functions only if NADPH as specific electron and H-donor is present. Similar enzymes may well function also in the molluscan tissues. If a suitable coupling exists between the electron transfer from NADPH to the fatty acids and the phosphorylating mechanism, ATP may be synthesized during the saturation of double bonds of the fatty acid chain.

Previous experiments have revealed some evidence for the existence of such a coupling. It has been shown that specific organelles containing a lipochrome pigment (cytosomes) are present in molluscan tissues of high anoxic tolerance [25]. The structural transformation of the cytosomal lipids into membranes during anoxia might offer the necessary compartments for the realization of a coupling [27]. The cytosomes contain also some respiratory enzymes such as cytochrome oxidase and succinic dehydrogenase [21, 30]; furthermore, they display an energy dependent accumulation of divalent cations during anoxia which can be inhibited by KCN and dinitrophenol (DNP), i.e. the cytochrome oxidase and the coupled phosphorylating mechanisms are most probably involved [9, 22]. Therefore, one can assume that the cytosomes are the sites of the anoxic energy production, performing an electron transfer from NADH or NADPH to the cytochromes, however, the final electron acceptor is not the molecular oxygen but a cytosomal substance of high electron acceptor capacity [23, 24, 29]. These results suggest the physiological reality of the "anoxic endogenous oxidation" mentioned in the introduction. It should be noted that a substantially similar mechanism was found in polymelanosomes of vertebrates where the electron acceptor function was performed by the melanin (it is the best biological electron acceptor molecule, see for Ref.: [16]), maintaining the same level of oxidative phosphorylation in anoxia as measured in the well-prepared mitochondria in the presence of oxygen [2].

It is obvious that one has to expect drastic changes in the fatty acid content when the duration of anoxia exceeds the tolerance limit of the animal, if the unsaturated fatty acids represent really the cytosomal electron acceptor. In the case of *Anodonta cygnea*, under the experimental conditions used by us, this tolerance limit is at 7—10 days [24]. In Experiment 2 we used animals responding to the mechanical stimulation of the mantle edge with a contraction of the adductors, i.e. they were still living, however, near to their anoxic tolerance limit. In the total lipids of these animals a considerable decrease of fatty acids was observed, whereas the total lipid concentration decreased to a lower extent. These results suggest that the termination of anoxic tolerance is accom-

panied by the exhaustion of the unsaturated fatty acid reserves. It should be noted that at this time considerable carbohydrate reserves are still available [24], therefore, carbohydrate shortage cannot be the reason for the termination of the anoxic tolerance.

The question arises why the saturated fatty acids show no higher accumulation during the prolonged anoxia. It is known that the poikilothermic animals possess a regulatory mechanism assuring a saturated to unsaturated ratio necessary for the maintenance of a sufficiently low melting point of their lipids [4, 8, 10, 11]. This regulation may function even during anoxia eliminating the excess of saturated fatty acids. Experimental evidence has shown that the molluscs are able to transform rather rapidly the excessive palmitic acid (16:0) into hydrocarbons [19] and the hydrocarbons are always completely saturated in molluscan tissues. Unfortunately, this type of experiments was carried out only in normal aerobiosis, nevertheless, such a mechanism could explain the rather high total lipid content accompanied with the drastic decrease of the fatty acids in the total body.

The "anoxic endogenous oxidation" can explain numerous phenomena of the facultative anaerobiosis of molluscs. It does not exclude the coexistence of other pathways like succinate or propionate production, etc. [6], since it may function in the tissues being rich in pigment granules (cytosomes). However, much more experimental work should be done to reveal further details.

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EFFECT OF PERINATAL MONOSODIUM GLUTAMATE TREATMENT ON ENDOCRINE FUNCTIONS OF RATS IN MATURITY

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Abstract

Rats were injected subcutaneously with monosodium glutamate (MSG); either a single injection of 2 mg/g b.w. was given on the 2nd day of life or repeated daily injections of the same dose were given from the 2nd to the 10th day. Controls received saline. The growth of repeatedly treated females was slightly retarded from day 80. Repeated treatment caused a slight reduction in endocrine organ weights in maturity; however, endocrine functions tested between 120—150 days of life were similar in control and treated animals, e.g., AM—PM difference in plasma corticosterone levels, adrenocortical stress responsiveness and glucose tolerance test (in males) and normal vaginal cyclicity, compensatory hypertrophy of ovaries and presence of pituitary castration cells after spaying (in females). None of these treatments influenced the sexual activity and fertility of either sex. These data indicate that perinatal MSG treatment does not cause hypopituitary syndrome in the mature rat.

Introduction

It was first reported by OLNEY [9] that monosodium glutamate (MSG) administered to newborn mice destroyed not only the nervous elements of the retina, but also nerve cells of the arcuate region of the hypothalamus. This observation was subsequently contradicted by AREES and MAYER [3], who stated that lesions involved primarily microglial cells with no effect on the perikarya of neurones, and ADAMO and RATNER [2] and CARSON et al. [5] saw no morphological changes even by electron microscopy after early postnatal MSG treatment of rats. ABRAHAM et al. [1] found that the presence and nature of morphological changes depended on the species, dose and route of administration, e.g., in mice, small doses affected only glial cells and large doses caused additional neuronal damage, while similar doses were ineffective in monkeys. On the other hand, OLNEY's original observation was confirmed by OLNEY and SHARPE [12] in one rhesus monkey, by BURDE et al. [4] in mice and rats, and by EVERELY [6] in rats. The observation that the arcuate region is affected by early postnatal MSG treatment is supported by our recent investigation [LENGVÁRI and KOSARAS, unpublished]: a single subcutaneous injection of 2 mg/g MSG on the second day of life caused striking ultrastructural

changes in arcuate neurones in rats. The most striking events were the rapid swelling of neuronal dendrites and cell bodies and the lysis of cytoplasmic organelles followed by nuclear pyknosis. The earliest signs appeared within two hours; 24 h after the treatment, the affected region seemed to be normal except for a marked accumulation of glycogen in glial and ependymal cells. Few, if any, signs of nerve cell degeneration were seen in our material. Therefore, with the exception that nerve cell degeneration was rarely, if ever, noted in our laboratory, our findings are consistent with the electron microscope observations of OLNEY [10]. OLNEY [10] and PEREZ and OLNEY [13] reported a marked decrease in the number of neurones after MSG treatment in mice, and OLNEY and HO [11] observed "necrotic neurone counts"; however, a precise description of these observations, or quantitative data, are absent from these presentations.

The arcuate region is considered to be a primary source of hypothalamic releasing factors. It is of interest to determine, therefore, whether administration of MSG to newborn animals could alter endocrine function in these animals in maturity. OLNEY [9] originally reported marked obesity in mice treated perinatally with MSG, associated with infertility of females and "mild adrenocortical hypertrophy" and reduction of the number of cells and the mass of adenohypophysis. REDDING et al. [16] treated newborn rats from days 2 to 10 of life. When these animals reached 110 days of age, both obesity and a reduction in endocrine organ weight were observed. Hypophyseal GH and LH was less than in the controls, while TSH content was unchanged. As endocrine organ weight and/or pituitary hormone content do not provide sufficient information on the functional capacity of the endocrine system, we have in these studies attempted to further elucidate the effect of perinatal MSG treatment on endocrine functions of rats.

Material and method

First litters of inbred Wistar-strain rats were used. Litter size was adjusted to 8—10 on the day of birth. One group received a single sc. dose of MSG (2 mg/g) on day 2 of life, and the second group was treated daily with 2 mg/g MSG sc. from days 2—10. Controls of both groups were injected sc. with an equal volume (0.1—0.2 ml) of saline. The animals were weaned on day 30, and according to sex, caged 7 per cage under standard environmental conditions (25 ± 1 °C; relative humidity, 60%; lights on, 4 AM-6 PM). Commercial rat pellets and tap water were available *ad libitum*. Nasoanal length and body weight of animals treated daily with MSG and corresponding controls were measured every 10th day up to the 120th day.

After 120 days of life, endocrine functions were tested: (a) Diurnal plasma corticosterone rhythm was investigated in control and treated males, the animals bled at 8 a.m. and 6 p.m. In another group of control and treated males, the rise of plasma corticosterone after combined stress was tested as follows: at 8 a.m. 1.5 ml blood was taken under light ether anesthesia by heart puncture, and immediately 5 mg formalin was injected sc. into the hind foot. One h later, a second blood sample was taken. The measure of ACTH secretion in response to stress was illustrated by the difference in the corticosterone content of the two blood samples. Plasma corticosterone concentration was determined fluorometrically. (b) A glucose tolerance test was performed on control and treated males; the animals received 1 g/kg b.w. glucose through a gastric tube after the first blood sample was taken by cutting a vessel of the tail. Additiona

blood samples were withdrawn 0.5, 1, 2, 3 and 4 h later. Blood glucose level was determined by the orthotoluidine method. (c) Time of vaginal opening was noted, and vaginal smears were taken from the 50th to 120th day of life daily. On day 120 of life control and treated females were divided into three groups. *Group 1.* Control and treated females were caged with normal males. They became pregnant and were allowed to give birth. Litter size was noted after parturition and at time of weaning. *Group 2.* Control and treated females were hemispayed, and compensatory hypertrophy of ovaries was investigated 30 days later. *Group 3.* Control and treated females were spayed on day 120 and sacrificed 30 days later. Pituitaries were fixed, embedded, sectioned and stained by Mann's method, and the presence of castration cells was investigated. Ovaries of animals of each group were sectioned frozen and stained with Scharlach-haematoxylin.

Males of treated groups were caged with normal females and their fertility investigated. The testes of some treated and control males were subjected to routine histological examination. Body weight and endocrine organ weights of each animal were determined at autopsy.

Results

A single subcutaneous injection of 2 mg/g MSG on the second day of life, or daily injection of 0.1—0.2 ml of saline from the second to the 10th day, resulted in approximately 20% mortality during the first 30 days of life, a result comparable to the spontaneous mortality in this strain up to the weaning age. On the other hand, after repeated daily injections of the same dose of MSG only 54.9% survived to day 30. Sex difference was not a factor in the survival rate. There was no significant difference in appearance between surviving control and treated animals, and gross behaviour of treated animals was normal in every respect.

Growth characteristics of repeatedly treated animals are illustrated in Fig. 1. As shown, weight and body length of control and treated males are

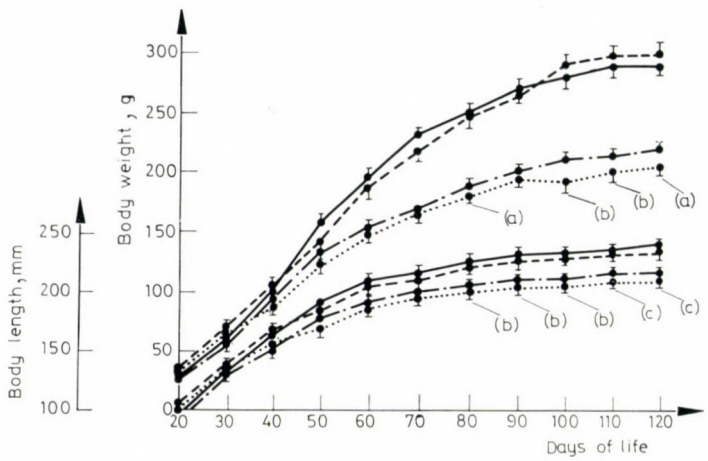

Fig. 1. Weight gain and longitudinal growth of control and daily MSG-treated male and female rats. ●———● control males; ●– – –● MSG-treated males; ●–·–·–● control females; · · · · · MSG-treated females. Vertical bars indicate SE. Significant differences between treated and respective control groups are indicated as (a) $P < 0.05$; (b) $P < 0.01$ and (c) $P < 0.001$

similar, while growth of treated females was slightly retarded from day 80. Furthermore, there is a correlation in reduction of body weight and length, e.g., the Lee index for treated and control groups was similar.

A single injection of MSG on the second day of life had no effect on endocrine organ weights of 120—150-day old animals (Table 1). Repeated daily

Table 1

Effect of perinatal MSG treatment on endocrine organ weights of mature rats

Group	Pituitary		Adrenal	
	mg	mg/100 g b.w.	mg	mg/100 g b.w.
A) *Single injection*				
1. Male NaCl	8.37[1] (18)[2] ± 0.84	3.23 ±0.08	40.41 (18) ± 1.71	14.94 ± 1.01
2. Male MSG	9.03 (21) ± 0.24	3.50 ±0.11	36.95 (21) ± 1.42	14.19 ± 0.45
3. Female NaCl	12.76 (12) ± 0.56	5.31 ±0.25	58.51 (6) ± 2.11	23.12 ± 0.86
4. Female MSG	12.04 (20) ± 0.38	5.18 ±0.14	50.79 (8) ± 4.54	21.30 ± 0.84
B) *Repeated injections*				
1. Male NaCl	9.14 (15) ± 0.43	3.40 ±0.15	40.88 (15) ± 4.48	15.22 ± 0.78
2. Male MSG	8.13 (15) ±0.29	*2.89*[4] *±0.11*	35.85 (15) ± 1.31	*12.76*[4] *± 0.46*
3. Female NaCl	14.43 (17) ± 0.50	5.72 ±0.25	63.79 (9) ± 3.09	22.31 ± 0.69
4. Female MSG	*12.23*[5] (25) *± 0.55*	*5.13*[3] *±0.18*	*55.31*[3] (12) *± 2.49*	*20.39*[3] *± 0.64*

	Thyroid		Gonad	
	mg	mg/100 g b.w.	mg	mg/100 g b.w.
A) *Single injection*				
1. Male NaCl	12.81 (10) ± 0.47	4.94 ±0.25	3070 (10) ± 112	1012 ± 73
2. Male MSG	11.62 (14) ± 0.47	4.74 ±0.17	3070 (10) ± 135	1015 ± 56
3. Female NaCl			89.20 (6) ± 9.20	33.15 ± 0.34
4. Female MSG			72.31 (8) ± 7.90	30.53 ± 2.50
B) *Repeated injections*				
1. Male NaCl	13.63 (6) ± 1.14	4.37 ±0.52	3270 (6) ± 110	1050 ± 80
2. Male MSG	13.10 (6) ± 0.95	4.65 ±0.31	*2730*[5] (6) *± 140*	970 ± 81
3. Female NaCl			94.77 (9) ± 4.78	33.35 ± 1.80
4. Female MSG			*79.58*[3] (12) *± 4.74*	29.50 ± 1.72

[1] Mean ± SE; [2] Number of animals; [3] $P < 0.05$, as compared to NaCl treated respective controls; [4] $P < 0.02$; [5] $P < 0.01$

Table 2

Effect of perinatal MSG treatment on daily fluctuation of plasma corticosterone of mature male rats

Group	Plasma corticosterone (μg/100 ml)		
	AM	PM	
A) *Single injection*			
1. NaCl	10.8 ± 0.61[1] (4)[2]	24.4 ± 2.20 (4)	$P < 0.001$
2. MSG	11.2 ± 0.83 (4)	26.4 ± 0.83 (5)	$P < 0.001$
B) *Repeated injections*			
1. NaCl	11.6 ± 0.84 (5)	28.1 ± 1.64 (5)	$P < 0.001$
2. MSG	14.8 ± 0.84 (5)	28.8 ± 2.20 (5)	$P < 0.001$

[1] Mean ± SE
[2] Number of animals

Table 3

Effect of perinatal MSG treatment on plasma corticosterone stress response of mature male rats

Group	Plasma corticosterone (μg/100ml)		
	Resting	Stress	
A) *Single injection*			
1. NaCl	14.3 ± 1.72[1] (10)[2]	36.2 ± 2.35	$P < 0.001$
2. MSG	11.0 ± 1.43 (12)	33.3 ± 1.64	$P < 0.001$
B) *Repeated injections*			
1. NaCl	18.7 ± 1.64 (5)	72.5 ± 4.42	$P < 0.001$
2. MSG	18.7 ± 1.86 (6)	70.7 ± 4.48	$P < 0.001$

[1]
[2] Mean ± SE
Number of animals

injections from 2—10 days of life reduced the pituitary and adrenal weights corrected to 100 g b.w., and the absolute gonadal weight of both sexes, while the thyroid weight of males was unaffected. All the changes, however, were at the borderline of statistical significance.

Neither a single injection of MSG nor repeated injections affected the daily fluctuations of plasma corticosterone levels (Table 2). Rise of the plasma corticosterone level after combined stress was comparable in treated animals and their respective control groups (Table 3); however, animals injected daily had higher resting and stress levels as compared to single-injected rats.

The glucose tolerance test, performed on day 120 of life, revealed no apparent difference between control and treated animals (Fig. 2).

Average time of vaginal opening was 38.3 days in controls and 38.6 days in continuously treated females. No difference in cyclicity between control

and treated animals was seen, based on vaginal smears taken from the 50th to 120th days.

Each female in every group became pregnant within two weeks when caged with normal males, and they gave birth to litters at normal term. The size of litters tended to be smaller in the treated groups, but the differences were not significant. As shown in Table 4, compensatory hypertrophy of ovaries occurred in both control and treated groups 30 days after removal of the first

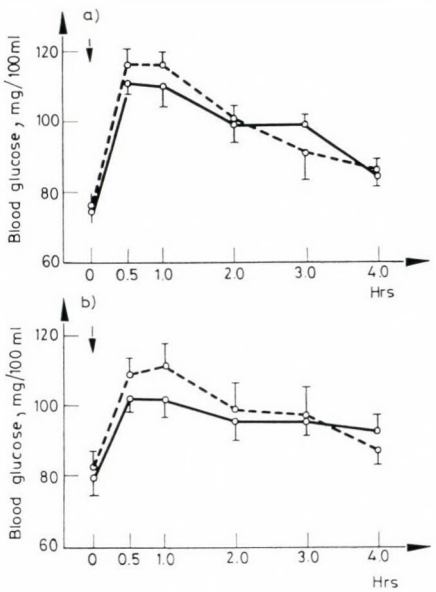

Fig. 2. Glucose tolerance test of single injected (a) and daily injected (b) male rats. ———— controls; ○ - - - - ○ MSG-treated. Administration of 1 g/kg glucose indicated by arrows. Vertical bars show SE

Table 4

Effect of perinatal MSG treatment on compensatory ovarian hypertrophy of mature female rats

	No. of animals	1st ovary	2nd ovary	
		mg/100 g b.w.		
A) *Single injection*				
1. NaCl	6	17.1 ± 1.09[1]	24.6 ± 1.66	$P < 0.05$
2. MSG	12	19.0 ± 0.71	26.4 ± 1.06	$P < 0.001$
B) *Repeated injections*				
1. NaCl	8	14.5 ± 0.57	21.4 ± 0.10	$P < 0.001$
2. MSG	13	12.2 ± 1.16	18.7 ± 1.55	$P < 0.01$

[1] Mean ± SE

ovary. Castration cells were present in the pituitaries of females of each group 30 days after spaying. Ovaries of control and treated females were similar histologically; i.e., all contained fresh corpora lutea. Testis weight of males given a single injection of MSG was similar to that of controls, while repeated perinatal MSG injections reduced the testicular weight (Table 1). No histological difference was seen, however, and when the MSG-treated males were caged with normal females, they showed normal sexual activity and fertility.

Discussion

In the present experiment a single injection of 2 mg/g MSG given to rats on the second day of life had no toxic effect, while a similar dose given daily from the second to 10th day resulted in a more than 2-fold increase in mortality during the first 30 days of life. THURSTON and WARREN [17], using a similar treatment pattern, found that the mortality rate in glutamate-injected mice was twice that of controls; similarly, a marked degree of toxicity was noted by PRABHU and OESTER [14].

Obesity is thought to be a characteristic result of early postnatal MSG treatment [9, 16]. The nature of obesity, however, was different in these two experiments. While OLNEY's mice had higher body weight and lower body length as compared to controls, REDDING et al. [16] found both body weight and nasoanal length reduced in treated rats, although the Lee index for these treated animals was higher than that for the controls. A growth suppression has also been reported by PRADHAN and LYNCH [15] in rats after MSG treatment, but only the body weight data are given in their publication. Obesity was absent in our treated rats, although our repeatedly treated females showed some signs of growth retardation. The mechanism of development of obesity or growth retardation is not known; food intake [9, 15, 16] or altered TSH secretion [16] might not be responsible. The normal glucose tolerance test, as seen in our present studies, excludes the possibility of affected insulin secretion. According to REDDING et al. [16] growth retardation in MSG-treated rats possibly resulted from impairment of growth hormone secretion, although poor direct evidence was given for this hypothesis.

A single subcutaneous injection of 2 mg/g MSG did not alter the endocrine organ weights. Continuous daily treatment with the same dose between days 2—10 of life had some effect in this respect. These results are generally in accordance with the data of REDDING et al. [16], although reduced endocrine organ weights were more pronounced in their treated rats.

Organ weights, however, do not reflect their function in every respect. In the present experiment diurnal fluctuation of plasma corticosterone was

similar in control and treated rats, and there was no difference in stress response; these two parameters are more sensitive than adrenal weight in connection with ACTH secretion.

OLNEY [9] stated that postnatally MSG-treated female mice were infertile. This was subsequently contradicted by ADAMO and RATNER [2] in rats. In the present work, treated females cycled normally, were fertile and showed the usual maternal behaviour. Although ovarian weights of treated animals were reduced, the histology of ovaries was similar to that of controls. The facts that compensatory hypertrophy of ovaries occurred in treated animals and that castration cells appeared in their pituitaries after spaying indicate that these animals were capable of enhancing the secretion of gonadotropic hormones. Interestingly, altered male gonadal function has never been seen in MSG-treated animals, and a similar result was obtained in the present experiment.

Present data clearly indicate that perinatal MSG treatment does not significantly affect the functional capacity of the endocrine system in rats. Reduced endocrine organ weights and pituitary tropic hormone content may reflect a reduced basal secretion of hypothalamic releasing factors; it is of more importance, however, that this basal secretion shows cyclicity (ACTH as well as gonadotropins) and capacity of enhancement.

There appears to be a discrepancy between the morphological picture of the arcuate region after acute MSG treatment and nearly normal endocrine functions in adulthood. For this several explanations may be given. It is possible that MSG does not cause the death of affected nerve cells. This hypothesis is supported by our recent results (LENGVÁRI and KOSARAS, unpublished], viz., relatively few signs of nerve cell degeneration were seen in rats after acute MSG administration. Conversely, OLNEY [10] frequently found degenerated nerve cells in the arcuate nucleus in mice, although these divergent results may be attributed to the difference in species. If it is accepted that MSG-induced nerve cell damage is irreversible, then the possibility that not every neuron is damaged should not be excluded; in our material we have frequently seen virtually unaffected neurones among the altered arcuate cells. OLNEY and HO [11] reported a reduction in the number of nerve cells in the arcuate region after postnatal MSG treatment of mice, but not the complete absence of arcuate neurones, an observation which may mean that the remaining cells increase their releasing factor production and, as a result, the pituitary tropic hormone secretion is almost normal. A further explanation may be that the hypophysiotropic area [see 7] which is responsible for the production of releasing factors is not identical with the region affected by MSG.

Further studies may be indicated to prove any of these hypotheses; however, one final conclusion may be drawn: early postnatal MSG treatment does not cause, at least in the rat, a hypopituitary syndrome.

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THE EFFECT OF MELATONIN AND CORPUS PINEALE EXTRACT ON SERUM ELECTROLYTES IN THE RAT

SHORT COMMUNICATION

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The pineal body is an ancient organ. In addition to its generally accepted regulatory influence on the gonads [7, 13], it seems to act on the function of the endocrine glands of ecto-endodermal origin [6]. Its regulatory action is mainly inhibitory, thus, it inhibits iodine uptake into the thyroids [1, 3, 5, 9, 10] and the function of cells in the Langerhans islets [2, 3] adrenal medulla [3], and parafollicular cells [4]. Furthermore, removal of the pineal gland seems to decrease ^{3}H-methionine incorporation into the parathyroids of rats kept on a low-calcium diet [11]. Pineal regulation is older than pituitary regulation, as the pineal gland itself is older than the pituitary gland [14]. The glands of ecto-endodermal origin, which are subject to pineal regulation, take part in adaptational regulation [6]. One important factor of regulation is the serum electrolyte concentration, hence, it may be assumed that the pineal body also takes part in the regulation of the latter. We have studied this problem by analysing the effects of both melatonin and total pineal extract, since recent data speak in favour of other hormones of the corpus pineale besides melatonin [8].

Wistar CB male rats were used. The blood levels of Ca^{2+}, P and Mg^{2+} were determined by Joó [10] and RICHTERICH's [12] method and the "Merckotest Mg" (Merck, Darmstadt), respectively. Then one group of the animals was given an i.p. injection of melatonin (Koch—Light, 10 μg/100 g body weight), the other group an i.p. injection of pineal extract (NBC, Cleveland, 10 mg/100 g body weight) suspended in saline. Thirty, 60 and 180 min after treatment, the serum electrolyte values were determined again. The results were evaluated by Student's "*t*" test.

Table 1 shows that both melatonin and the pineal extract act in the same way, viz., by decresing the levels of Ca^{2+} and Mg^{2+} and increasing the phosphate values. The effect develops slowly, a significant difference appears after 3 h in the case of melatonin, and after 60 min in the case of the pineal extract. Taking into account the similar tendency, it seems likely that melatonin is the active substance in the extract, too. The quantitative difference may be ascribed to a higher melatonin content of the pineal extract. In the case of the Mg^{2+} values the pineal extract showed values at the border of significance

Table 1

Effects of melatonin and pineal extract on the blood levels of inorganic Ca^{2+}, P *and* Mg^{2+}

Group Treatment	Time, min	Ca^{2+} mg/100 ml	Significance	P mg/100 ml	Significance	Mg^{2+} mg/100 ml	Significance
Melatonin	0	9.08	—	5.03	—	2.09	—
	30	8.51	—	5.28	—	1.89	—
	60	8.81	—	5.33	—	1.78	—
	180	7.66	$P < 0.01$	5.94	$P < 0.05$	1.58	$P < 0.01$
Pineal extract	0	9.57	—	5.43		2.43	—
	30	9.59	—	5.37	—	2.14	—
	60	8.14	$P < 0.05$	6.46	$P < 0.05$	1.97	$0.1 > P > 0.05$
	180	8.11	$1 > P > 0.05$	6.62	$P < 0.02$	1.99	$0.1 > P > 0.05$

after 60 min and 3 h, however, it did not deviate quantitatively from the melatonin effect, only the spreading was higher.

Our earlier experiments [11] also point to a possible role of pineal body in influencing the Ca^{2+} and P levels in the rat. From our present experiments this becomes evident for the Mg^{2+} level, too. However, we cannot arrive at an unequivocal conclusion because removal of corpus pineale increases the number and activity of parafollicular C cells [4], whereas it decreases those of the parathyroids [11].

Since the two glands are antagonists, it remains open whether the changes in electrolyte levels are due to an inhibition of the one gland or to a stimulation of the other. Another open question derives from the fact that release of labelled P into the blood circulation is increased as a consequence of pinealectomy, whereas melatonin increases the total P content of blood. Hence, we only arrive at concluding that the electrolyte concentrations are changed as described for the action of pineal hormone; for the elucidation of the mechanism further experiments are necessary.

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RECENSIONES

DeRobertis, E.D.P., Saez, F. A., DeRobertis, Jr., E.M.F.: **Cell biology** *Saunders Company*, Philadelphia—London—Toronto (1975), pp. 615.

The investigations in the field of cell structure and function, have developed very intensively in the recent years. Many articles and monographs have been published in this topic. However each of the individual works deals with a very narrow field. For this reason it is now nearly impossible to survey the ever increasing mass of literature although it would be essential for both university students and researchers.

This book, presenting a good survey of the most recent literature on cell biology, helps to smooth away this difficulty.

These results are systematized very didactically. Since the first edition of the book appeared in 1946, the authors have not only enlarged the book with a high number of data, but have also developed its system.

In the first chapter a short survey of cell biological and general cytological researches, and in the second part the most modern biochemical observations concerning the structure and functions of the cell, are presented. The third part summarizes the histochemical methods used in the cell biological research. The fourth and fifth parts deal with the different membranes and organelles. The cellular basis of cytogenetics is the subject of the sixth part.

The recent molecular-biological aspects developing perhaps most dynamically, can be found in the seventh part.

In spite of the predominance of the morphological and biochemical data, the book reflects a physiological point of view.

Each of the parts consists of seven chapters. Thus, the total number of chapters is 25. In each chapter there are subchapters. The summaries at the end of the subchapters are very instructive.

Good understanding is supported by 49 tables and 366 figures. These and the good typography praise the careful work of the Saunders Company.

L. Kovács (Budapest)

Organ culture in biomedical research
Ed. Michael Balls and Marjorie Monnickendasm. Cambridge University Press, Cambridge—London—New York—Melbourne (1976) pp. 570, £ 22.0.

Organ culture — as described by Dame Honor Fell, the pioneer of organ culture techniques — is the maintenance of tissues in a differentiated functional state in a nutrien medium *in vitro*.

The Festschrift for Dame Honor Fell on Organ Culture in Biomedical Research is the first published symposium of the British Society for Cell Biology (8—11 April, 1975).

The first chapter by Dame Honor Fell deals with the history of organ culture. In the following chapter G. M. Hodges gives precise information on general methodology. The chapter on experimental embryology by R. Dubois et al. describes mainly the problems of all interactions in morphogenesis. Other papers are devoted to several organs in culture, such as kidney, skin, periodontium, joints, nerves, bone, etc. A special attention is paid to studies on enzyme and hormone production by, as well as effects of hormones on, organ cultures. The organs investigated include pituitary gland, mammary gland and placenta, ovary, prostate and pancreas.

Organ cultures may be used as *in vitro* models for studying all invasiveness (D. M. EASTY and G. C. EASTY) as well as for *in vitro* carcinogenesis experiments (E. A. DEFRIES and L. M. FRANKS).

A new attempt in experimental cancer chemotherapy is the production of organotypic cultures of human tumours (E. WOLFF et al.) and the investigation of the responses of these cultures to cytotoxic drugs and hyperthermia (DICKSON J. A. and M. SUZANGAR).

Other papers report on the effect of viruses as well as irradiation on organ cultures.

The book deals with some of the most important problems of organ culture and gives the recent data on the theoretical and technical development in this field for research workers.

B. SZENDE (Budapest)

BEEVERS, L.: **Nitrogen metabolism in plants**
Edward Arnold (Publishers) Ltd., London (1976), pp. 33, £ 6.95.

The present volume on Nitrogen Metabolism in Plants was badly needed, since the last book on the same topic was published in 1959. The text is divided into nine chapters: 1. Nitrogen nutrition; 2. Amino acids; 3. Amino acid metabolism; 4. Purines, pyrimidines, nucleosides and nucleotides; 5. Nucleic acids; 6. Proteins; 7. Nitrogen metabolism in seeds; 8. Nitrogen metabolism during fruit ripening and leaf senescence. Clearly, the first six chapters are devoted to basic, general problems of nitrogen metabolism, whereas the last two are dealing with specific aspects of nitrogen metabolism in higher plants. However, it should be stressed that, even in the "general" section (first 6 chapters), great attention is devoted to problems and/or processes which are characteristic specifically of (higher) plant tissues. This is especially evident in chapters 5 and 6 (nucleic acids and proteins) in which the usual overgeneralization, based on results of the much more advanced bacterial field, is carefully avoided. One has to acknowledge the critical attitude of the authors making specific statements, all over the text, as to the problem what is valid and what is not valid for the higher plant tissues from vast literature on nitrogen metabolism in bacteria and mammalian tissues. Still, the text is coherent and lucid. The bibliography is "selective" (more than 700 citations) and remarkably up-to-date (covers the literature up to 1974—75) for a book. The volume will be an excellent source of information both for undergraduate and graduate students as well as for their teachers. The carefully balanced amount of information contained by the book, the excellent style (easy to read and understand), and the well-chosen figures make the reading of the book a must for the research workers as well, especially if their fields are not exactly in, but are related to, the area of plant nitrogen metabolism.

G. L. FARKAS (Szeged)

WILLIS, I. C.: **A dictionary of the flowering plants and ferns**
Cambridge University Press, London (1973), pp. 1245. £ 10.0.

The first edition of this dictionary was published in 1897 and the eighth revised edittion in 1973. This volume includes all generic names published from 1753 onward, and all family names published after appearence of the Genera Plantarum of DE JUSSEIN in 1789. The names of taxa are arranged in alphabetical order, but at the end of the dictionary they are ordered according to the BENTHAM—HOOKER and ENGLER—PRANTL's system. The editor in the text frequently uses mathematical sign but sometimes not in mathematical meaning (for example $\sim$ sign is applicable in "alternative" senses). It may be pointed that in the brief characterization of families and genus the editor uses morphological abbreviation without giving any explanation for them. Probably, he regards them to be well known abbreviations, but they are not so.

The usefulness of the dictionary has increased by the fact that when the editor could not decide about the valid name of a given taxon, he has given it at two places in an alphabetic order, but at the latest case the first given name is also in parenthesis. The "Preface" gives a good information for using the system of the dictionary and about the literature from which the necessary corrections have been made in this edition. The Pteridophyta was edited and corrected by R.E. HOLTUM, who adapted the classification of PICHI—SERMOLLI (1958), but sometimes instead of the above mentoined system, he has used the COPELAND (1947) system specially for generic names. The dictionary is very useful for the botanist. It gives a short but good information about the higher taxons, including their economic importance as well, besides the main characteristic features.

J. SZUJKÓ-LACZA (Budapest)

Flora Europea. Vol. 4.
Ed. TUTIN et al. Cambridge University Press, London (1976), pp. 505, £ 25.0.

This volume which contains the second part of Sympetalae — Plantaginaceae to Compositae (also Rubiaceae), presents a synthesis of all the national and regional floras of Europe, following ENGLER's system.

Besides the brief keys and description of the taxa, information is given about their geographical distribution. Maps are given at the end of the volume indicating the exact boundary between the given geographical region. The respective countries are also reffered to whithin the region: On this maps the Editors have interpreted Europe in its traditional sense. Only those taxons have been included which have their representatives in Europe. In case of families which have only one or few or an atypical member, a particular sign has been used after the species. In case of genera Taraxacum and Hieracium, having many apomictic species, the flora does not contain a complete enumeration of species. They are just summerized and grouped in the description and the key.

The purpose of Editors was to give a complete flora of Europe. They have taken informations from every basic and standard floras. However, they could not eliminate the main problem, which originates from the differences in the authors' opinion regarding the floras or monographs of genera.

The Flora Europea is a good example of international organization in co-operation. We hope, that the taxonomical probleme arising out of it will give an inspiration for further collaborative study with a view to provide a more exact and upto-date information about the similar and dissimilar characters of families, genera and species.

J. SZUJKÓ-LACZA (Budapest)

WILLIAMS, R. F.: **The shoot apex and leaf growth**
A study in quantitative biology. pp. 256, Cambridge University Press, London (1975), pp. 256, £ 6.50.

The author summarizes the results of the V. H. BLACKMANN school and successfully develops them from the mathematical point.

BLACKMAN's famouse paper on the analysis of plant growth appeared in 1919, this has been developed since in two directions by his pupils, among others by the author himself (1), the method of plant growth analysis has been used for quantification of the single plant organs (2), the results of growth analysis have been subjected to distribution analysis. The latter method has been useful in the three-dimensional approach of plant growth on the shoot axis.

Phyllotaxis, viz., the situation of the single leaves, is specified as an estimation concerning a geometric problem.

The author considers the "everywhere present" so-called Fibonacci spiral fundamental, although the occurrence of parallel and contra-directional double spirals, are not unusual.

Besides the parameters of phyllotaxis, the differences between the plastochron ratio and phyllotaxis indices have been determined with double logarithmic transformation.

The author and other members of this school have used histogenetic and quantitative methods in investigating the development of shoot apex. This process is excellently illustrated in anatomic preparations and 3-dimension diagrams.

The volume will be very useful for teaching taxonomics and morphology though unfortunately the author absolutely neglects the results of Troll's famous school.

The volume is excellently edited and the printing credits the publishers.

J. SZUJKÓ-LACZA (Budapest)

Ed. J. E. TREHERNE: **Insect neurobiology.**
Frontiers of biology. Vol. 35. North-Holland Publishing Co., Amsterdam—Oxford (1974), pp. 450, Dfl. 115.

Insect neurobiology is one of the most tremendously growing parts of biological sciences. Therefore, it is a difficult task to select the most important information from the enormous complexity of our present knowledge in this field. The monograph, written by leading experts, represents a successful solution of this problem. It provides a comprehensive treatise of data concerning the structure and function of the insect's nervous system in an accessible form.

The book consists of seven chapters. The first of them deals with the organization of the insect's nervous system including its central and peripheral parts and aspects of its development. In the following chapters the problems of axonal conduction and synaptic transmission in the central nervous system are covered. Separate chapters are devoted to the environment and function of insect nerve cells, to nerve-muscle transmission and to the neurosecretion. In the last chapter the neural basis of insect behaviour is analysed in detail.

All parts of the monograph are written at a high level. The volume is well illustrated, its technical presentation is excellent. The lists of references the make easy to survey the subject. It may be most useful for all biologists interested in neurology and entomology.

J. Kovács (Budapest)

Tiernahmen und Zoologische Fachwörter unter Berüksichtigung Allgemeinbiolögischer, Anatomischer und Physiologischer Termini (Animal names, zoological technical terms in relation to general biological, anatomical and physiological terminology)
VEB Gustav Fischer Verlag, Jena (1976), pp. 507, M. 19.

The volume is a dictionary of zoological definitions, containing over 1000 headwords, consisting of 6 chapters. The first part describes the origin of designaters. The first part describes the origin of designations used in zoology. The rules of forming, pronouncing and spelling of the most often used latin and greek expressions are discussed. The origin and rules of forming, terms based on names of authors are explained (for example: Gasser's ganglion — ganglion Gasseri). Finally the correct way of using these in german, their abbreviations and other designations are discussed.

The second is an encyclopaedical part, presenting terms of anatomy and physiology in ABC order. The headwords indicate the lingual origin of the term, the german translation is given and a short definition.

The third part is a german—latin vocabulary of zoological names, followed by an author index and a chapter displaying zoological taxonomy. The volume is completed by a short list of references.

The volume is easy to handle, it is a very well got-up edition, the definitions are very concise, presenting the reader the most essential information. The volume is recommended to german knowing zoologists, university students, editors of books and journals on zoology

J. Kovács (Budapest)

Moffat, D. B.: **The mammalian kidney**
Cambridge University Press, London—New York (1975), pp. 263, £ 9.]

The structure and function of the kidney had been described by many authors, but most of them analysed the questions of morphology and function, separately. Recently, because of the ever increasing amount of physiological and morphological data, writing a book, containing both aspects of the question, has becoma actual.

Prof. D. B. Moffat's book analyses at high level the above mentioned correlations of these features.

The first, introductory chapter summarizes the terminology as well as the testing methods, used in the analysis of the organ.

The next chapter contains a general morphological examination of the organ. The vascular system plays and extraordinary role in the function of the kidney. This one is described in the third chapter. The functions of the different parts of organ are briefly surveyed in the 4th chapter.

The forthcoming 4 chapters give a detailed light-scanning-transmission electron microscipic, as well as schematic description of the glomeruli, proximal tubuli, distal nephron and the juxtaglomerular apparatus. In close connection with the structure the author described the functions of the above mentioned parts.

Because of the great importance, the author paid attention to the question of the immature kidney.

In the final part of the work, the future of the different examining methods is given.

At the end of each chapter there is a good reference list. The cover part, the good typography and the photography praise the work of Cambridge University Press.

L. Kovács (Budapest)

ETZOLD, G.: **Aufgaben und Organisation eines metodischen Zentrums in der molekularbiologischen Forschung**
Akademie-Verlag, Berlin (1976), pp. 52, M. 6.

A summary of experience gained in the past few years and the principles embodied in the formation of a new research establishment are described in this booklet. The Methodological Centre for Molecular Biology in Berlin-Buch (GDR Academy) is the collection of advanced instrumentation and data processing hardware and software. The personnel of the Centre is specialized in the first line on applying and advancing particular methods and their contemporary evaluation. It certainly requires a strong and very high level scientific leadership to assign priorities to proposals coming from research people outside the Centre. However, it is even more obvious that the rapid increase in the need for sophisticated instrumentation in molecular biology requires appropriate changes in the organization of research work. A methodological centre appears to be potentially a very rational solution for a smaller national territory. Sharing the use of very expensive equipment and the best use of its capacity before it becomes obsolete is certainly achieved in such an institution. The author describes the background, the advantages and the pitfalls in establishing such a centre.

F. B. STRAUB (Szeged)

DOBZHANSKY, T., HECHT, M., STEERE, W. C.: **Evolutionary biology. Vol. 8**
Plenum Press, New York and London (1975), pp. 396, $. 29.40.

This is the 8th volume of the publication "Evolutionary Biology", a series which is based on the conviction that evolution represents a unifying principle in the life sciences. This last volume testifies to the above aim. There are five articles in the book. Genetic differentiation during the specialization process by F. J. AYALA. Protein variation in natural populations of animals by J. R. POWELL. Chemical basis of mutation by V. N. SOYFER. The classical case of character displacement by P. R. GRANT and Chromosome evolution in the caudate *amphibia* by A. MORESCALCHI.

Each of these papers are worth to read not only for specialists of the given field but also for those interested in general biology, both students and teachers. One obtains a better understanding of the process of evolution with the help of these studies which take into consideration results obtained by various methods — traditional as well as biochemical, cytological, etc.

The chapter about mutation is an up-to-date and comprehensive one, helpful information about the molecular events of genetic variability.

G. SZABÓ (Debrecen)

RIBE, M. A., ERAUT, M. R., SNOOK, R. K.: **Basic biology course. 7. Enzymes.**
ambridge University Press, London (1976), pp. 104, £ 2.75.

Similarly to the other volumes of this series this also serves the programmed teaching of biology. This volume is involved with intracellular regulation mechanisms. May an order of rank be made, then this is perhaps the best book of the series. An excellent chemical basis necessary for understanding the enzyme reactions is presented. The reader, the student is presented with many examples, thus the course of the whole mechanism is explained — from the historically interesting and not entirely dismissible Key-book theory to the most modern concepts (induced fit hypothesis; strain theory).

Many diagrams and curves demonstrate and explain the dynamism of enzyme function.

Reading this volume, even readers with poor knowledge of the subject will be able to understand the substance of enzyme function.

G. CSABA (Budapest)

TRIBE, M. H., ERAUT, M. R., SNOOK, R. K.: **Protein synthesys. BBC 9** £ 6
TRIBE, M. A., TALLAN, I., ERAUT, M. R., SNOOK, R. K.: **The ecology game. BBC Organism and envirounent.** £ 10
TRIBE, M. A., PEACOCK, D.: **Metabolism and mitochrondria. BBC, 8** £ 8½ **Cell membranes BBC 5** £ 5
Cambridge University Press, London (1976), pp. 142, 179, 103, 119.

Further four volumes of the basic biology course have been published. The 5th volume is involved with the cell membrane including its structure and function. The cell membrane is demonstrated both as a passive and an active factor, particularly in relation to the problem of phagocytosis and pinocytosis. The history of membrane models is also displayed, these are all, including the most modern model (Singer—Nicolson), demonstrated on both schematic diagrams and electron microscopic photos. The dynamics of membrane function are also discussed, diagrams and tables display the diffusion rate and penetration capability of the single substances. Endocytosis is demonstrated on film squares.

The 8th volume is involved with energy transformation and the role of mitochondria. The necessity of metabolism in living organisms, the basic components of this and the synthesis of molecules from these components are demonstrated. Respiration is discussed in most detail as photosynthesis has already been demonstrated in the 6th volume. Based on these, the regulation of metabolism and the role and structure of mitochondria are discussed in detail. The 9th volume is involved with synthesis of proteins. First the general structure of proteins are reviewed, the structure and function of DNA is also discussed. The genetic code is described in detail, further on, the translation and amine acid activation, the role of transfer RNA in the synthesis of proteins are demonstrated. Finally the operon hypothesis is discussed.

All volumes are completed with recording tapes, slides and films, an excellently documented question–answer system is an enormous help to the student in acquiring this vast amount of knowledge. The dictionaries at the end of the volume are also an aid to the student.

The Ecology game displays the mode of detecting an assumed environmental soiling. In this game test papers are distributed which are collectively solved by groups of students. These test papers contain information on the ecological system (alga, bacteria and invertebrates) whose biological balance has been disturbed, further on the quantitative changes of detergents, oil impurity, metal ions found in the single species etc.

This simulation of research work, as the authors state also themselves, does not substitute actual studies, however, from certain aspects this method represents a lot more. Naturally, this volume is also based on the 4th volume (Ecological principles) and it is assumed that students have already acquired the knowledge of this previous volume. The volume used for the Ecology game is a guide for both the tutor and the students. The game is a wonderful source for proving the importance of the ecological balance and for displaying how large the danger is of upsetting this balance, further on it is also a great aid for memorizing data.

G. CSABA (Budapest)

GRESHAM, G. A.: **Primate atherosclerosis**
Monographs on atherosclerosis. Vol. 7.
S. Karger, Basel—München—Paris—London—New York—Sidney (1976), pp. 101, figs 6, $ 19.0.

The author, who has many years of experience in the subject of atherosclerosis, discusses the problems of using primates in research work on atherosclerosis. This subject is discussed in a short volume consisting of only 95 pages including a list of references. The problem is discussed in a very clear and comprehensive way, rarely encountered in this field. The volume contains unusually few, only 6 illustrations, which also proves that this monograph is actually a rational review of present literature on this subject. Four chapters are involved with the whole problem. First the known data are shortly reviewed. This is followed by a discussion of the function of the normal blood vessel wall. In this part a few photos demonstrate the structure of the endothel. A striking point is that the author does not discuss — as many other authors do — the significance and role of the adventitia. The next chapter reviews the problems of using primates for this research work. Atherosclerosis has been found to occur spontaneously in these animals, making the evaluation of experiments rather difficult. This problem is even

more complex in relation to captive animals as these lesions occur more often in these compared to their free companions. Experimental results are presented in detail. Author discusses the dietetic experiments in most detail: the initial difficulties arising in the first part of these experiments are also described; when the evaluation of the found lesions was difficult because of the possibility of spontaneous occurrence of these.

Authors have proved the so-called fatty streak to be a pre-phenomenon of atherosclerosis. Further on, experiments have supported the possibility of other regressions, although these were not easy to induce in primates as in rabbits.

In agreement to the multifactorial principle experiments were performed in hypoxic conditions, and in a series of carbohydrate, vitamin and hormone experiments. These experiments clearly separated the spontaneous morphological findings from the experimentally induced ones. Author constantly stresses that when a morphological lesion is discovered, biochemical changes will already be present. The early stage of lesions, according to the author is probably due to some dietetic factor, however these early lesions always determine the site of cholesterin deposits. Therefore, he stresses the significance of all haemodynamic changes which may occur and also those occurring in juvenile vessel injuries occurring during the period of growth.

The effect of drugs on this course is also discussed. Three sites of action are discussed, however no great future is prophesed for any of these in the therapeutics of atherosclerosis. The drug reducing permeability may be of importance. The conclusion of the book is very surprising, as the author states that in the order of primates only in one genus: in man are the vessels of various organs affected by atherosclerosis, and only in man does thrombosis occur as a complication. In all other experimental animals only parts of these specifically human illness are reproducible. Even so all data acquired from various sources are extremely significant.

This volume will be of interest and use to both clinicians and pathologists.

H. JELLINEK (Budapest)

Symposium on the Muscle

Edited by E. Bíró and M. Garamvölgyi

(Symposia Biologica Hungarica 18.)

The "European Muscle Club" is an institution which functions without having been formally organized, and which once a year invites the European muscle researchers, biochemists, physiologists, etc. to assemble. The aspects of their symposium now are primarily the regulating role of calcium, and the mechanical features of the muscle. Two examples selected at random were the address by Professor Pringle of Oxford, who delivered and address on the mechanical characteristics of the flying muscle of insects, and the address by Professor Hamoir of Liège who held a lecture on the research developments in the field of various calciumbinding proteins, the parvalbumens. The 36 short lectures related to the main subjects are included either as unabridged lectures or as abstracts.

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Edited by J. Szegi

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von B. Buda

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THE EFFECT OF CYCLIC AMP ON THE MATURATION AND DEGRANULATION OF MAST CELLS

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(Received 1976—01—07)

Abstract

In short-term tissue cultures dibutyryl cAMP inhibits the cortisone-induced degranulation of mast cells. The effect of methylxanthine was found to be similar but developed at a slower rate.

The increase of cAMP level also affected the maturation of mast cells, as the ratio of cells of mixed granulation increased, compared to the alcian blue- and safranin-positive cells. This indicates inhibition of the maturation of mast cells, which in the present model inhibited degranulation.

Introduction

Since Sutherland's discovery [2] it has been recognized that cAMP plays a role as a second messenger in the cell by transmitting hormone-mediated information from the cell membrane. Furtheron, the cAMP mechanism is involved in secretion function of gland cells [6]. The mast cell is a specific one-celled endocrine gland [5] which secrets its products — the content of its granules — heparin and histamine, in mice and rats also serotonin, into its environment by degranulation. As the mast cells have proved to be suitable models for observing secretion activities, numerous authors [1, 6, 7] have applied biochemical methods for studying the histamine release of mast cells under the influence of cAMP and of substances regulating cAMP concentration in the cell. The histamine release by mast cells was found to be reduced by cAMP [1, 6, 7], although contradictory results have also been disclosed on the subject [9]. However, there are no morphological data at our disposal explaining how this decrease is involved in the degranulation process, and how cAMP is involved in the preceding process, of granule maturation. Our present work aimed at finding an answer.

Material and method

Peritoneal mast cells of adult, about 200 g in body weight, Wistar CB male and female rats were used. Five mg/100 g body weight/day cortisone [Adreson-Organon] was injected into 54 rats for 3 days. On the 4th day 10 ml MC solution (T. 199-solution enriched with the precursors of granule components) + calf serum (9:1) was injected i.p. into the rats under ether narcosis. After 5 min the fluid was withdrawn from the abdominal cavity and the fluids ob-

tained from several rats were pooled. The pool was pipetted into Bellco tubes containing glass plates. The cells were allowed to sediment for 2 h at 37 °C, hereafter the medium was changed. Three groups of tissue cultures were given different media as follows: (1) control nutrient medium; (2) a medium containing 5×10^{-4} *M* dibutyryl cAMP (Aldrich-Europe, Beerse, Belgium) and (3) nutrient medium containing 10^{-4} *M* 1-3-dimethylxanthine (Diaphyllin, Richter). 1, 3 and 24 h hereafter the plates were fixed in Carnoy fixative and were stained with the alcian blue-safranin technique. 200 mast cells per plate were counted and classified according to maturity: alcian blue-positive granulation; mixed granulation; safranin-positive granulation; degranulated. A hundred and seventy tube cultures were examined. The results were evaluated with Student's "t" test.

Results

After 1 h of cAMP treatment, the ratio of alcian blue-positive cells to safranin-positive cells remained unchanged compared to the control, however, the number of cells with mixed granulation was highly increased at the expense of the degranulated. Methylxanthine treatment resulted in a statistically not significant decline of safranin-positive cells in the population, and a significant increase in cells with mixed granulation.

Three hours after the onset of the treatment, the ratio of cells with mixed granulation was still elevated in both the cAMP-treated and the methylxanthine-treated group, and degranulated cells were correspondingly fewer, compared to the control. No changes were observed in any of the other cell types.

Twenty-four hours after the onset of treatment, a significant change was hardly observable. Only methylxanthine caused a significant increase in the number of degranulated cells.

Results are shown in Table 1.

Table 1

Percentage distribution of peritoneal mast cells of rats treated with cortisone in short-term cultures

Additional treatment		Alcian blue+	Mixed	Safranin+	Degranulated
Control	1 h	9.75	9.85	37.13	43.34
cAMP	1 h	9.67	49.15	36.8	4.25
Methylxanthine	1 h	8.81	15.61	25.42	51.33
Control	3 h	14.39	6.32	42.93	36.33
cAMP	3 h	11.71	33.85***	48.04	3.37*
Methylxanthine	3 h	10.59	42.66***	40.0	6.83
Control	24 h	5.57	1.11	39.68	53.94
cAMP	24 h	9.92	3.75	38.01	48.98
Methylxanthine	24 h	4.55	1.37	25.71	68.38**

* $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$.

Discussion

In earlier experiments in which cAMP-induced histamine release from mast cells was studied by biochemical methods, either 48/80 or allergic (anaphylactic) reactions were used for inducing degranulation. In our own experiments [3, 4, 5] massive cortisone treatment resulted in degranulation of mast cells and stimulated the rate of their maturation. Therefore, we chose cortisone degranulation as a model for experiments: cortisone was administered *in vivo* and the effects on cortisone-degranulation of cAMP and of the cPDE (cyclophosphodiesterase enzyme) inhibitor methylxanthine were observed *in vitro*. The precursor content of the medium supported the maturation of the mast cells in culture. The dibutyryl derivative of cAMP was used because this, unlike purified cAMP, penetrates cells and thus increases the intracellular cAMP content [2].

Our experiments unambiguously support the results of previous biochemical studies [1, 2, 7], *viz.*, that dibutyryl cAMP treatment results in an increase of cAMP, which in turn reduces the degranulation of mast cells. Although the changes after one hour treatment were statistically not significant, it is clear that the number of degranulated cells dropped to one tenth. The results of the 3-h cAMP treatment were significant. The cPDE-inhibiting effect of methylxanthine was less clear. One hour after the onset of treatment it resulted in an increased degranulation, and only after 3 h of treatment was a reducing effect observed, and even this was not significant. At 24 h, when the cAMP effect was already equalized, *viz.*, the cultures treated with cAMP had reached the control level, methylxanthine induced a statistically significant increase in degranulation. Naturally, this does not contradict the assumption that the effect of methylxanthine, *i.e.*, of the indirect increase of the cAMP level, is essentially identical to the effect of exogeneous cAMP. It develops somewhat slower and, perhaps, less consistently. Goth and Johnson [6] have already drawn attention to the uncertainty of the effect.

The maturation of mast cells proceeds as follows: young cells are alcian blue-positive, these develop into cells of mixed granulation and, finally, into safranin-positive mature cells [5]. Cortisone treatment accelerates this course and, although cortisone itself does not induce degranulation (this has been proved by our microcinematographic analysis [4], it sensitizes the cell to all degranulating factors, *viz.*, mechanical, chemical, *etc.*, ones. According to our previous experiments [4], in the first place safranin-positive cells degranulate, thus, in cortisone-treated animals the degree of degranulation is higher, as due to the more rapid maturation the younger cell forms are already safranin-positive and are thus ready to degranulate. The 1 h and 3 h control results of our present study are in agreement with previous [3, 4, 5] observations.

Thus, the rise of cAMP level [8] inhibits degranulation by preventing

the maturation of cells of mixed granulation into safranin-positive cells. In this relation our experiments were unambiguous, the results regarding cAMP and methylxanthine were significant, although a certain delay was experienced in the case of methylxanthine.

We found lymphoid precursors of mast cells among the cells of the peritoneal fluid [3], and the medium contained all the substances necessary for granule formation. Thus, any influence of cAMP on mast cell formation would change the ratio of alcian blue-positive cells. However, such a change did not occur, indicating that cAMP is not involved in mast cell formation. It is involved in the maturation of mast cells causing retardation of this process. It seems furthermore probable that in short-term experiments for example when mature mast cells are examined in anaphylactic models [7], histamine release may be affected in some other way too.

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COMPARATIVE LIGHT AND ELECTRON MICROSCOPICAL STUDIES ON THE ARGYROPHILIC STRUCTURES OF *EUGLENA VIRIDIS*

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(Received 1976—09—13)

Abstract

(1) A method for the electron microscopical investigation of Algae is described. (2) The silverlines of *Euglena viridis* are oriented in the same helical manner as the pellicular stripes and are linked by very fine argyrophilic structures. Accordingly, the silverlines are continuous over the whole cell, even in its anterior and posterior parts, where the silverlines and pellicular stripes are reduced in number. (3) Electron micrographs of silvered *Euglena viridis* clearly show that the silverlines are located in the posterior part of the pellicular stripes, just underneath the plasmalemma. At the same site, material of fibro-granular appearance is present in specimens which have been prepared in the conventional way. This kind of silver deposition could be observed in dry- and wet-silvered *Euglena viridis* alike. (4) The argyrosomes are not identical with the muciferous bodies. In ultrathin sections, they appear as subpellicular vacuoles with electron-dense content. (5) The electron micrographs of the pyrenoids show a strictly localized silver deposition at the lamellae of the chloroplasts which penetrate the pyrenoids. (6) In the anterior and posterior regions of *Euglena viridis*, there are more microtubuli below the pellicular stripes than in the central region of the cell. This is interpreted as an effect of the reduced number of pellicular stripes in these regions. The shape of the pellicular stripes is correlated with the state of movement of the cells. (7) The argyrophilic structures were not affected by colchicine, but in many specimens they were partially or completely destroyed by cytochalasin B. It is therefore suggested that the silverlines of *Euglena* are cytochalasin sensitive filaments. Rupture of the pellicle by mechanical pressure showed similar results: in many specimens, the silverlines appeared broken into pieces or transformed into a fine network. (8) The structure and function of the silverline system of euglenoid flagellates is discussed. It is supposed that the silverlines are filaments which may have a neuroid function.

Introduction

It was two years after the discovery of the silverline system in ciliates by KLEIN [29] that JIROVEC [25] succeeded in demonstrating, by the dry silvering method, silverlines in certain euglenophyceans. The argyrophilic lines regularly followed the pellicular stripes. The line system staining with opal blue proved to be identical with that demonstrable with silver impregnation [27], comp. also [9]. Soon thereafter, JIROVEC's [25] findings were confirmed by KLEIN [30], who discovered a continuous silverline network in *Gonium* sp. and *Volvox* sp. This finding, however, has not been confirmed by others since then. JIROVEC [25, 27] and KLEIN [30, 32] considered the sylver-

line systems of flagellates to be a fibrillar pellicular structure conveying impulse. However, they could not exclude a supporting function. KLEIN [30] and DEFLANDRE [12] called attention to the fact that the silverline system of euglenophyceans is covered by a rigid pellicle, therefore, a supporting function cannot be its primary task.

HALL [20] and JIROVEC [27], the first authors who used the wet silvering method of CHATTON and LWOFF [8] in studying euglenophyceans, emphasized that by this procedure other cell organelles may also be impregnated, therefore, the pictures should be cautiously interpreted. CHADEFAUD [4] has mentioned that in some cases the silverlines appear not as fibrils but as impregnated pellicular stripes. KÜSTER [33] extended this statement to all euglenophyceans. He is convinced that the impregnated pellicular stripes share nothing with the silverlines described in *Gonium* sp. and *Volvox* sp. Later CHADEFAUD and ARLET [6] refused the fibrillar nature of the silverlines and noted true fissures in the pellicle, a view which has been taken over by HOLLANDE [22] and by PRINGSHEIM [40]. The silverlines must be built up of rows of linearly arranged argyrophilic granules which are more or less connected with one another. Furthermore, in *Peranema trichophorum*, the argyrophilic substance must lie in the fissures of the pellicle [5]. CHADEFAUD [5] has suggested that, in fact, the silverlines might not be pellicle elements. CHADEFAUD and ARLET [6] were the first to recognize that the pellicular stripes are not argyrophilic, they only contain argyrophilic granules called argyrosomes by them.

Recently POCHMANN [39] has demonstrated a gradually variable impregnability of the silverlines in *Phacus pyrum* and *Lepocinclis* sp. and emphasized the fragility of the argyrophilic lines. He raised the question whether there are in the area of gyri mucous substances, cytoplasm fragments or enzymes which, being reductants, might bring about artefacts. More recently DE HALLER [21] has suggested on the basis of electron microscope studies that the cisterns of the endoplasmic reticulum beneath the pellicular stripes are responsible for building up the silverlines.

This brief historical review shows that we have little information about the structure, location and function of the silverlines. At least part of the problems have been solved in the present study.

Material and methods

Masses of *Euglena viridis* (determined according to [28, 40, 21] were found in a sewage highly contaminated by kitchen sewage near Linz. For light and electron microscopy, cells were taken with a spatula from the surface of the sample and poured with the fixative into a centrifuge tube.

(1) *Light microscope preparations.* Argyrophilic structures were looked for by the use of a dry [14] and a wet [11] silvering method.

(2) *Electron microscope preparations.*

a) Three-minute fixation according to CHAMPY (prescriptions, *v.* [11, 17]). The fixative was removed by washing for 60 min with twice changed Da Fano mixture [11, 17]. The preparations were dehydrated in an alcohol series and in propylene oxide (30 min) and embedded in EPON 812.

b) Wet-silvered *Euglena viridis* was carried up to 50% alcohol in an alcohol series, then removed by a razor blade from the slide, together with the gelatine layer. Then, dehydration in alcohol and propylene oxide (30 min) was continued. The silvered specimens in gelatine layer were embedded in EPON 812.

c) After silver reduction, dry-silvered *Euglena viridis* cells were overlaid by distilled water for 5 min. The cells, on the top of the protein layer, were separated from the slide with a razor blade, dehydrated in an alcohol serie transferred into propylene oxide (for 30 min) and embedded in EPON 812.

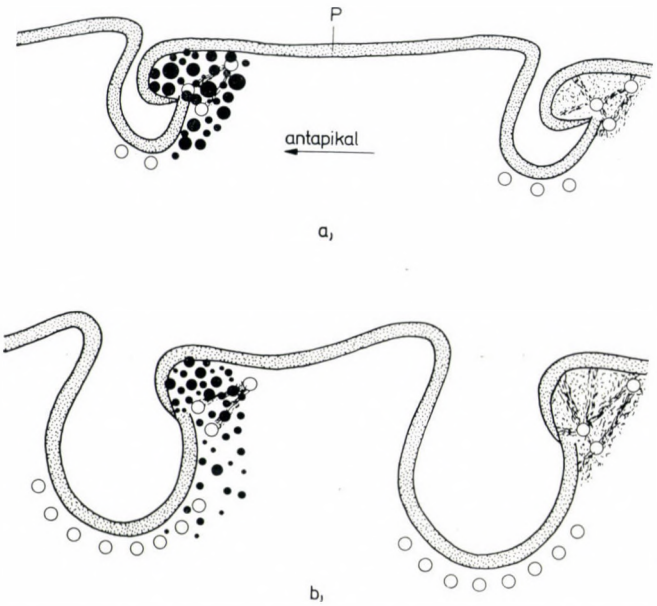


Fig. 1a, b. Location of the silverlines and the pellicular microtubuli in the middle, apical and antapical regions of *Euglena viridis.* A schematic drawing. The silverlines lie in the posterior (antapical) part of the pellicular stripes, tightly beneath the three-layered plasmalemma (P).

All electron microscope preparation procedures except silvering were carried out in a centrifuge tube. In preparing thin sections we used a diamant knife in a Reichert OMU-2 microtome. The thin sections were transferred to carbonized pioloform-filmed slides. The preparations were counterstained in an ethanolic (50%) uranyl acetate solution and Reynold's lead citrate [41] for 15 min each. A Zeiss EM 9S apparatus was available for electron microscopy.

(3) *Experimental methods*

a) Treatment with aqueous colchicine solution (5%) for 10 min or 2 h. After treatment the cells were dry-silvered.

b) Treatment with 50 μg/ml cytochalasin B solution for 10 min, 1 h or 2 h. For this purpose, cytochalasin B was dissolved in DMSO, 1 mg in 0.5 ml, and diluted with distilled water up to 5 ml.

c) Mechanical destruction of cells by pressure. One drop containing many *Euglena viridis* cells were pressed between the slide and a coverslip until the majority of the cells had burst. Then the preparations were dry-silvered at intervals of 10 sec, 2 min and 10 min.

Results

Light microscopic studies

Figs 2 and 3 show the silverline system in a partially and a fully contracted *Euglena viridis*. The silverlines run in a spiral form and, like the pellicular stripes, are separated from each other by a distance of 1 μm. In relaxed cell regions, the silverlines are somewhat farther from each other than in contracted cell areas; the silverlines are bound together by transversally running silverlines at irregular distances (Fig. 2, 3 arrows). The transversal lines show very weak argyrophilia and are only seen in the best preparations. KLEIN [30] described similar silverlines in *Euglena* sp.

In the centre of the cells, there are argyrophilic lines, some 40—50 in number. Owing to the fusion of silverlines, this number is reduced to its half (comp. [25, 34]) at the apex and antapex, where uninterrupted, Y-like fusioning figures are seen (Figs 2 and 3).

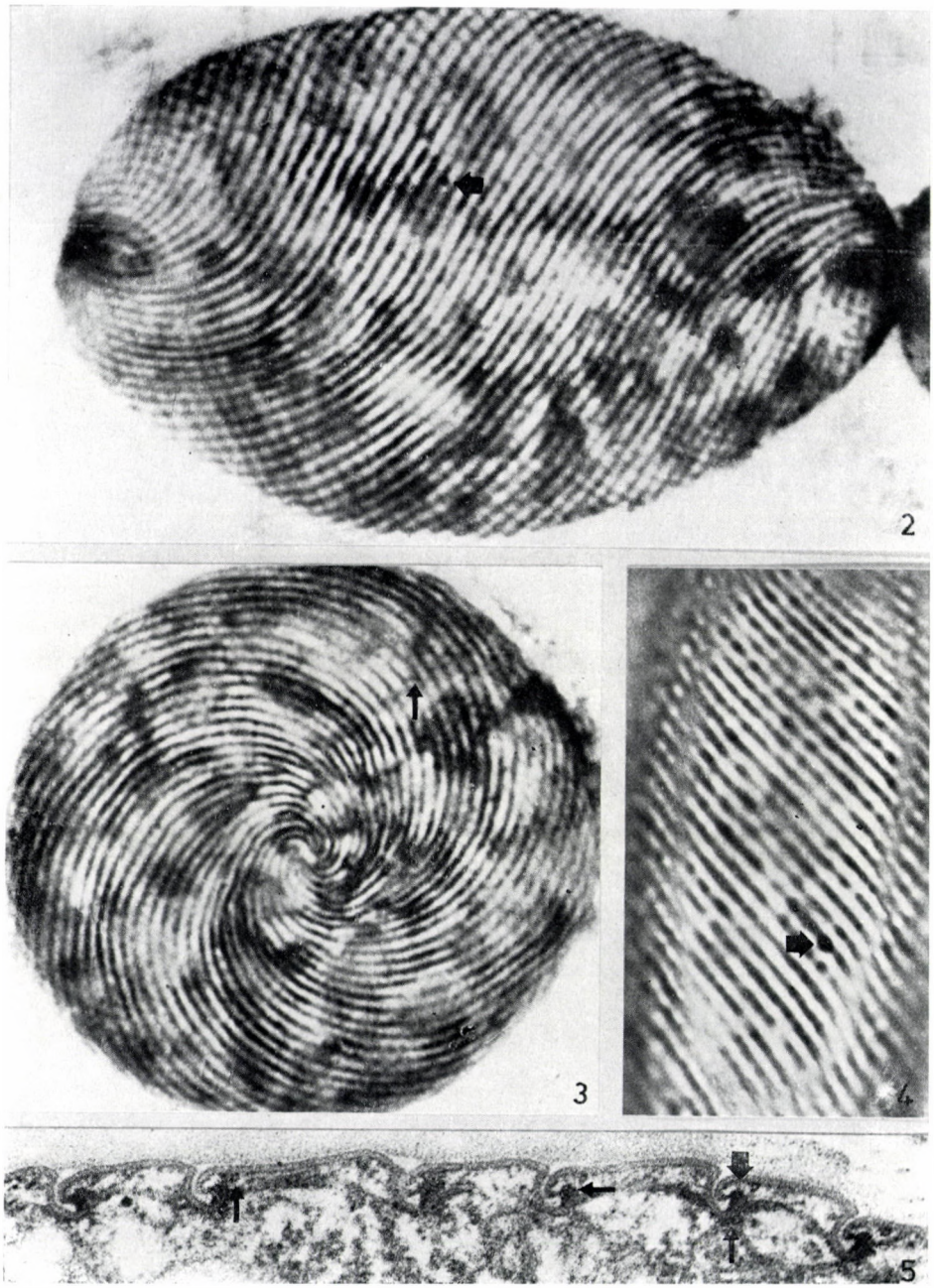
The dry-silvered silverlines appear uneven in structure. They are quite fine in some places and thick and coarsely granulated elsewhere. Sometimes they lose continuity (Figs 2 and 3). The argyrophilic substance is often drop-like (comp. [30]). This irregularly variable picture is certainly due to artefacts. Presumably, the uneven impregnability led KLEIN [30] to supposing the existence of a two-component silverline system in euglenophyceans. However, JIROVEC [25] as well as myself failed to observe such a structure. Furthermore, unlike CHADEFAUD and ARLET [6], we have never seen silverlines built up of tightly packed granules. There is no doubt that the silverlines are continuous in structure (Figs 2 and 3) as also shown in the electron micrographs. The existence of broken silverlines as claimed by POCHMANN [39] is unlikely because the silverlines follow the cell in all of its movements without showing breakage (Figs 2 and 3). The argyrosomes of *Euglena viridis* are of little size and occur sparsely. They lie as a rule tightly beside the silverlines (Fig. 2).

Fig. 2. The silverline system of a partially contracted *Euglena viridis*. The spirally running silverlines, which turn down at the edge of the cell, appear distinctly thick and are reduced in number by fusion in the antapical region (right side). Arrow points to an argyrosome. Dry silver impregnation. ×2000

Fig. 3. Antapical view of a fully contracted *Euglena viridis*. The numerical reduction of silverlines ensues as a result of further fusions of two silverlines each time. The silverlines are connected by very fine argyrophilic lines with each other (arrow). Dry silver impregnation. ×2000

Fig. 4. Part of the silverline system of a non-contracted *Euglena viridis*. The irregular thickness of the silverlines is especially emphasized by this method of preparation. Arrow points to an argyrosome. Wet silver impregnation. ×2000

Fig. 5. Cross-section of the pellicle in the middle region of *Euglena viridis*. The pellicular stripes are variable in height, width and shape and are strictly correlated with the state of cell movement. The microtubuli (arrows) running tightly beneath the three-layered pellicle are partially recognizable. Note the fibrogranular substance between the three outer microtubuli and the pellicle (thick arrow, comp. *Fig. 1a*) Method *a*, ×56,000



Wet-silvered preparations showed essentially the same picture (comp. Figs 2 and 3 with Fig. 4 and see ref. [27]), except for the fine transversal silverlines found between the spirally running silverlines. These could not be observed. Furthermore, silverlines dissolved in small granules and discontinuity of them occurred more frequently by this impregnation. The argyrosomes, on the other hand, appeared more pronounced in wet-silvered preparations (Fig. 4, arrow). Weak argyrophilia often occurred around the paramylon granules as well.

Electron microscope studies

E. viridis as prepared according to method a. In preparations fixed by CHAMPY's method, the ultrastructure remained as clear as after simple OsO_4 fixation. Even the pellicular microtubules were distinctly visible.

The fine structure of the pellicular stripes essentially corresponds to that of other *Euglena* species [3, 35, 36, 37]. As described by DE HALLER [21], they are limited towards the environment by a triple membrane the middle of which appears less contrasted. This middle zone disappears in the area that is overlapped by stripes (Figs 1*a*, *b*, 5), and it was not clear whether the outer zones of this membrane, too, end here or, whether they bind the single stripes together as it is generally supposed. There lies more medially in the stripes a thin fibrogranular zone transversally to the length of stripes. Supposedly [34], these are formed by the teeth. Tightly nearby, or sometimes deeper, many, more or less spherical vacuoles are seen, which may correspond to channels of the endoplasmic reticulum [1, 18, 43] or, more probably, muciferous bodies [34] (Figs 12, 15 M). The vacuoles are limited by a single membrane and appear empty.

The sections from the apical and antapical areas have proved that the numerical reduction of pellicular stripes, in accordance with LEEDALE's suggestion [34], is due to fusion of stripes (Fig. 7). The observation that the silverlines in the area of fusioning stripes run without being interrupted ac-

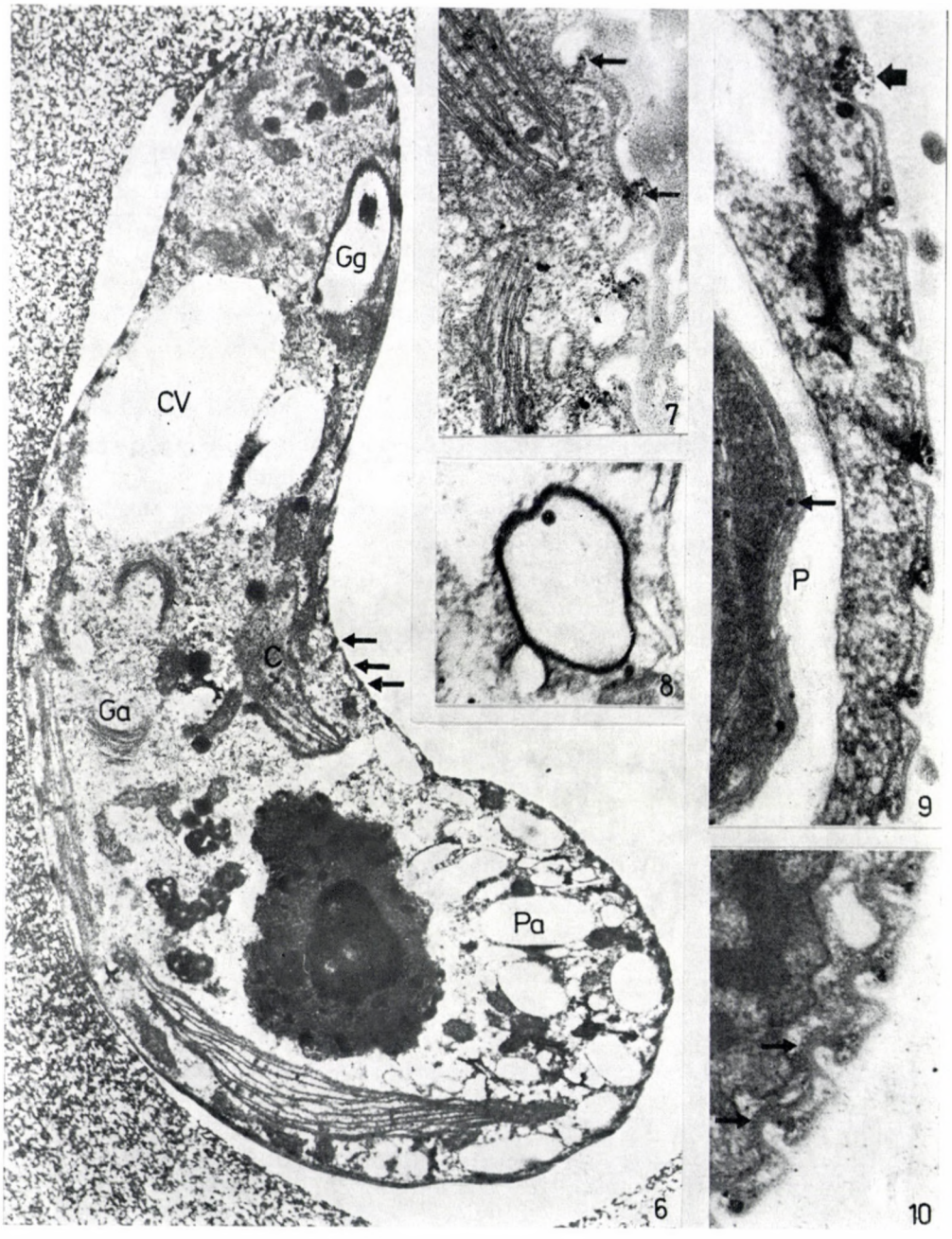
Fig. 6. Longitudinal section of a silvered *Euglena viridis*. The silverlines run in the posterior part of the pellicular stripes. The nucleus with a large and many small nucleoli, paramylon grains (Pa), chloroplasts (C), the Golgi apparatus, the ciliary cavity (Gg) and the contractile vacuole (CV) are to be recognized. Method *b*, ×9000

Fig. 7. Cross-section of the pellicle in the antapical fusion zone of pellicular stripes and silverlines. The silverlines (arrow) at pellicular stripes in fusion have not fused yet. Method *b*, ×48,000

Fig. 8. A very fine silver precipitate is visible on the membrane enclosing paramylon grains. Method *b*, ×48,000

Fig. 9. This cross-section clearly shows that the silverlines (thick arrow) lie in the posterior part of the pellicular stripes, in the zone of microtubuli. Note the sporadic silver aggregates on the chloroplast lamellae of the pyrenoids (P). Method *b*, ×48,000

Fig. 10. Cross-section in the apical area of *Euglena viridis*. Microtubuli have markedly increased in number. The silverlines lie beneath the pellicle. Method *b*, ×48,000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cords well with this suggestion. However, all these are inconsistent with the data published by GUTTMAN and ZIEGLER [19], who believe that the apparent reduction in the number of pellicular stripes is due to simple growing down by crossing stripes.

An analysis of many cells being in different states of moving has shown that the height, the width, and the shape of the pellicular stripes must be correlated with the state of cell movement. The stripes in weakly contracted or non-contracted regions are wide and flat (about 450×100 nm) and only slightly depressed at the middle (Figs 5, 9), whereas those in contracted cells are depressed and higher (about 300×300 nm) and more narrowed at the middle (Figs 10, 11). Pellicular stripes, even those lying in closely adjacent regions, on the other hand, are often very different in height, width and shape (Figs 5, 12). This suggests that individual stripes can change their shape independently. These observations clearly show that when studying the function mechanism of metabolic movements we should taken into account the configurational changes of the pellicular stripes as already suggested by GUTTMAN and ZIEGLER [19], who investigated configurational changes by raster electron microscopy.

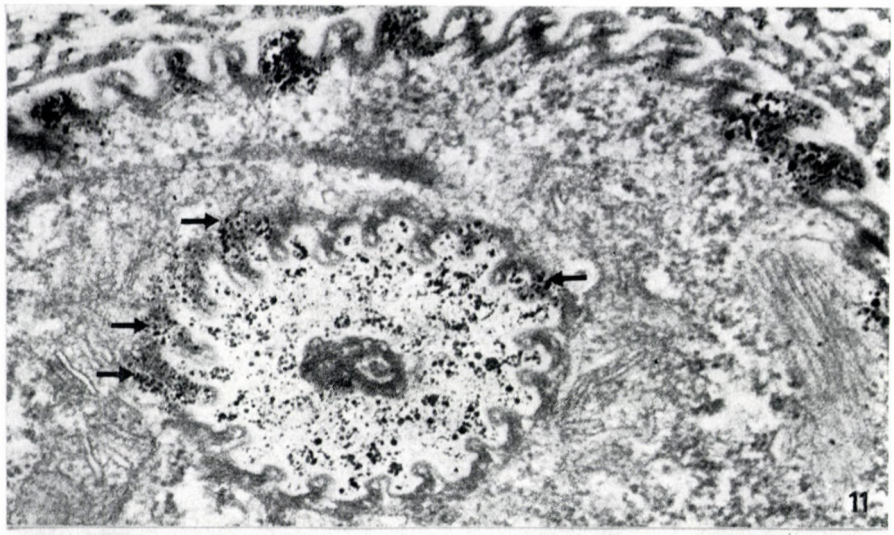
The pellicular microtubuli, except those in the area of the channel, had been described as evenly arranged, *e.g.*, [3, 35, 36, 37]. Surprisingly, I have found that the microtubuli in different cell regions are differently arranged. Figs 1*a* and 5 show the arrangement of microtubuli in the central region, where no fusion of pellicular stripes occurs. A very similar arrangement was described as typical by DE HALLER [21] in *Euglena viridis* and by MIGNOT [36] and ARNOTT and WALNE [1] in *Euglena gracilis*. Around the microtubuli, there lies a more or less contrastable fibrogranular substance. Especially the three outer microtubuli are bound together with one another and with the pellicle by well-defined bridges (Figs 1*a*, *b*, 5; comp. [36]). Figs 1*b* and 10 show cross sections in the apical and antapical regions of a cell. It is clear that unlike the 2 or 3 microtubuli in the central part of the cell (Fig. 5, arrows) 8 or 9 are seen in these regions (Fig. 10, arrows). I attribute the increased number of microtubuli beneath the pellicular stripes to that here the pellicular stripes are reduced

Fig. 11. Cross-section in the channel region. Owing to the irregular silver impregnation, only few pellicular stripes appear impregnated. The channel is filled by gelatine in which many small silver aggregates are embedded. Nevertheless, silverlines at some pellicular stripes have been impregnated even here (see, *e.g.*, arrows). Method *b*, $\times 48{,}000$

Fig. 12. This picture impressively shows cross-sectioned silverlines, muciferous bodies (M) and an argyrosome (A). A pellicular stripe carries small silver aggregates on both sides. Method *b*, $\times 56{,}000$

Fig. 13. Numerous silver aggregates are seen only in the centre, at the large argyrosome (A) lying tightly beneath the pellicle. Note the droplet-like structures (arrow) with very fine-granulated silver precipitates outside the pellicle. It may correspond to muciferous bodies. Method *b*, $\times 44{,}000$

Fig. 14. Cross-section through a dry-silvered *Euglena viridis*. Silver aggregates occur only beneath the pellicle. Method *c*, $\times 44{,}000$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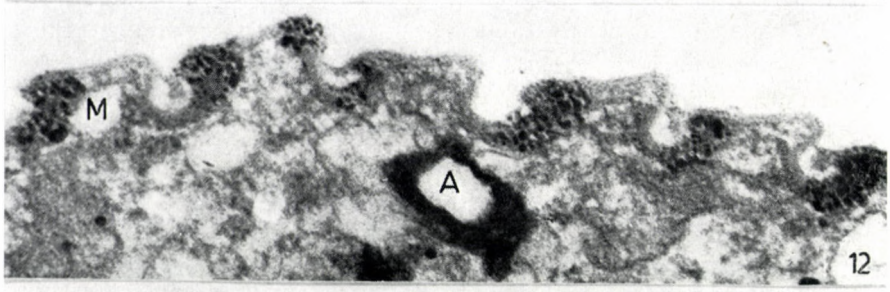
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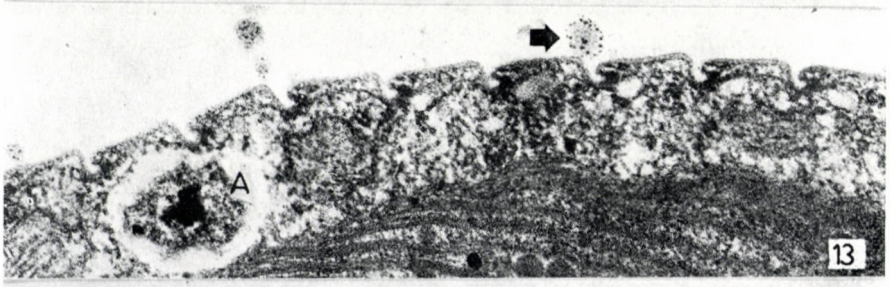
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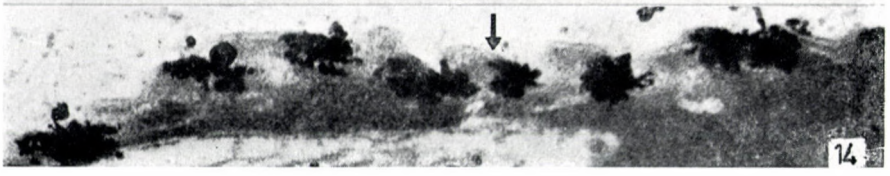
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in number, whereas it is only farther that the microtubuli are reduced by fusion.

E. viridis prepared according to method b. The structural state of the samples is markedly affected during preparation. Consequently, a great number of cells should be examined to establish the location of the argyrophilic structures.

(a) *Silverlines.* As shown in Figs 7, 9, 10, 12, 13 and 15 the silverlines lie tightly beneath the plasmalemma, *viz.*, in the area of microtubuli where the fibrogranular substance is to be found. In serial sections, the silverlines appear as continuous structures consisting of round silver aggregates 5—50 nm in diameter. In exactly cross-sectioned pellicular stripes, they are more or less distinctly spherical, 80—140 nm in diameter, and indistinctly limited towards the cytoplasm. The zone of silver deposition extends in general from the outer apex of the pellicular stripe to the microtubuli situated between two stripes each (Figs 6, 9, 10, 12, 15). The same is seen in obliquely (Fig. 15) and longitudinally sectioned stripes. In the apical and antapical regions, where the pellicular stripes are markedly tapering, the whole stripes may be filled by silver aggregates (Fig. 11). This may be attributed to an insufficiently fine impregnation. Silverlines were demonstrated in the region of channel (Fig. 11), they probable end in the basal bodies of cilia. Very rarely, we found pellicular stripes which produced silver deposition on both sides (Figs 12, 14, arrow). We cannot decide whether these deposits corresponded to real silverlines or to accidental silver depositions. The second alternative seems to be more probable because in light microscope preparations of *Euglena viridis* the distance between silverlines proved to be very stable. It is clearly seen in the Figures that among the numerous pellicular stripes possessing silverlines several stripes may occur which do not elicit silver aggregates (Figs 9, 11, 13). This phenomenon may be attributed to an incorrect silver impregnation, not to lacking silverlines.

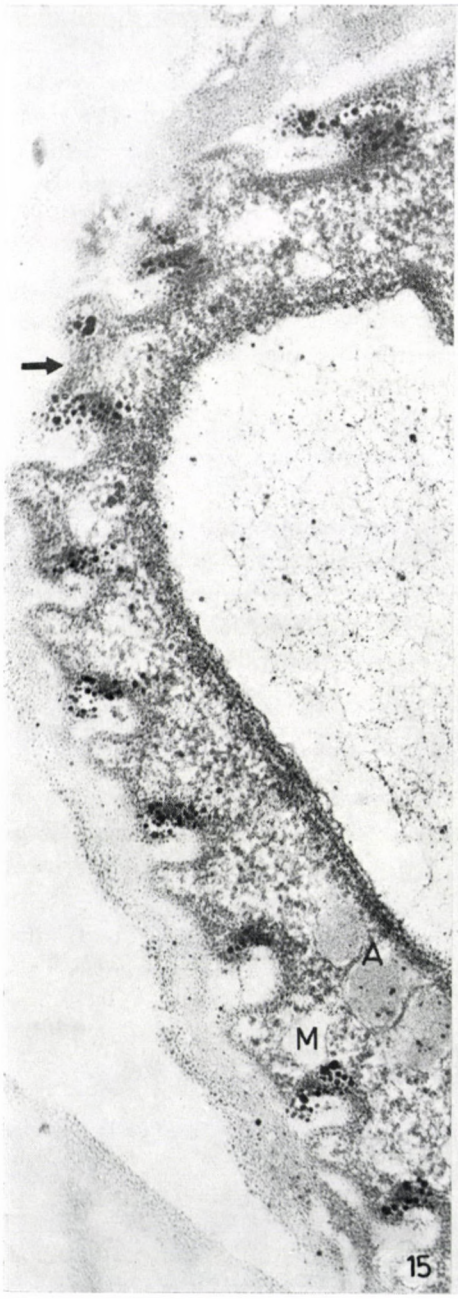
(b) *The argyrosomes.* The argyrosomes of *Euglena granulata* were identified by ARNOTT and WALNE [1] on the basis of comparative light and electron microscopic investigations. The argyrosomes in this species are pores opening externally; they contain a highly osmophilic core. However, these authors

Fig. 15. Section through the apical region. Obliquely and cross-sectioned silverlines and muciferous bodies (M). Note the silver deposition on both ends of a pellicular stripe (arrow). The eye spot (A) shows no argyrophilia. Method *b*, ×53,600

Fig. 16. Part of the silverline system of an *Euglena viridis* after one hour treatment with colchicine. No changes in the silverlines. The chloroplasts, appearing as dark spots, are highly argyrophilic. Dry silver impregnation. ×3000

Fig. 17. Part of the silverline system of an *Euglena viridis* treated for 1 h with cytochalasin B. Granular dissociation of the silverlines. Dry silver impregnation. ×3000

Fig. 18. Part of the silverline system of an *Euglena viridis* treated with cytochalasin B for 2 h. The silverlines are fully destroyed. Only irregularly distributed coarse-granular silver aggregates are seen. Dry silver impregnation. ×3000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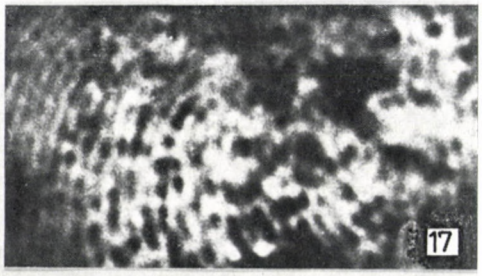
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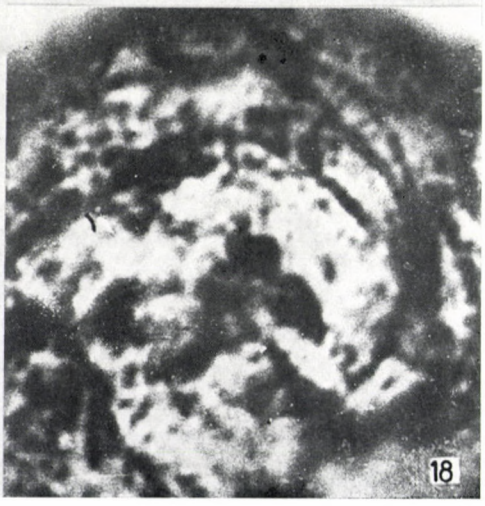
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did not examine silvered specimens, therefore, could not localize the site of silver deposition.

The argyrosomes of the *Euglena viridis* strain examined by us are few in number and less distinct than those of *Euglena granulata* and *Peranema trichphorum*. Supposedly, Figs 12 and 13 (A) show cross-sections through argyrosomes. Irregular spherical bodies are seen with distinctly denser silver deposition. They lie at the middle of two pellicular stripes tightly beneath the pellicle. No connection with the pellicle could be demonstrated. The size and the location of these argyrophilic bodies suggest that they are not identical with the muciferous bodies. DE HALLER [21] has described subpellicular vacuoles with electron-dense core, of the same appearance, in *Euglena viridis*. I suppose that muciferous bodies may also be slightly argyrophilic, for sometimes many small droplets with fine silver deposition occurred (Fig. 13, arrow) in the immediate vicinity of the pellicle. These droplets may represent muciferous bodies.

(c) *The pyrenoids and the paramylon granules.* In the pyrenoids, where light-microscopically no argyrophilia was seen, very fine, strictly localized silver deposition was demonstrated (Fig. 9, arrow). Silver aggregates occurred between the broad stroma bundles lying most frequently on the chloroplast lamellae, outside the pyrenoid.

Sometimes there was another argyrophilic zone demonstrable around the paramylon granules. The silver was deposited on the outer side of the membrane surrounding the paramylon grains (Fig. 8).

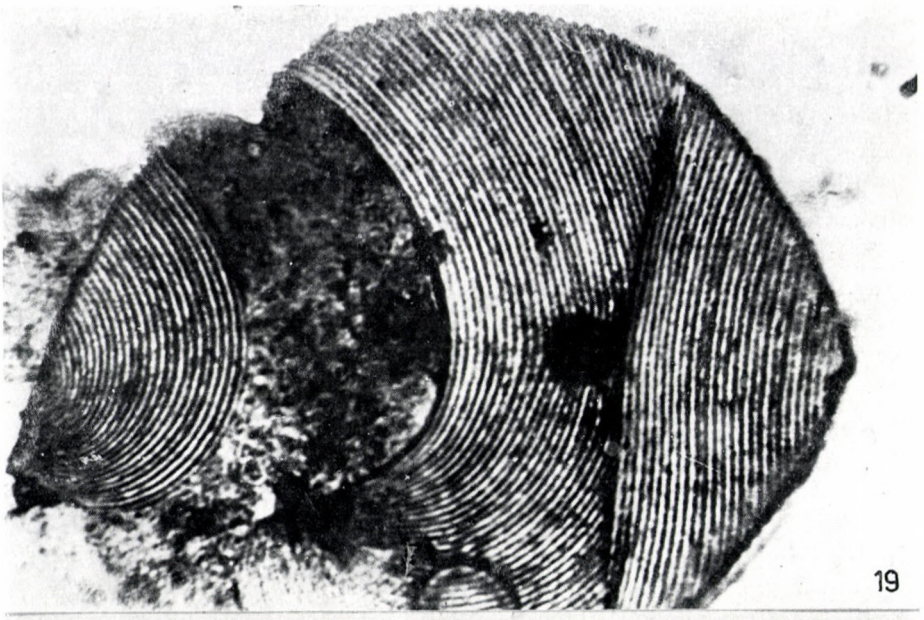
(d) *Cell organelles without specific argyrophilia.* There was no specific argyrophilia at any other cell organelle (*e.g.*, nucleus, chloroplasts, Golgi apparatus, stigma) (Figs 6, 15). The silver aggregates in these organelles were very small and irregularly arranged.

(e) *Euglena viridis prepared according to method c.* The same results were obtained as with method *b*, except that the state of the specimens prepared with method *c* was much worse. Fig. 14 clearly shows the silverlines immediately beneath the plasmalemma. It is therefore evident that the same structures are impregnated by both methods.

Fig. 19. Dry silver impregnation of an *Euglena viridis* 5 sec after squatching. No changes in silverlines of the disrupted cell. ×2000

Fig. 20. Dry silver impregnation of an *Euglena viridis* 30 sec after squatching. The silverlines have been partially disrupted into pieces (see, *e.g.*, arrow). ×2000

Fig. 21. Dry silver impregnated *Euglena viridis* 10 sec after squatching. The cell has been disrupted and at the site of splitting (arrow) the silverlines have disintegrated to form a very fine meshed network. ×3000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Experimental studies

Effect of colchicine. Colchicine affected neither the structure nor the movements of *Euglena viridis.* Only chloroplasts with an increased argyrophilia were frequently seen (Fig. 16).

Effect of cytochalasin B. After two-hour treatment, cyto-chalasin B caused in silverlines a partial destruction in 50%, and total destruction in 5%, of the specimens. The specimens with destroyed silverlines ceased euglenoid moving. The first changes, *viz.*, granular disintegration of silverlines in many specimens appeared one hour after cytochalasin B had been added (Fig. 17). Later the silverlines disappeared, leaving behind an irregularly distributed silver precipitate (Fig. 18). In about 50% of the specimens, the silverlines remained apparently intact by the end of the second hour of cytochalasin treatment, suggesting the existence of a distinct individuality in cytochalasin sensitivity.

Effect of mechanical pressure. Owing to pressure, the pellicle bursts and the cell contents flow out. When cells were silvered immediately after burst, the argyrophilic substance appeared unchanged in the majority of the specimens (Fig. 19). Latticed disintegration (Fig. 21) or breaking into pieces (Fig. 20) of silverlines at the site of disruption was seen in some 10% of the disrupted specimens. If the preparations were silvered 5 to 10 min after burst, only rudiments of silverlines were observed, owing to postmortal changes. The silverline system in the surviving *Euglena viridis* specimens appeared normal.

Discussion

Location, structure and function of the silverline system in euglenophyceans

The present investigations have made a number of the hypotheses mentioned in the introduction clear. It is now obvious that the silverline system in euglenoid flagellates is identical neither with pellicular splits [5, 6, 22, 40] nor with the pellicular stripes [4, 33]. Mucous substances [39] and the canaliculi of the endoplasmic reticulum [18, 21] can also be excluded. Only the hypotheses of JIROVEC [25] and KLEIN [30], postulating a fibrillar nature of silverlines and the POCHMANN's [39] generally formulated hypothesis suggesting that the impregnable structure corresponds to cytoplasmic elements or enzymes, have remained to be discussed. A strict definition of silverlines as pellicular structures should first of all exclude their being cytoplasmic elements. However, it cannot be excluded that enzymes are impregnated even though there is no information as to their nature and it is unlikely that such

a stable and regular arrangement exists without any morphological basis. SOMMER and BLUM [43] succeeded in inducing pellicular phosphatase activity near the silverlines, but the impregnable substance cannot correspond to the phosphatase, latter being located outside the plasmalemma.

The present results fully agree with JIROVEC's [25] and KLEIN's [30] view, *i.e.*, with the fibrillar nature of the silverlines of euglenophyceans. This hypothesis is supported by the following observations: (*i*) fibrogranular substance has been demonstrated in the place of the silver deposition (Fig. 5, comp. [36]); (*ii*) cytochalasin B does, colchicine does not, destroy the argyrophilic lines. SILVERMAN and HIKIDA [42] have shown that the euglenoid movement and the pellicular microtubuli are influenced by colchicine but very slightly, whereas cytochalasin B is known to distroy microfilaments first of all [10]; (*iii*) on mechanical destruction of the cells, at least part of the specimens showed a reticular disintegration of silverlines, a phenomenon typical of cytochalasin B-sensitive filaments [24]; (*iv*) the structurally and morphologically very similar silverline system of ciliates (*v.* below) is a fibrillar structure of the cortex [13, 15, 16, 17].

No essentially new information has been obtained as regards the function of the silverline system. It can nevertheless be excluded that it is a supporting [27, 30] or elastic structure [5]. Such differentiations would be clearly visible by conventional electron microscopy. As already emphasized by KLEIN [31] and JIROVEC [27], the close connection of the silverlines with the basal bodies as well as their morphological arrangement is suggestive of a neuroid function. POCHMANN [39], on the other hand, believes that euglenophyceans, having no cilia, do not need an impulse-conveying system. Similarly, JAHN and BOVEE [23] consider the existence in euglenophyceans of a "nervous system" very unlikely. However, unequivocal observations have proved that the euglenoid movement is strictly controlled by environmental factors [38] and the euglenophyceans are able to control their metabolic activities [23]. We believe that the question put by JAHN and BOVEE [23], *viz.* "where such a coordinative, initiative system morphologically resides or is morphologically distributed, and how it operates is only theoretical and still a mystery", might be answered by supposing that the silverline system is this co-ordinative system. There is no doubt that, owing to its morphology and location (Figs 2, 10, 12), the silverline system is predestined for a co-ordinating function. The fact that, perhaps through the destruction of the co-ordinating silverlines, cytochalasin B caused an inhibition of the euglenoid movement points to the same direction.

Comparison of the silverline system of euglenophyceans with those of ciliates

Silverline systems have been discovered, besides ciliates and euglenophyceans, in all the other groups of *Protista, viz.*, in *Pyrosomida* and *Trypanosoma* by JIROVEC [26], in *Dinoflagellata* by BIECHELER [2] and CHATTON [7] and in *Heliozoa, Amoeba, Sporozoa* and *Oscillatoria* by KLEIN [31] and FOISSNER [14]. The silverline systems are very similar to each other in their light-microscopic structure, though, they are variable in shape.

As to the silverline systems of euglenophyceans and ciliates, the differences are very slight. Among the many common features the following deserve mentioning: (*i*) deposition tightly below the pellicle; (*ii*) more or less fibrillar character; (*iii*) similar size; (*iv*) connection with locomotor organelles; (*v*) silverlines form a continuum in the cell.

POCHMANN [39] disagrees with the similarity between ciliate and euglenophycean silverline systems because of differences in dividing processes and lack of multiplication by copulation. However, his arguments are not convincing because *(a)* there are no observations on the division of the silverline system in euglenophyceans available, *(b)* a negative marker like lack of copulation cannot be accepted as proof.

*

Acknowledgements

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MONOAMINE LEVEL AND PERIODIC ACTIVITY IN 6-HYDROXYDOPAMINE TREATED MUSSELS *ANODONTA CYGNEA* L.

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Abstract

In the central nervous system of the mussel (Anodonta cygnea) 6-hydroxydopamine (6-OHDA) causes a significant and prolonged decrease in the dopamine (DA) and noradrenaline (NA) concentration. The decrease of serotonin (5HT) level did not exceed 25 per cent and was observed only on the 2nd and 3rd day after the treatment. Parallel with the alteration of the monoamine level, there is a marked change in the activity of the animals. Two phases of the effect of 6-OHDA can be distinguished. During the first phase, not only the catecholaminergic but, presumably, also the serotoninergic system is injured. The long-lasting effect of the 6-OHDA administration is reflected in the predominance of the active periods and in the absence of rest periods of the animal.

Introduction

The behaviour of the fresh-water mussel *Anodonta cygnea* is characterized by a distinct periodicity involving regular alteration of active and rest phase of the adductor muscles and other organs [17, 23]. The rest period is maintained by the prolonged tonic contraction of the adductors, whereas during active period the adductors are relaxed and perform fast rhythmic contractions.

Earlier investigations showed that serotonin plays a mediator role in the relaxation of some molluscan "catch" muscles like the adductor muscle of *Anodonta* [21, 25] and the retractor muscle of *Mytilus* [27]. The presence of dopamine and noradrenaline in the nervous tissue of *Anodonta* was also demonstrated [10]. In accordance with the activity and rest, serotonin and catecholamines change in an opposite way in the CNS of *Anodonta* [25], which is an analogy of the circadian variation of serotonin and noradrenaline levels described in the CNS of vertebrate animals [6, 15, 18, 19, 20].

In vertebrates the administration of 6-OHDA produce depletion of catecholamines and selective degeneration of adrenergic neurons [3, 4, 12]. This property of 6-OHDA opens a new tool in the examination of behaviour, suggested to be controlled by catecholaminergic neuronal system. In the present study, we treated animals with 6-OHDA to obtain further information about the correlation between the periodic activity and the catecholamines in the CNS.

Methods

Experiments were carried out on fresh-water mussels *(Anodonta cygnea* L.*)* weighing approx. 200 g. 6-OHDA was dissolved freshly in physiological saline [16] containing 0.5 mg/ml ascorbic acid. The first group animals received 25 mg/kg 6-OHDA hydrochloride. The second group received 10 mg/kg 6-OHDA on the first day and 7.5 mg/kg on each of the third and fifth days. The 6-OHDA was injected in 1 ml solution into the foot muscle, while control animals received ascorbic acid in physiological solution. For the monoamine measurements, animals were killed from the 2nd up to the 47th day, both of the control and the treated group. The monoamines were estimated in the united cerebral, visceral and pedal ganglia in five experiments and the changes of concentration measured after 6-OHDA treatment are expressed in per cent of the control. 5HT level was measured according to the method of SNYDER et al. [26], while DA and NA according to ANTON and SAYRE [1, 2].

The activity of the animals was recorded by mussel actograph [22]. Measuring the duration of the active and rest periods before and after treatment, we expressed the effect of the ascorbic acid and 6-OHDA on the activity of the animals by the mean values of time duration as described earlier [8]. The experiments were carried out from November to February.

Results

Effect of single doses of 6-OHDA on the monoamine level

During the experimental period, the mean 5HT, DA and NA concentrations in the CNS of *Anodonta* were 39.6 μg/g, 27.6 μg/g and 2.6 μg/g, respectively. Following 25 mg/kg 6-OHDA treatment, 25 per cent decrease of the 5HT level of the ganglia was detected on the second and third days but it was restored on the 4th day and remained at the control level during the investigated period (Fig. 1). A significant decrease in the DA level started on the 2nd day and reached a maximum, 64 per cent, value on the 10th day. By the 36th day the DA level remained still at 50 per cent as compared to the control. The NA level decreased to a somewhat lesser extent: a marked decrease ensued on the 2nd day, its maximum reached 36 per cent and it did not restore within 36 days (Fig. 1).

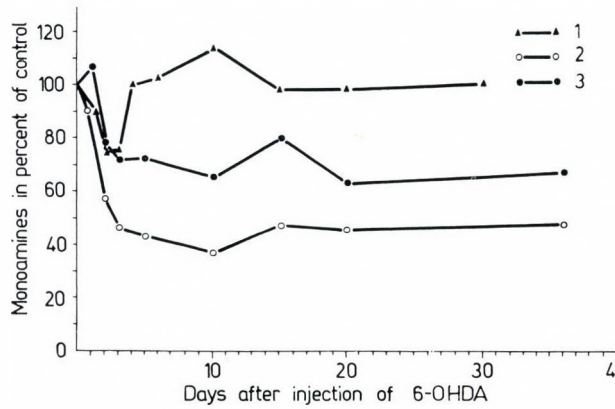

Fig. 1. Effect of 25 mg/kg 6-OHDA on the serotonin (1), dopamine (2) and noradrenaline (3) level of the CNS of *Anodonta*

Effect of repeated 6-OHDA administration on the monoamine level

The 5HT level was not influenced by a single 10 mg/kg dose of 6-OHDA, but after repeated application (7.5 mg/kg) a 20 per cent decrease was observed on the 2nd day. The extent of the decrease was not enhanced by the third dose (7.5 mg/kg); on the contrary, the 5HT level reached the control value on the 5th day and remained at this level with a fluctuation of ±10 per cent during the observation period (Fig. 2). The DA level decreased to 50 per cent following

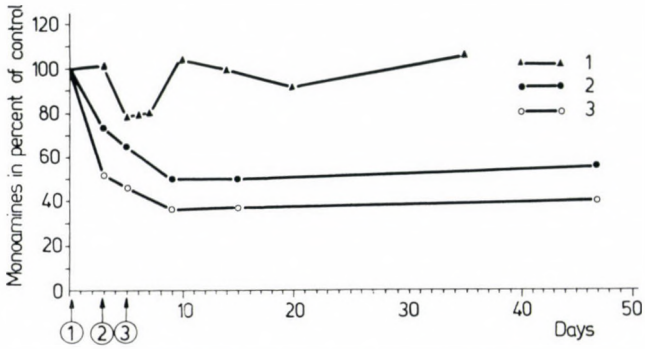

Fig. 2. Effect of repeated 6-OHDA administration on the serotonin (1), noradrenaline (2) and dopamine (3) level of the CNS of *Anodonta*. 1, 2 and 3 arrow marks 10, 7.5 and 7.5 mg/kg 6-OHDA, respectively

the first dose. Further 10 per cent decrease was detected as a result of the 2nd and 3rd doses and the 60 per cent decrease of the DA level remained unchanged up to the end of the experimental period (Fig. 2). The NA level decreased similarly to the DA level, but to a lesser degree; the maximum decrease was about 50 per cent. The control level was not reached during the experimental period, there was still a decrease of 44 per cent on the 47th day (Fig. 2).

Effect of 6-OHDA on the activity

The rhythmic and periodic activities of the animals were markedly influenced by a single dose of 6-OHDA (25 mg/kg) as well as by repeated treatment. Following an injection of 6-OHDA, an active period of long duration appeared in every case. The duration of the first active period was longer by 280 per cent then the duration of active periods before the administration (Table 1). During this first active period, the number of the fast, rhythmic contractions increased as compared with the activity registered before treatment (Fig. 3). In contrast to this, the duration of the first rest period decreased after 6-OHDA treatment. The duration of the 2nd and 3rd active periods did not differ from the values obtained before the treatment, while the duration of

Table 1

Mean (± S.E.M.) duration of active and rest periods in hours

	6-OHDA treatment (25 mg/ kg) n = 15		Control injection n = 10	
	active	rest	active	rest
Before treatment	22.7±4.2	6.3±1.9	20.9±5.1	6.2±1.1
After the treatment				
1st period	64.8±1.1*	5.4±1.0**	19.6±2.1	6.2±1.6
2nd period	26.2±7.2	5.6±0.9**		
3rd period	24.8±5.8	6.1±0.6	19.1±5.1	7.0±1.8
4th—15th periods	21.1±5.0	6.6±0.8		

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

the 4th—15th active periods showed a small, non-significant, decrease as compared with the control. The duration of the 2nd rest period was still shorter than the mean of the rest periods before treatment, but the duration of the 3rd and further periods were similar to the values obtained before 6-OHDA administration. As compared with the pretreatment values, the number of the fast rhythmic contractions did not show any change during the second and further active periods.

In the case of repeated 6-OHDA administration a lasting active period appeared again after the second dose, but the fast, rhythmic contractions were fewer in number and the time of the relaxation following these contractions became longer (Fig. 4c). After the third dose, the activity was characterized by the appearance of long-lasting active periods, by the moderate increase of the number of the fast, rhythmic contractions and by a considerable prolonged relaxation time following the fast contractions (Fig. 4d).

During the 47-day-long period of observation, the activity was characterized by an alteration of 2—3 day-long active periods and a few hours short resting ones. The rhythmic contractions, characteristic of the active period, were hardly visible (Fig. 4e). Starting from the second or third week, the lasting tonic contraction of the adductors characteristic of the control during the rest period did not ensue, instead, the muscle remained in a semi-relaxed state without the appearance of rhythmic contractions.

In control animals, the administration of 5HT in the water evokes the immediate appearance of a 3—5 days long active period with markedly increased fast, rhythmic contractions (Fig. 4f). When the animal was treated with 6-OHDA repeatedly, the effect of 5HT occurred only 3—4 h after adding the 5HT. The evoked active period was of 3—4 h duration and only a moderately increased frequency of weak rhythmic contractions was observed (Fig. 4g).

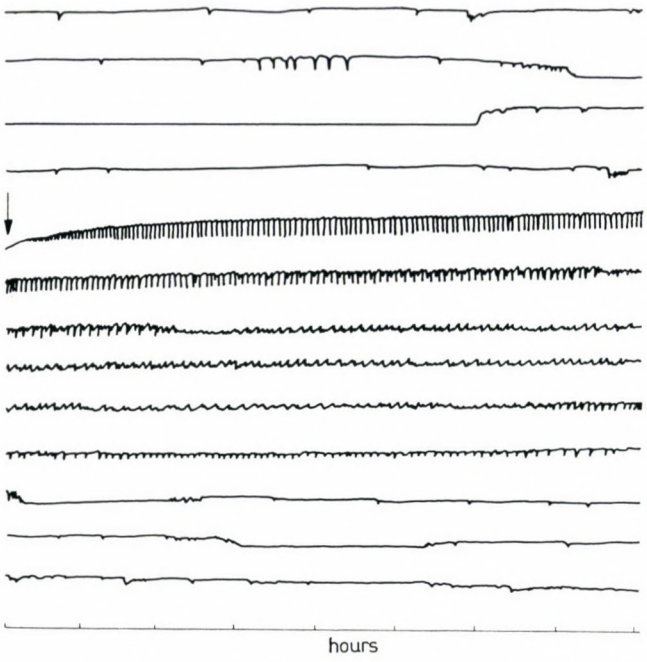

Fig. 3. Effect of 25 mg/kg 6-OHDA on the activity of the animal. Record is continuous, the drug was given at the arrow. At upward movements of the recording lever the shells opened. Straight line at low position marks the rest period

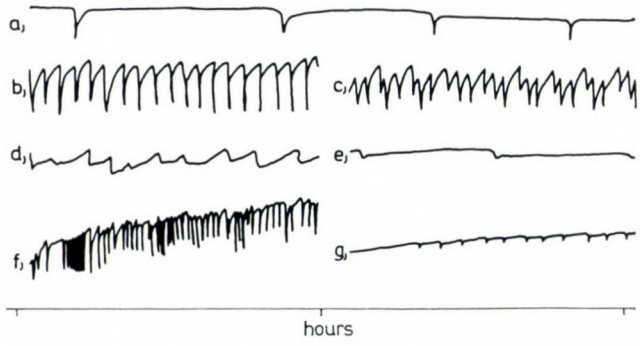

Fig. 4. Effect of repeated 6-OHDA administration on the activity of the animal at different times. *(a)* Control active periods; *(b)* active period 2 h after 10 mg/kg 6-OHDA injection; *(c)* active period after 6-OHDA (7.5 mg/kg) injection; *(d)* active period after 3rd 6-OHDA (7.5 mg/kg) injection; *(e)* rhythmic contractions during an active period on the 35th days after repeated injections of 6-OHDA; *(f)* effect of 10 μmol/l 5HT on the activity of a control animal; *(g)* Effect of 10 μmol/l 5HT on the 36th day after the repeated injections of 6-OHDA

Discussion

It has been unequivocally proved by our results that the monoamine level of the ganglia and the activity of the animals are markedly influenced by 6-OHDA administration. Two phases of the effect can be distinguished in the activity: an early one, very similar to the effect of exogenous 5HT [21, 25], and a long-lasting one, observed after 6-OHDA administration, which does not resemble the effect of any already investigated pharmacon affecting the monoamine level [8]. Following 6-OHDA treatment [5, 7, 28], a significant and lasting decrease, similar to that observed in vertebrates, occurred in the DA and NA levels of the nervous tissue of the fresh-water mussel. The decrease of the 5HT level occurred only at the beginning of the treatment. The duration of the long active period (64.8 h) evoked by the treatment coincides with the decrease of the 5HT level of the ganglia. This suggests that the applied concentration of 6-OHDA might mobilize 5HT at the ganglionic or neuromuscular level and, as a consequence, the long-lasting active period as well as the increase in the frequency of rhythmic contraction ensued.

It should be noted that under physiological circumstances at the beginning of the active period the 5HT level of ganglia declines, whilst it rises in the muscle [24]. It was also reported that the relaxation of molluscan muscles can be significantly increased by exogenously administered 5HT [14] and that the 5HT is responsible for the relaxation of the muscle contracted tonically [27]. Since the 5HT is synthetized only in the nervous system, and not in the muscle [9], the increased 5HT of the adductor muscle can take its origin from the ganglia during the active period by an active transport.

According to investigations performed on vertebrates, the selective neurotoxicity of 6-OHDA might be realized on catecholaminergic neurons [3, 4, 11], but for larger doses 5HT depleting effects have also been described [13]. In the fresh-water mussel, the extent of 5HT liberation was not higher than the fluctuation observed under physiological conditions, being connected with the periodicity of the activity [24]. This alteration is analogous to that observed in the rat brain following 6-OHDA treatment, where, in addition to the decrease of the catecholamine level, the 5HT synthesis and the diurnal fluctuation of the 5HT level are also influenced [7].

Earlier pharmacological investigations showed that the prolongation of active periods in mussels is caused by the predominance of the serotoninergic system, while the appearance of resting periods is a consequence of the predominance of the catecholaminergic system [8, 25]. The effect observed after repeated administrations of 6-OHDA, especially absence of the rest periods, when low DA and NA levels were measured, are obviously due to the damage of the catecholaminergic neurons. The active periods occurring at this time differ from the active periods observed at the beginning of the effect of 6-OHDA

in the absence of frequent, fast, rhythmic contractions. In connection with the first phase of 6-OHDA effect, it can be supposed that not only the catecholaminergic, but also the serotoninergic, neurons are directly affected, while during the late effect probably not only the CNS, but also the neuromuscular junctions in the adductor muscles are damaged. The absence of the tonic contraction and the weak muscle responses at 5HT administration refer to the latter possibility.

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CHANGES IN CHROMOSOME COMPLEMENT IN LONG-TERM PEA CALLUS CULTURES

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Abstract

A prolonged callus culture from pea *(Pisum sativum* L. var. *Kiir)* cotyledons subcultured for 7.5 years on Torrey's solid medium was examined cytologically. The initially (and up to 3.5-year cultivation) predominantly diploid pea callus strain changed into triploid. The frequency of aberrant ana- and telophases increased during 5 year cultivation from 9 to 40 per cent and thereafter returned to the initial rate maintaining it at least for one year of subculturing. Some possible mechanisms of chromosomal variation tendencies *in vitro* are discussed.

Introduction

The karyologic variability of plant cells and tissues during *in vitro* cultivation is well-known. Both heteroploidy and chromosome aberrations in callus cultures of different species have been reported [3, 4, 8, 10, 12, 14, 15, 16, 22, 24, 26, 31]. It has been shown that even the callus clones of single-cell origin cultured on the same medium and under the same conditions are unstable in chromosome number [5, 13].

However, the surveys considering the possible regularities or trends in chromosomal changes of callus cultures are rather contradictory. While some authors are of the opinion that there is no regularity in variation of chromosome complements under culture conditions, others refer to the presence of certain tendencies in chromosomal variation during prolonged cultivation. Thus, some investigators have reported a trend to polyploidy [13, 20, 23, 24], others to haploidy [12, 15]. Initially karyologically unstable callus cultures have subsequently obtained a stabilized ploidy level state [11, 19, 22, 24], while initially stable callus strains have lost stability in the course of long-term culturing [4, 27]. Besides, a fluctuation of ploidy level in prolonged callus culture has been observed [7, 15].

The inconsistency in the results of cytological observations is partly explicable by the diversity of nutrient media and/or methods of subculturing, partly by the diversity of the investigated objects. Nevertheless, we believe that too much emphasis has been given to irregular deviations in nuclear

behaviour of excised plant cells, and there have been few investigations on the changes in the chromosome complement during continuous subculturing under stable conditions.

The present study was undertaken as an attempt to get more information about the chromosome complement behaviour of plant cells in a long-term culture.

Materials and method

The callus tissue under investigation was derived from pea *(Pisum sativum* L. *var. Kiir)* cotyledons and subcultured up to 8 years on Torry's solid medium supplemented with 2.4-D 1 mg/l, adenine 0.25 mg/l, casein hydrolysate 0.5 g/l, vitamins by White, and sucrose 20 g/l.

The material for cytological observations was taken from 15, 30, 45, 60 and 90-day-old subcultures of six different passages and fixed in a 3:1 alcohol-acetic acid solution. Squash preparations were made by the standard acetic orcein method.

Results

The investigated pea callus strain belonged to the normal type of undifferentiated callus tissues, exhibited a low growth rate (with transfers of 2 to 3 months intervals, and mitotic index $<2\%$), and had a nodular structure (Fig. 1).

To study the chromosome complement dynamics of a prolonged callus culture we examined the ploidy level and the aberration frequency of the pea callus strain during 8-year subculturing.

The results of our observations on the chromosome number changes are summarized in Fig. 2. The histogram represents $2\times$ and $3\times$ cells frequency exclusively as far as the number of metaphases with any other ploidy level ($\times$, $4\times$, etc.) did not exceed 9 per cent of the total number of the counted metaphases. As it was difficult to make completely accurate chromosome counts the aneuploid cells were accounted to the cells with the closest basic ploidy level. The basic ploidy level of each of the six passages under investigation (the age of which varied from 0.5 to 7.5 years) was deduced from karyotypic analyses of 100 to 200 metaphases in 15, 30, 45, 60 and 90-day-old subcultures. Thus, we recorded a total of 400 to 1000 metaphases to determine the mean value of ploidy level for each culture passage of the corresponding age.

Figure 2 shows a definite change in the basic ploidy level of the pea callus cells during the 7.5 years of culturing. The callus strain characterized up to 3.5 year cultivation as a predominantly diploid tissue ($2\times$) became subsequently a triploid one. The triploid (and near to $3\times$ aneuploid) metaphase cells made up about 80 percent of all observed metaphase cells in the 7.5-year-old pea callus culture. Figure 3 shows some typical metaphases with $2\times$ and $3\times$ chromosome complements.

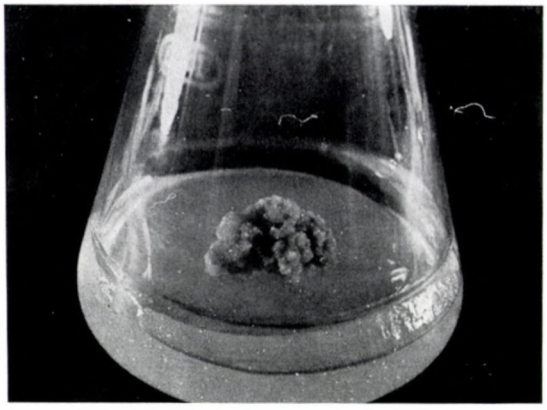

Fig. 1. The nodular pea callus

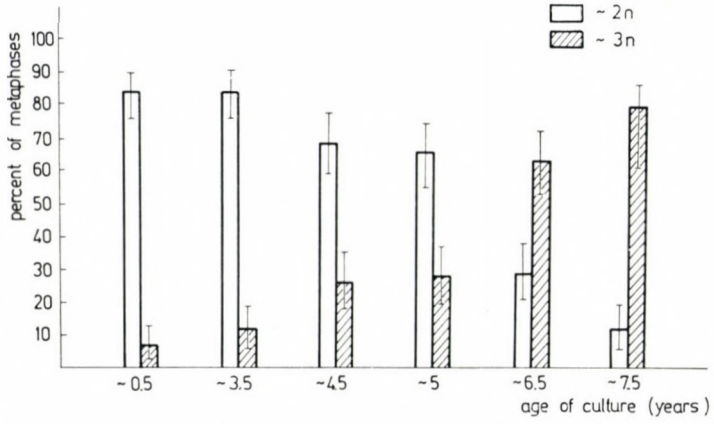

Fig. 2. Changes in basic ploidy level of pea callus cells during prolonged cultivation

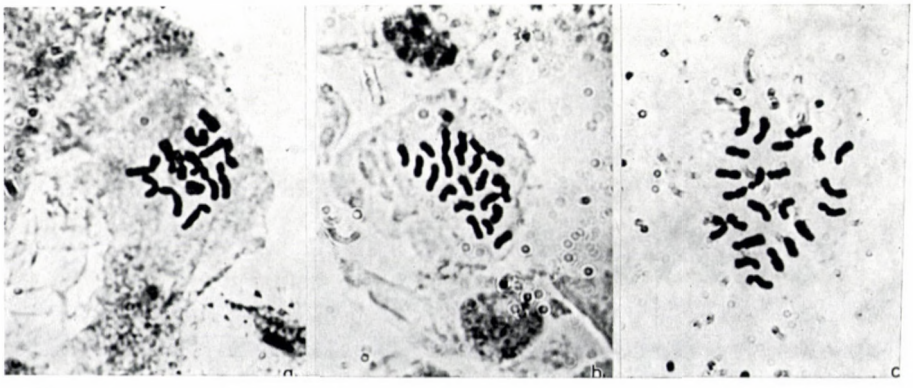

Fig. 3. Karyotypes of pea callus cells. *a* = Normal diploid cell; *b* = triploid cell; *c* = aneuploid cell. ×1570

The rate of chromosome aberrations was estimated by counting the number of aberrant ana- and telophase figures on which bridges or fragments were seen (Fig. 4). To characterize the dynamics of chromosome aberration rate during prolonged cultivation we determined the percentage of aberrant ana- and telophases in six different culture passages at various ages over the 7.5 year period. Similarly to the determination of the basic ploidy level, the rate of aberrant ana- and telophases of each passage was determined by count-

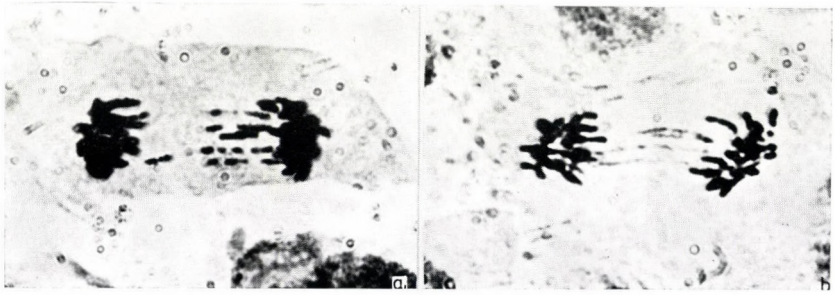

Fig. 4. Aberrant anaphase and telophase in pea callus cells. ×1570

ing these phases in 15, 30, 45, 60 and 90-day-old subcultures, per 100 ana- and telophases each time. The results are presented in Fig. 5.

Up to 5-year cultivation, the chromosome aberration rate of the callus strain exhibited a tendency to increase: the frequency of aberrant ana- and telophases increased from 9 per cent to 40 per cent, subsequently, it returned to the initial level, and remained there at least for a year.

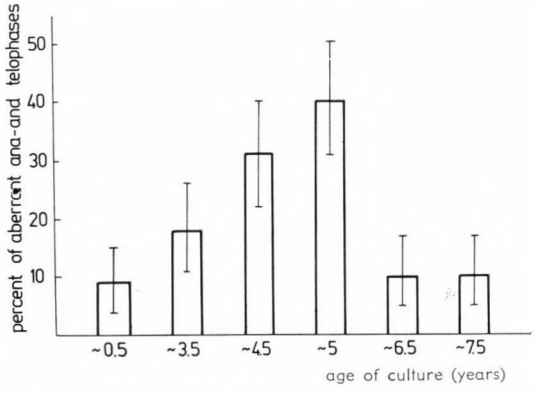

Fig. 5. Changes in chromosome aberration frequency of pea callus cells during prolonged cultivation

Discussion

We have shown that there may be some definite trends in the karyologic variation of a prolonged callus culture even in the case of such a polysomatic plant species as *Pisum sativum*. Regular changes characterized both the ploidy level and the chromosome aberration rate of the pea callus strain. It is noteworthy that the changes in the karyologic variation trends became evident only after a long period (4 to 5 years) of subculturing. A tendency to triploidy did not appear during the first 3.5 to 4 years of subculturing. On the other hand, the same callus culture obtained a stabilized rate of chromosome aberrations only after cca 5 years. This aberration rate (cca 10 per cent) coincides not only with that of the first culture passages but also with the spontaneous aberration rate in the intact root tips of the investigated pea variety. Therefore it may be considered as an actually stable state.

We are far from the conclusion that always and in all conditions the prolonged callus cultures subsequently obtain a definite stable state of ploidy level and/or aberration rate. It may only be suggested that the karyologic variability of callus cultures is less irregular than usually considered and in certain cases some tendencies or trends may occur during a long-term cultivation. Besides, stabilization as well as instabilization of the chromosomal complement of cultured tissues as well as changes in the established tendencies may take place.

As to the possible mechanisms and causes of chromosomal variation tendencies *in vitro* there are many different opinions. It has been postulated that the nuclear behaviour of plant cells *in vitro* is largely a reflection of their potentialities *in vivo* [6, 13, 17, 18, 26]. Therefore, the presence of chromosomal variability in polysomatic species (pea, tobacco *e.a.*) contributes to the corresponding deviations in tissue cultures of these species and, *vice versa*, plant species characterized by a stable chromosome content of its tissues *(C. capillaris, Paeonia, Helianthus)* maintain such a stability also *in vitro*. However, there are notable exceptions. *In vivo* polysomaty is not a prerequisite for it to occur *in vitro*. Heteroploidy of callus cultures may be due to endoreduplication, endomitosis or various mitotic disturbances directly stimulated by culture conditions [1, 2, 7, 10, 21, 22, 23, 26]. Imperfections and irregularities in the processes of chromosomal reproduction and division provide the basis for the karyotypic variability of cell and tissue cultures. It has been suggested that the hormones, primarily exogenous auxins, in the nutrient medium, affect the nuclear behaviour in tissue cultures [14, 21, 29, 30]. Yet, there is a sufficient base for assumption that cultural conditions not only promote chromosomal instability of callus cells, but may also have a selective effect on karyotypically heterogenous callus cell population [1, 14, 19, 25, 26, 28]. The ploidy level of callus culture depends not only on the pre-existence of polysomaty or the rate

of polyploidization under culture, but also on the selective proliferation rate of mixoploid callus cell population. The cell lines with a less fit karyotype would be replaced by those with a higher fitness.

Our investigation system does not permit of distinguishing the primary origin of triploid cells from favoured triggering of triploid cells into mitosis. But the presence of little portion of triploid cells already in the second passage of the investigated pea callus strain, and the slow continuous increase in the frequency of triploid cells during a long-term cultivation give evidence of the selective fitness of this ploidy level. The nature of selective factors remains obscure as far as the pea callus tissue has been subcultured on the same medium and under the same conditions. It should only be supposed that some endogenous factors of intra- or intercellular character were in action. Besides, our previous investigations on several other pea callus strains have shown that even the culture strains of similar origin grown under apparently similar conditions may diverge and reveal various tendencies in chromosomal variation [9]. These findings are in agreement with those reported by BUTCHER et al. [4].

Finally, it should be pointed out that, though there is no rigid regularity in chromosomal variability of different tissue cultures and we are far from understanding the fundamental causes of this phenomenon, some trends have been observed in chromosomal variability of long-term plant tissue cultures permitting us to examine some new aspects of nuclear behaviour *in vitro*

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INHIBITORY EFFECT OF ADRENALINE AND HYDROCORTISONE ON THE GROWTH OF *ALLIUM CEPA* ROOTS

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Abstract

The roots of *Allium cepa* were allowed to grow in distilled water containing 10^{-4} *M* adrenaline hydrochloride or 2×10^{-3} M hydrocortisone sodium succinate. Adrenaline inhibited the growth of roots; they decreased in length, number and total dry weight. The total amount of DNA in the roots was reduced much less than that of extracellular root components after adrenaline. Also hydrocortisone treatment resulted in a considerable decrease of the length and dry weight of *Allium cepa* roots. Both DNA and extracellular root components were influenced.

Introduction

It is now well-established that the administration of glucocorticoids results in the suppression of somatic growth of immature animals [3, 4]. The inhibitory effects of these hormones on DNA synthesis and mitosis can be also demonstrated in the cells maintained in tissue culture [1]. The question arises whether glucocorticoids are specific tools used by animals to control cell division or whether their action is only nonspecific interference with the mitotic process. We have examined if hydrocortisone influences the growth of plant tissue that has never come into contact with adrenal hormones.

Another adrenal hormone, adrenaline, has been reported to slow down the growth of certain animal tissues [5, 6]. Therefore, it has been included in our study on plant tissue growth.

Material and method

Allium cepa onions weighing approximately 25 g were used. Dry roots were removed and the lower parts of the onions were immersed in distilled water, in adrenaline hydrochloride (Adrenalinium chloratum solutum Spofa) solution or in hydrocortisone sodium succinate (Hydrocortison solubile Spofa) solution. 6 onions were included in each group. The solutions were exchanged 3 times a week.

On the 7th day of the experiment, the number and the length of the roots were determined. The roots were allowed to dry at 60 °C, then extracted with cold 1 *M* perchloric acid for 72 h in order to remove soluble compounds that would interfere with DNA determination. The roots were then hydrolysed for 30 min in 0.5 *M* perchloric acid at 70 °C and DNA content was determined according to BURTON [2].

Results

The roots growing in 10^{-4} *M* adrenaline hydrochloride were by approximately 70% shorter than the control ones (Table 1). The reduction in root dry weight was even more pronounced (about 76%). A decrease in root number in 10^{-4} *M* adrenaline-treated onions was found in both experiments but it was not statistically significant.

Table 1

Influence of adrenaline and hydrocortisone on the growth of Allium cepa roots

Experiment No.	Treatment	Root length (mm)	Root number	Root dry weight mg/onion
1.	—	43.8± 7.9	40.3±26.4	52.8±37.5
	10^{-4} *M* adrenaline HCl	13.4±10.1*	19.3±17.8	12.5±19.3*
	2×10^{-3} *M* HCS	16.9± 5.4*	40.5±29.0	21.9±13.6
2.	—	18.2± 9.9	78.2−17.4	40.5±24.8
	10^{-4} *M* adrenaline HCl	5.0± 8.1*	66.5±36.9	9.3± 6.7*
	2×10^{-3} *M* HCS	7.5± 1.6*	69.7±25.9	11.6± 3.8*
3.	—	21.6± 9.1	74.6±18.6	43.3±21.3
	10^{-5} *M* adrenaline HCl	23.7± 7.6	71.6±21.3	40.1± 6.1
	2×10^{-4} *M* HCS	16.8± 2.9	57.9±29.3	37.2±18.8

HCS . . . hydrocortisone sodium succinate. Means ± standard deviations are indicated. Asterisks denote statistically significant ($P < 0.05$) results

2×10^{-3} *M* hydrocortisone sodium succinate was found to be almost as effective as 10^{-4} *M* adrenaline in blocking root growth. The number of roots, however, was decreased only slightly.

10^{-5} *M* adrenaline and 2×10^{-4} *M* hydrocortisone had no significant effect on root growth.

DNA content in root dry matter, which approximately corresponds to the percentage of cells in the roots of an onion, was increased after 10^{-4} *M* adrenaline treatment (Table 2). This indicates that the balance between cell

number and extracellular material was disturbed. The total amount of DNA did not change significantly.

2×10^{-3} *M* hydrocortisone had similar effects as 10^{-4} *M* adrenaline but the increase in DNA content per g dry weight was not so high and the total amount of DNA was decreased by about 50%.

10^{-5} *M* adrenaline and 2×10^{-4} *M* hydrocortisone caused no significant changes in DNA content.

Table 2

Influence of adrenaline and hydrocortisone on DNA content in Allium cepa roots

Experiment No.	Treatment	DNA (μg/onion)	DNA (mg/g dry weight)
1.	—	397±322	7.6± 1.1
	10^{-4} *M* adrenaline HCl	128±156	13.9± 4.2*
	2×10^{-3} *M* HCS	262±169	12.9± 4.1*
2.	—	492± 90	14.5± 5.1
	10^{-4} *M* adrenaline HCl	512±401	53.7±14.0*
	2×10^{-3} *M* HCS	203± 89*	17.4± 2.8
3.	—	403±122	10.1± 2.0
	10^{-5} *M* adrenaline HCl	429±110	10.7± 1.8
	2×10^{-4} *M* HCS	334±177	8.3± 3.8

HCS . . . hydrocortisone sodium succinate. Means ± standard deviations are indicated. Asterisks denote statistically significant ($P < 0.05$) results

Discussion

We have found that adrenal hormones inhibit the division of the cells in *Allium cepa* root tips. Some functions of these cells, namely, the production of extracellular material is negatively affected, too. These findings suggest that the inhibition of animal cell division by glucocorticoids or by adrenaline is not specific and, thus, is not a physiological means of growth control.

It can be argued that the hormone concentrations used in our experiments are very high. Additional experiments will have to be done to elucidate why these concentrations are necessary. For instance, the transport of hydrocortisone into the cells and to the primary sites of its action in cell nuclei as well as the interaction of adrenaline with the adenylate cyclase system are likely to play an important role in the inhibition we have observed and should be the subject of further experiments.

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EXPERIMENTAL INVESTIGATIONS ON HYPOKINESIS OF SKELETAL MUSCLES WITH DIFFERENT FUNCTIONS I.

CHANGES IN MUSCLE WEIGHT, PROTEIN AND CONTRACTILE PROPERTIES

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Abstract

In New Zealand white rabbits the right hind limb was immobilized in full extension with a plaster cast. The free left hind limb served as control. The masses of both the tetanic m. gastrocnemius and the tonic m. soleus considerably decreased as a consequence of immobilization for 5, 10, 14, 28 or 42 days. The decrease was more marked for the m. soleus. The water content of the muscle did not change substantially in the course of the atrophy. The total protein and myofibrillar protein contents of the immobilized muscles fell significantly. The tonic m. soleus atrophised sooner and was more extensive than the m. gastrocnemius. Superprecipitation of the myofibrillar proteins of the immobilized muscles decreased by 20—25% compared to the controls. The experimental model is considered suitable for further biochemical and ultrastructural investigations relating to the development of atrophy and to regeneration.

Introduction

The close correlations between the functioning of organs and their morphological and biochemical structures have long been known. The consequences of use and disuse are particularly striking in the skeletal muscles, in which systematic and enhanced functioning leads to a relatively rapid, well-visible and measurable mass increase and metabolic modification [2, 3, 9, 15, 16, 17, 20]. In contrast, constrained disuse results in the atrophy of the musculature, and in appreciable changes in the chemical constituents [6, 8, 12, 14, 21, 22, 25, 27].

A study of the physiological and diochemical features of atrophies induced by restricted use of innervated muscles may provide information valuable for both basic biological research anb the clinician and may enable us to outline the roles of use and of the mehanic cal activity of the muscle in the homoeostasis of materials which are of prim ary in the muscle function; further, the clinician is supported by such studies in selecting the best method for the prophylaxis and therapy of muscular atrop hies of non-neurogenic origin.

Detailed investigation of muscular atrophy caused by disuse is, moreover, of current importance, for the avoidance or moderation of the consequences of the reduced movement activity of astronauts under conditions of weightlessness during prolonged space flights can be attempted rationally only in the exact knowledge of the inducing processes.

Inactivation of muscles can be brought about in various ways under experimental conditions. It may be achieved, for example, by transection of the medulla [5, 13], by tenotomy [8, 11, 22, 33, 36], and by different forms of immobilization, with a plaster cast, with splinting and with articular fixation [5, 12, 21, 27, 34].

Under terrestrial conditions, plaster cast immobilizations appears to be the most convenient method for simulating the reduced functioning of muscles in the state of weightlessness. A study of the metabolic effects of fixation with a plaster cast may provide valuable information for the clinician, as such a procedure is frequently employed in the therapy of bone fractures and limb deformities.

In the experimental series reported in the present paper, we used biochemical, physiological and morphological methods to study the tonic and tetanic muscles in rabbit hind limbs immobilized with a plaster cast; we hoped to obtain new data on the pathogenesis of the non-neurogenic atrophy of skeletal muscles.

Material and methods

The New Zealand rabbits *(Oryctolagus domesticus)* used originated from the same strain; they were adult animals of both sexes, weighing 3000 $\pm$ 200 g. Eighty animals were employed in this series. They were subjected to mild anaesthesia with intravenous pentobarbital (Nembutal^R) (30 mg/kg), and the right hind limb was fixed in full extension with a plaster cast. The full extension is essential, as literary data [12, 25, 31] indicate that the extent of the atrophy and the rate of its development are considerably influenced by the length of the immobilized muscle and the magnitude of the articular angle (*i.e.*, the position of the limb). The plaster cast was checked daily and was corrected if necessary (*e.g.*, if the animal had damaged it by gnawing it, or if it had loosened). Five, 10, 14, 28 or 42 days following the fixation, the animals were decapitated, exsanguinated, and two functionally different muscles, the tetanic m. gastrocnemius and the tonic m. soleus, were isolated and excised. The corresponding muscles of the contralateral, non-immobilized limb of the same animal were used as control. In preliminary experiments on 10 intact animals, it was established that the parameters measured in the m. gastrocnemius and the m. soleus in the right and left hind limbs of a given rabbit were nearly identical.

The water contents of the muscles were determined by drying 80—100 mg muscle samples to weight-constancy at 106 °C.

Protein was determined with a micro-Kjeldahl method. 40—50 mg muscle samples were hydrolysed in conc. H_2SO_4 for 12 h, with a mixture of SeO_2, K_2SO_4 and $CuSO_4$ as catalyst. The NH_3 content of the lysate was absorbed in 0.01 N HCl and determined titrimetrically.

The myofibrillar proteins were extracted by the method of HELANDER [15].

The natural actomyosin preparations for the superprecipitation experiments were produced by the method of EBASHI [7]. Prior to the actomyosin extraction, the sarcoplasmic proteins were extracted from the minced rabbit muscle with 0.05 M KCl, and the actomyosin was then extracted with 0.6 M KCl alkalinified with 0.01 M $NaHCO_3$. The crude actomyosin extract was purified by dilution with bidistilled water, and was freed from Ca^{++} by

treatment with EDTA-Tris buffer. The assay medium contained 60 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 20 mM Tris-maleate buffer of pH 6.74.

Superprecipitation of actomyosin suspensions was studied turbidimetrically at 660 nm, in a 1 cm cell, with a Spektromom 360 spectrophotometer. The actomyosin protein content was 0.5 mg/ml, and the total volume of the test sample 2.9 ml. Following measurement of the basic extinction, 0.1 ml 5 mM neutralized ATP solution was added to each reaction mixture, and the extinction was measured at 1 min intervals from then on. Before each measurement, the suspensions were mixed with a plexi microstirrer to ensure homogeneity.

Results

The m. gastrocnemius of the intact rabbits weighed 13—15 g, and the m. soleus 1.5—2 g. After immobilization for 28 days, the masses of both muscles were reduced, as clearly seen even with the naked eye (Fig. 1).

The experimental weight data (Fig. 2) showed that after 14 days of immobilization the m. gastrocnemius had decreased in mass by 21% on the average, and after 28 days by 33%. The corresponding decreases for the m. soleus were 52% and 62%, respectively, compared with the contralateral, non-immobilized control muscle.

The weights of the muscles were measured again after immobilization for 42 days. At that time the animals were strongly emaciated, and the non-immobilized contralateral muscles, too, were appreciably atrophied. This atrophy had also resulted from disuse, for when either of the hind limbs of the animal was immobilized, movement of the other hind limb was also restricted. In the first few weeks the animal compensated the restricted activity of the non-immobilized limb by several periods of temporary restlessness each day and violent attempts to move. As a consequence of this, the non-immobilized

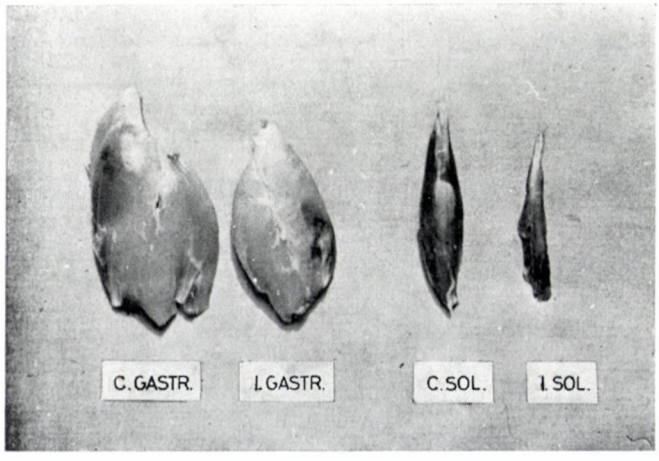

Fig. 1. Atrophy of immobilized rabbit muscles on the 28th day of hypokinesis .C. Gastr. = = Control m. gastrocnemius. I. Gastr. = Immobilized m. gastrocnemius. C. Sol. = Control m. soleus. I. Sol. = Immobilized m. soleus

contralateral muscles did not significantly change in weight during an immobilization period of 14 or 28 days. In the 5th or 6th week of immobilization, the rabbits gave up the compensatory movements, and their food intake decreased. The general debility is accompanied by a 25—35% decrease in weight of the muscles of the contralateral nonimmobilized limb. Although this behaviour may vary by animal species the data obtained by various authors [26, 34] after immobilization for longer than 30—35 days must be accepted with reservation. We limited to 28 days the maximum duration of immobiliza-

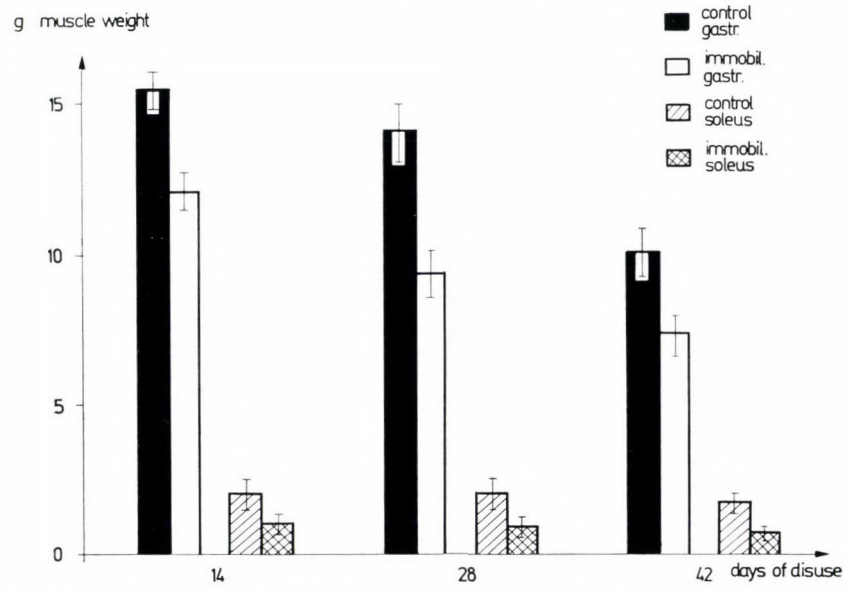

Fig. 2. Effect of immobilization on the weights of the m. gastrocnemius and the m. soleus (the data for the 14 and 42-day experiments represent the averages of 8 measurements, and those for the 28-day experiment the averages of 16 measurements)

tion, which, being free of side-effects, is suitable for comparison with the controls to 28 days. From five experiments in each case, it was established that the weight of the m. soleus had decreased by 22% by the 5th day after immobilization, by 41% by the 10th day. The corresponding weight losses for the m. gastrocnemius were lower, 8% and 15%, respectively.

A role may be played in the reduction of the mass of the muscle by loss of water. Accordingly, the water contents of control and immobilized muscles were determined. Although the water content slightly increased in certain cases following immobilization, this was not characteristic. On the basis of our experiments we can exclude the possibility that the loss of weight of the immobilized muscle was due to loss of water.

Our results as regards protein loss are shown in Figs 3 and 4.

Figure 3 shows that after 14 days the immobilized tonic m. soleus contained 30% less total protein compared to the control. In the m. gastrocnemius the decrease in total protein was only 10%, yet, this change may be regarded as significant. After 28 days, the corresponding total protein loss was 36% and 20%, respectively.

The myofibrillar protein concentrations of the muscles decrease at about the same rate as the total protein.

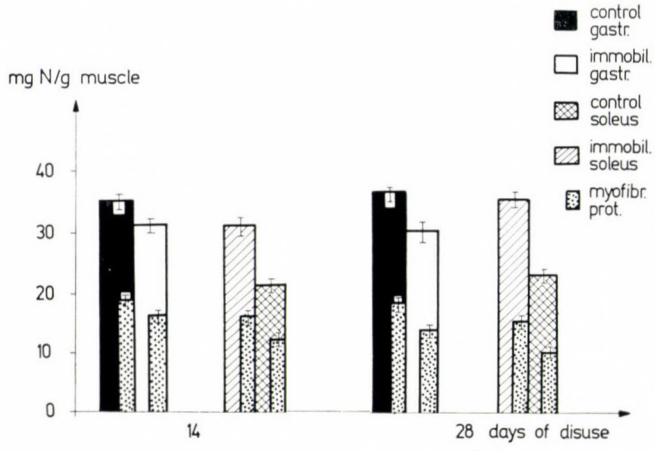

Fig. 3. Effect of immobilization on the total protein concentrations of the m. gastrocnemius and the m. soleus (mg N/g muscle)

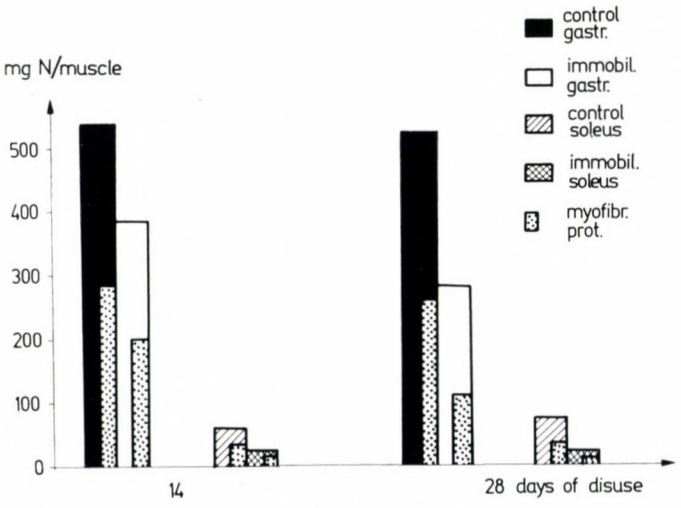

Fig. 4. Effect of immobilization on the total protein contents of the m. gastrocnemius and the m. soleus (mg N/muscle)

If the quantitative changes in the protein are referred to the total muscle, is even more striking.

The results of superprecipitation experiments with myofibrillar protein extracts prepared from the m. gastrocnemius are presented in Fig. 5.

The curves clearly illustrate that the turbidity with ATP was by about 25% less in the myosin preparation obtained from the immobilized m. gastrocnemius than in the preparation from the control m. gastrocnemius. Individual experiments could not be performed with the m. soleus because of their low masses. Pools from several soleus muscles gave similar results as the individual m. gastrocnemius specimens.

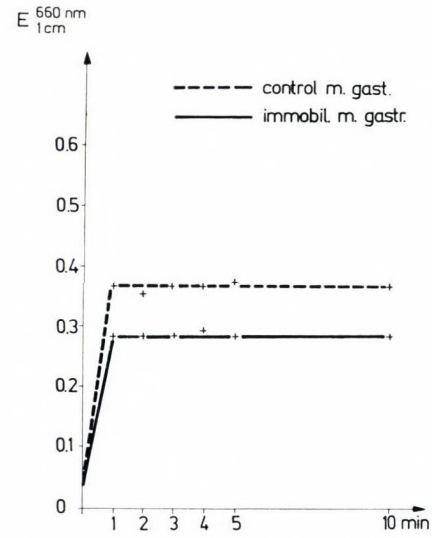

Fig. 5. Superprecipitation of the actomyosin of the immobilized m. gastrocnemius (the representative curve from 10 experiments)

Discussion

The structural and functional changes following muscular atrophy have been investigated on various experimental models. The applicability of the models is limited by the effects accompanying the atrophy.

Muscular atrophy can frequently be observed as a consequence of extensive degenerative pathological processes and traumatic injuries of the medulla. Similar conditions can be induced in animal experiments by medullary transections in various segments. From a study of the skeletal muscles innervated by the segments situated below the transection, however, a complete picture of the direct consequences of loss of function may be accepted only with reservations, for the heat regulation is disturbed in the affected area, and homoiother-

mia can be maintained only by artificial heating. Experiments in which an appropriate heating was not provided or the degree of the hypothermia of the affected areas was not reported cannot be evaluated correctly.

Numerous authors have dealt with muscular atrophy following peripheral denervation. It has been proved that the differentiation of the fibres of the skeletal muscles and the maintenance of their structure need an undisturbed neural supply. Peripheral denervation in animal experiments is a reliable model for the investigation of those human diseases caused by either pathological processes or traumatic injuries of peripheral nerves. The non-denervated, contralateral, symmetrical muscle is suitable for comparative purposes. It was established in this way that the mass of the muscle considerably decreases as a result of denervation. Fundamental studies have also been made on the functional, structural and biochemical changes occurring in the myofibrils [1, 4, 10, 14, 18, 19, 23, 24, 28, 30, 32, 34, 35]. The inability of the muscle to function is an important factor in inducing the metabolic, morphological and functional changes following denervation. Besides the cessation of the neural trophic effects must not be neglected in the development of neurogenic atrophy. Fibrillation, an increased acetylcholine sensitivity and prolonged chronaxy are commonly observed after denervation of muscle.

None of these phenomena are observed if the muscular dysfunction is due to tenotomy. Experimental tenotomy is an adequate model for the study of those processes which follow rupture of a tendon, due to excessive physical exertion, sporting activity, etc. Such experiments have shown that, as regards the skeletal muscles, after tenotomy the tonic muscles are more markedly atrophied than the tetanic ones [8, 11, 22, 33, 36]. Disuse seems to predominate in inducing post-tenotomy symptoms. Besides, the muscle loses the load ensured even in rest by the interconnection with the skeleton. Thus, the proprioceptive impulses maintained by the resting tone disappear almost completely, the resting length of the muscle is extended, and the tenotomy is frequently followed by contractions lasting for various periods.

In accordance with the above, aneurogenic atrophy of the skeletal muscles due to dysfunction may perhaps be induced most appropriately by experimental immobilization of a limb with a plaster cast. Clinically, this procedure is extensively employed in the therapy of bone fractures. A detailed investigation of muscular atrophy caused by disuse is, furthermore, of current interest: the avoidance or moderation of the consequences of the reduced movement activity in the state of weightlessness needs the exact knowledge of the pathological processes. On this basis, immobilization seems to be the most suitable method of simulating disuse due to weightlessness under terrestrial conditions. Immobilization has already been employed with various animals, *e.g.*, cat [6, 11, 21], guinea-pig [34], rat [26, 27], rabbit [12], *etc.* Although the consequent symptoms vary by species it may be concluded that the fibre-type of the

muscle is the main factor determining the mode and the extent of the atrophy and, also, the changes in the metabolism and in the biochemical composition of the muscle. The tonic muscles are selectively more sensitive to disuse, though, immobilization considerably affects all fibre-types. Accordingly disuse is a substantial factor in muscle pathology.

In the currently beginning systematic experimental series of investigations on rabbits, we are attempting to acquire new data on the atrophy of skeletal muscles subjected to disuse. In the first publication, relating to the general biological parameters, we have reported that, essentially in agreement with literary data, immobilization caused appreciable changes in both the tetanic m. gastrocnemius and the tonic m. soleus. We have confirmed that tonic muscle are more sensitive to immobilization. The decrease in both the mass and the protein content of the muscle was more announced in tonic muscles.

We have performed experiments in which the contractile properties of immobilized muscles were examined on Sherrington preparations. It seems worth-while to mention that the tonic muscles exhibited a greater decrease in strength than did the tetanic m. gastrocnemius; the decreased muscle-strength was nearly proportional to the decreases in the mass and the myofibrillar protein content of the muscle.

Our basic experiments also draw attention to the fact that the weakening in contractile strength of the muscle is not only a consequence of the decreases in the muscle weight and protein content. On the basis of the superprecipitation experiments we assume that the quantitative changes in the protein content are accompanied by changes in the submolecular structure of the myofibrillar proteins. Otherwise we would not be able to interpret our observation that in preparations obtained from immobilized muscles the reduced tendency of actomyosin suspensions to undergo superprecipitation.

The muscle weight and protein content proved to be readily followable parameters, suitable for investigation of the development of muscular atrophy. From our experiments the conclusion was drawn that an analysis of the fully developed 28-day atrophic state appears to be the most convenient for studying the functional and morphological changes resulting from immobilization.

A more detailed multidirectional analysis of the changes due to immobilization and studies on the possible modes of delaying the development of atrophy and of promoting regeneration are in progress.

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EXPERIMENTAL INVESTIGATIONS ON HYPOKINESIS OF SKELETAL MUSCLES WITH DIFFERENT FUNCTIONS, II.

QUANTITATIVE CHANGES IN THE NUCLEIC ACIDS

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Abstract

The effect of hypokinesis on the quantitative changes in the nucleic acids was studied in functionally different muscles of rabbits on the 5th, 10th, 14th and 28th days following plaster cast immobilization of the limbs. As a consequence of the immobilization, the total amount of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) decreased in both the tonic and the tetanic muscles. The decrease was more marked in the tonic muscles. As a result of the hypokinesis, the RNA/DNA ratio increased in the m. gastrocnemius, and decreased in the m. soleus. The plaster cast immobilization has a greater effect on the homoeostasis of the nucleic acids of the tonic muscles than in the case of the tetanic muscles.

Introduction

In the first paper of this series [13] the effects of plaster cast induced limb immobilization on some biochemical and physiological parameters of functionally different skeletal muscles of rabbits have been published. The immobilization resulted in significant weight and protein losses in the limb muscles.

In connection with modifications of the protein metabolism following immobilization [3, 8, 9, 13], special attention should be paid to the nucleic acids, quantitative and qualitative changes in which may play a central role in the adaptive and pathological modifications of the mechanisms regulating the protein metabolism.

In the present work, a study has been made of the quantitative changes in the nucleic acids at various periods after plaster cast immobilization of limbs. Since functionally different muscles may react to immobilization-induced hypokinesis in different ways as regards their metabolisms, examinations were performed on the (tetanic, "rapid" or white) m. gastrocnemius and the (tonic, "slow" or red) m. soleus.

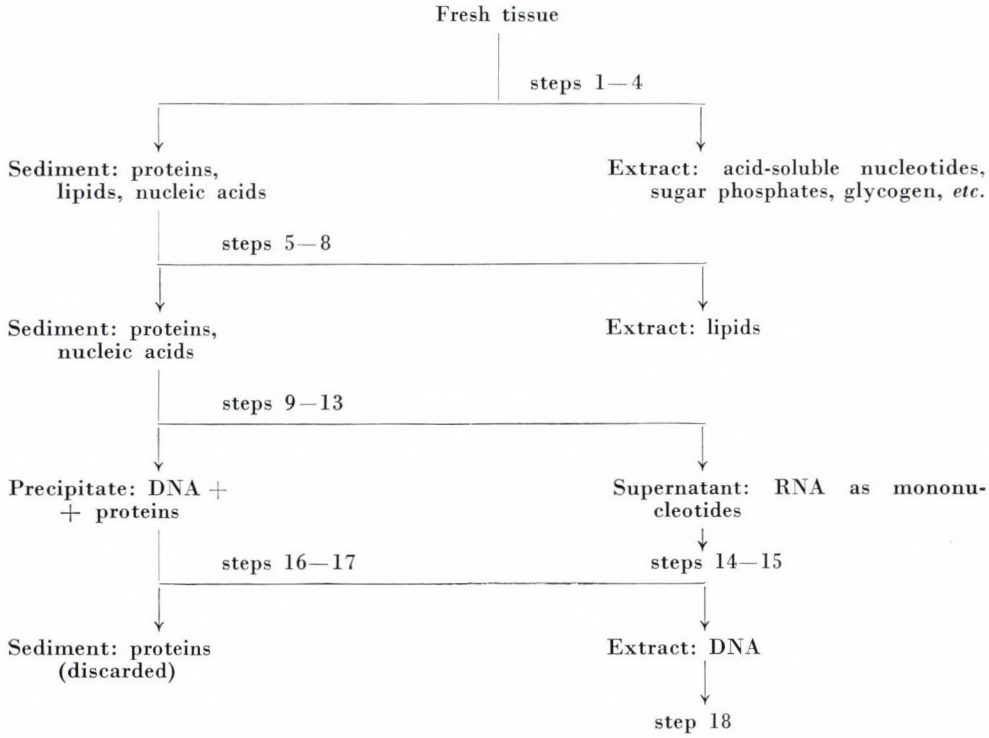
Material and methods

The effects of immobilization were examined on 21 New Zealand white rabbits *(Oryctolagus domesticus)* of both sexes, weighing 3000 ± 200 g. The muscles of two intact animals were used to control the immobilization stress effects affecting the entire skeletal musculature. The animals were maintained on a standard rabbit diet prepared by the Laboratory Animal Breeding Institute, Gödöllő.

The right hind limb was immobilized in full extension with a plaster cast [13]; the contralateral limb muscles served as controls.

On each of the 5th, 10th, 14th or 28th days following immobilization, a group of the animals was exsanguinated and processed. The m. gastrocnemius and the m. soleus were prepared, weighed and frozen in crushed solid CO_2; the muscles were processed within 2 hours.

Nucleic acids were determined in the following manner, by a variant of the combined method of SCHMIDT—THANNHAUSER and SCHNEIDER [10], adapted for muscle tissue:



Step 1 (homogenization): 300—500 mg muscle was cut up into fine pieces with scissors and homogenized in 5 ml ice-cold 5% trichloroacetic acid (TCA) in a Potter's glass homogenizer for 3 × 1 min. The homogenate was transferred into a 25 ml glass centrifuge tube containing 10 ml 10% TCA, which is situated in an iced-water bath

Steps 2—4 (Removal of acid-soluble components):

Step *2:* The homogenate was thoroughly mixed with a glass rod, left to stand for 20 min, and centrifuged at 1050 *g* at 4 °C for 10 min. The supernatant was poured off.

Step *3:* 10 ml ice-cold 10% TCA was added to the sediment, and step *2* was repeated.

Step *4:* Step *3* was repeated.

Steps 5—8 (Removal of traces of TCA, lipid extraction, dehydration):

Step *5:* 20 ml ice-cold distilled water was added to the precipitate, and centrifugation was performed as in step *2*. The water was poured off.

Step *6:* 3 ml cold 96% ethanol was added to the sediment, and the mixture was centrifuged. The alcohol was poured off.
Step *7:* 5 ml of a 3:1 mixture of 96% ethanol + ether was added to the sediment, the mixture was stirred, and heated in a 70 °C water bath for 20 min. This was followed by centrifugation at 1050 *g* for 20 min. The extract was poured off.
Step *8:* 3 ml ether was added to the sediment, and the mixture was held in a 40 °C water bath for 15 min. Centrifugation was performed as in step 7. The extract was poured off.
Step 9 (Preparation of tissue powder): The sediment was dried in a vacuum desiccator and powdered in a mortar.
Steps 10—13 (Separation of DNA and RNA):
Step *10:* 3 ml 0.5 *N* KOH was pipetted onto 50—70 mg tissue powder in a 10 ml glass centrifuge tube. A blank tube without tissue powder was also prepared.
Step *11:* The tubes were sealed with parafilm (Marathon, Neenah, Wisconsin), and placed in a 37 °C thermostat for at least 15 h.
Step *12:* The tubes were placed in iced water, and their contents first neutralized with 70% $HClO_4$ and then acidified with 3 ml 0.4 *M* $HClO_4$. The tubes were centrifuged and the supernatant was poured into a calibrated test tube.
Step *13:* The precipitate was washed with 3 ml cold 0.2 *N* $HClO_4$. The supernatants were combined.
Step 14: The volume of the solution containing the RNA nucleotides was made up to 10 ml with 0.2 *N* $HClO_4$.
Step 15: The ribose content of the solution was determined according to BIAL [1].
Steps 16—17 (Extraction of DNA):
Step *16:* 2 ml 0.75 *N* $HClO_4$ was added onto the precipitate, and the DNA was extracted for 20 min at 70 °C. The supernatant was transferred to a calibrated tube.
Step *17:* The extraction was repeated with 1 ml 0.75 *N* $HClO_4$. The combined extracts in the calibrated tubes were made up to 3 ml with the same solution.
Step 18: The DNA content of the solution is determined by the method of BURTON [2].

The Na salt of DNA (isolated from calf thymus L. Light, Colnbrook, England) and the Na salt of RNA (isolated from yeast BDH, Poole, England) were used as standards for the quantitative determination of the nucleic acids. Photometric measurements were made with a Beckman DB spectrophotometer.

In the statistical evaluation of the experimental results, the standard error (S. E.) of the mean was calculated with the S. D./$\sqrt{n}$ relation, and the significance of the difference between the experimental series with the Student's *t* test [12]. The significance levels were adapted from CRAMER [4].

Results

The DNA and RNA concentrations in the control muscles of the animals with immobilized limbs were similar to the values for the intact muscles until the end of the 2nd week of hypokinesis whereafter decrease somewhat (Tables 1, 5 and 7). Consequently, in the examination of the effects of immobilization for longer periods it is desirable to use muscles from intact animals as controls.

Up to the 28th day of the hypokinesis, the DNA concentration increased to an insignificant extent in the m. gastrocnemius (Table 1). In contrast, by the 10th day a significant increase, and by the 14th day a highly significant increase (38.3%) was observed in the m. soleus (Table 3).

In the examination period the total amount of DNA exhibited a decreasing tendency in both muscles (Tables 2 and 4). By the 14th and 28th days following limb immobilization, the decrease was more significant in the m. soleus.

Table 1

DNA μg/100 mg wet wt. in gastrocnemius muscles of rabbit legs immobilized with plaster cast

Period of fixation (days)	Number of muscles analyzed	Muscles from intact animals	Contralateral control muscles	Immobilized muscles	Difference %*	P**
		mean±S.E.				
—	2 a)	57.5±0.8				
—	2 b)	57.0±3.1				
5	3		55.0±1.1	56.7±1.1	+3.1	>0.2
10	3		52.8±0.9	54.8±2.9	+3.7	>0.5
14	7		59.0±2.0	61.0±2.2	+3.3	>0.2
28	8		54.6±3.8	59.8±5.6	+9.5	>0.4

Table 2

DNA mg/muscle in the immobilized gastrocnemius muscles

5	3		10.528±0.29	9.633±0.26	— 8.5	>0.05
10	3		9.185±0.95	7.902±1.21	—13.9	>0.4
14	7		9.165±0.6	7.426±0.57	—18.9	<0.005
28	8		6.746±0.67	5.530±0.36	—18.0	

Table 3

DNA μg/100 mg wet wt. in soleus muscles of rabbit legs immobilized with plaster cast

Period of fixation (days)	Number of muscles analyzed	Muscles from intact animals	Contralateral control muscles	Immobilized muscles	Difference %*	P**
		mean±S.E.				
—	2 a)	77.9±0.8				
—	2 b)	76.7±2.1				
5	3		78.5±0.9	83.0±0.7	+ 5.7	<0.026
10	3		82.9±2.8	100.1±1.4	+20.7	<0.01
14	7		81.8±2.7	113.2±4.5	+38.3	<0.001
28	8		83.9±3.0	143.6±4.6	+71.1	

* Percentage changes from contralateral nonimmobilized muscles.
** Significance of difference between control and immobilized muscles.
a) From the right legs, *b)* from the left ones

Table 4

DNA mg/muscle in the immobilized soleus muscles

Period of fixation (days)	Number of muscles analyzed	Muscles from intact animals	Contralateral control muscles	Immobilized muscles	Difference %*	P**
		mean ± S.E.				
5	3		1.666±0.14	1.442±0.22	−13.4	>0.4
10	3		1.725±0.14	1.613±0.14	− 6.5	>0.5
14	7		1.521±0.07	0.963±0.07	−36.6	<0.001
28	8		1.530±0.13	1.111±0.09	−27.4	

* Percentage changes from contralateral nonimmobilized muscles.
** Significance of difference between control and immobilized muscles.
a) From the right legs. *b)* from the left ones.

With the advance of time, the RNA concentration increased in the m. gastrocnemius (Table 5), but exhibited a decreasing tendency in the m. soleus. (Table 7).

The absolute quantity of RNA decreased in both muscle types, but this attained a statistically significant or highly significant extent only in the m. soleus (Tables 6 and 8).

As a result of the hypokinesis, the RNA/DNA ratio increased in the m. gastrocnemius (Tables 5 and 6), and decreased in the m. soleus (Tables 7 and 8). In the m. gastrocnemius a change was observed in this ratio after the 5th day of the hypokinesis.

Discussion

The experimental results indicate that plaster cast immobilization of the limbs has a more profound effect on the nucleic acid metabolism of the tonic muscles (m. soleus) than on that of the tetanic muscles (m. gastrocnemius). The neurogenic atrophy exerts an opposite effect on the nucleic acid content of the skeletal muscles. Hollósi and Lovas [6] observed a more pronounced decrease in the RNA content of the denervated tetanic muscles of the rat than in that of the tonic muscles. Their investigations prove that the nucleic acid metabolism of the tetanic muscles depends to a greater extent on the neuromuscular integrity of these muscles than in the case of the tonic muscles.

The effect of plaster cast immobilization can be compared with that of some other intervention suitable for inducing inactivation atrophy, *e.g.* tenotomy. Shchesno [11] observed a change of similar direction, but of greater extent, in the relative and absolute amounts of tenotomized rabbit muscles.

Table 5

RNA μg/100 mg wet wt. in gastrocnemius muscles of rabbit legs immobilized with plaster cast

Period of fixation (days)	Number of muscles analyzed	Muscles from intact animals	Contralateral muscles	Immobilized muscles	Difference % *	P**	RNA/DNA ratio		
		mean±S.E.					Intact	Control	Immob.
—	2 a)	134.1±3.9					2.33		
—	2 b)	133.1±2.7					2.33		
5	3		129.5±2.7	133.4±2.7	+ 3.0	>0.2		2.35	2.35
10	3		130.3±5.6	139.6±5.8	+ 7.1	>0.2		2.46	2.54
14	7		126.0±3.1	141.8± 3.3	+12.4	<0.005		21.3	2.32
28	8		94.3±5.1	146.7±13.2	+55.5	<0.001		1.72	2.45

Table 6

RNA mg/muscle in the immobilized gastrocnemius muscles

5	3		24.774±0.2	22.656±0.7	− 8.5	> 8.5		2.35	2.35
10	3		22.647±2.1	20.029±2.3	−11.5	>0.4		2.46	2.53
14	7		19.440±1.1	17.190±1.1	−11.5	>0.1		2.12	2.31
28	8		14.646±1.5	13.176±1.1	−10.0	>0.4		2.17	2.38

* Percentage changes from contralateral nonimmobilized muscles.
** Significance of difference between control and immobilized muscles.
a) From the right legs, *b)* from the left ones

Table 7

RNA μg/100 mg wet wt. in soleus muscles of rabbit legs immobilized with plaster cast

Period of fixation (days)	Number analyzed	Muscles from intact animals	Contralateral control muscles	Immobilized muscles	Difference % *	P**	RNA/DNA ratio		
		mean±S.E.					Intact	Control	Immob.
—	2 a)	223.0±0.9					2.85		
—	2 b)	222.4±1.5					2.89		
5	3		223.3± 2.8	219.1± 1.9	− 1.8			2.84	2.63
10	3		226.2± 2.9	223.7± 2.8	− 1.1			2.72	2.23
14	7		225.7± 6.0	216.5± 3.8	− 4.0	>0.05		2.75	1.91
28	7		216.9±18.6	188.0±13.0	−13.3	>0.2		2.58	1.30

Table 8

Total quantity of RNA mg/muscle in the immobilized soleus muscles

5	3		4.731±0.35	3.804±0.58	−19.6	>0.2		2.83	2.63
10	3		4.693±0.29	3.601±0.29	−23.2	>0.05		2.72	2.23
14	7		4.211±0.22	1.947±0.13	−53.7	<0.001		2.76	2.02
28	7		4.441±0.45	1.479±0.18	−66.7	<0.001		2.90	1.33

* Percentage changes from contralateral nonimmobilized muscles.
** Significance of difference between control and immobilized muscles.
a) From the right legs, *b)* from the left ones

The tonic muscles were similarly more strongly affected by the surgical intervention. HOLLÓSI et al. [5] and LISSÁK et al. [7] did not observe any change in the nucleic acid content of rat muscles up to the 12th postoperative day following tenotomy.

Further studies are necessary to elucidate the mechanisms inducing the quantitative changes in the nucleic acids during plaster cast immobilization. An understanding of the mechanisms modifying the nucleic acid and protein metabolisms may provide a possibility for selecting the most appropriate method in the prevention and therapy of immobilization atrophy.

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EXPERIMENTAL INVESTIGATIONS ON HYPOKINESIS OF SKELETAL MUSCLES WITH DIFFERENT FUNCTIONS, III.

CHANGES IN PROTEIN FRACTIONS OF SUBCELLULAR COMPONENTS

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Abstract

Changes occurring in the protein fractions of rabbits' immobilized skeletal muscles with different functions were studied. Disuse of the muscles resulted in a gradual reduction in the contractile proteins. The specific proteins of the tonic muscle (m. soleus) were degraded to a greater extent than those of the tetanic (white) muscle (m. gastrocnemius). Parallel with the decrease in the structural proteins the sarcoplasmic protein exhibited a relative increase. The tonic muscles underwent greater damage than the tetanic muscles, indicating that the dedifferentiation was more marked in the tonic muscle. The results are explained by the biological importance of the function and activity of the cell: disuse leads to changes in the physiological and biochemical characteristics of the muscle, and to dedifferentiation of the cells.

Introduction

Apart from the cell nucleus, the skeletal and heart muscles consist of well-distinguishable compartments: the myofibrillar elements performing the special function of contraction, and the sarcoplasm (soluble proteins). In addition, the muscle cells contain particles (mitochondria and lysosomes), and a specially organized network system, the sarcoplasmic reticulum.

The myofibrils consist of thin and thick filament and the associated Z membrane. Their biochemical components are the structural proteins: myosin, actin and regulation proteins. The sarcoplasmic compartment, or myogen as it was earlier termed, means the protein fraction extractable below pH 6.8 at low ionic strength [4]. This system is substantially more heterogeneous than the structural proteins; its components are mainly glycolytic enzymes (*cca.* 70%), creatine phosphokinase (*cca.* 5%) and myoglobin (1—2%), and also other enzymes of extracellular origin.

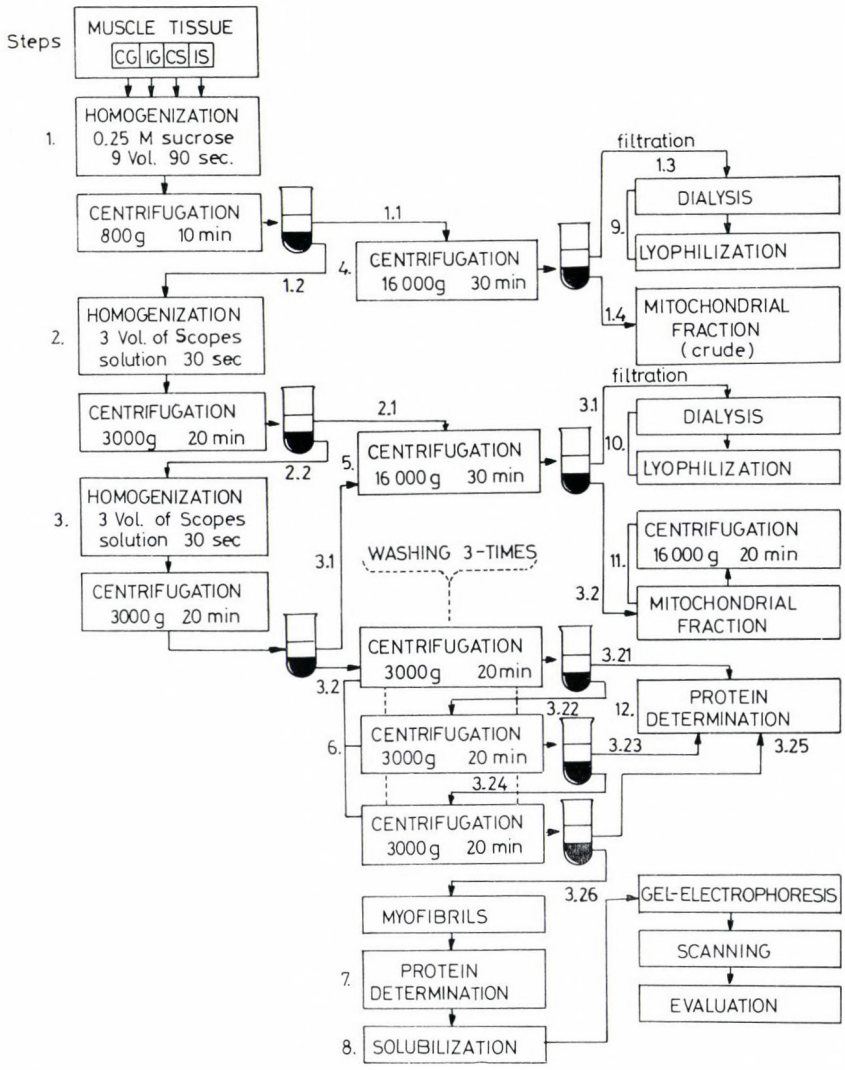
As regards the functioning of the muscle, each cell component plays an important role. Disuse presumably has a destructive effect on every protein. It was earlier demonstrated [6] that the masses of the immobilized rabbit m. gastrocnemius and m. soleus (in the following G and S) progressively decrease

in time; during 4—6 weeks they may lose even 50—60% of their original weights. After 14 days' disuse, the decrease in the protein content was, on the average, 10% for G and 30% for S; the corresponding values after 28 days' disuse were 20% and 36%.

It appeared reasonable to investigate the changes occurring in the compartments of the muscle cell and fibres under the same experimental conditions.

Table 1

Process of preparation



Abbreviations: C = control, I = immobilized, G = gastrocnemius, S = soleus muscle.
Arabic numbers: Code symbols for marking individual samples

Material and methods

The entire working process of the preparation was carried out in a cold-room or at 4 °C. Reference is made to earlier publications [3, 6] for details of the techniques of immobilization and muscle excision. Animals were exsanguinated 5, 10, 14 and 28 days after being put in plaster. The immobilized and the contralateral limb muscles were excised rapidly, freed from connective tissue, weighed and immediately processed. There were 5 rabbits in each group: 3 of these were immobilized, and 2 (from the same strain) served as controls. The 5- and 10-day experiments were performed twice, and the 14 and 28-day experiments three times (altogether 50 animals). In the latter, the limb muscles were processed bilaterally.

Solutions:

(1) 0.25 M sucrose solution for step *1*.

(2) Scopes solution. 0.1 M KCl — 2 mM EDTA, 3% glycerol. — 10 mM Tris-HCl pH 7.7.

(3) Washing solution: 0.01 M KCl — 5 mM Tris-HCl pH 7.7.

The course of the preparation is illustrated in Table 1.

In order to save work and ensure the identification of individual samples, we used a code notation. The letters C, I, G and S, written after the serial number of the animal, mean control, immobilized, gastrocnemius and soleus muscles.

For complete exposure and loss-free fractionation of the muscle tissue, the native muscle was homogenized with an MSE blender in 9 volumes of 0.25 M sucrose solution (step *1*). The homogenate was centrifuged for 10 min at 800 *g*. The supernatant (1.1) was recentrifuged for 30 min at 16,000 *g* to yield the soluble (1.3) and mitochondrial (1.4) fractions. The sediment (1.2) was suspended in 3 volumes of Scopes solution [5] and rehomogenized for 30 sec in a blender (step *2*). The myofibrillar fraction was sedimented out at 3000 *g*, and this step was repeated (step *3*). For removal of adsorbed sarcoplasmic proteins, the sediment was washed three times, on each occasion with 10 volumes of 0.01 M KCl — 50 mM Tris-HCl buffer (pH 7.7) solution (step *6*). All supernatants were centrifuged at 16,000 *g* to sediment out the particles (mitochondrial fraction) (steps *4*, *5*). The final supernatants (1.3 and 3.1) were combined, dialysed and lyophilized (steps *9*, *10*). The protein contents of the washing solutions were determined. The final sediment (3.2.6), which corresponds to the pure myofibril preparation, was weighed, and its dry matter content determined in three parallel samples (step *7*). This was about 5%. The particle fractions (1.4 and 3.2) sedimented at 16,000 *g* were suspended in Tris buffer of pH 7.4, corresponding to 1/20 part of the extracting solution, the final volume was measured, and the protein concentration was determined (step *11*). Using this method we lost negligible amounts of protein.

Results

M. gastrocnemius

Changes in the myofibrillar fraction

The myofibrillar protein content of the intact muscle was 114.2 ± 3.5 mg/g wet weight. The total protein and myofibrillar protein contents of the non-immobilized, contralateral muscles of the experimental animals exhibited only insignificant differences compared to the corresponding muscles of intact animals. In the following the experimental data are compared to the data for the intact animals.

In the early stage of immobilization (5 days) the absolute amount of structural proteins showed a slight decrease 110—113 mg/g (Fig. 1). This corresponds to a relative change of 3—4%. As a result of 10 or 14 days' immobilization, the myofibrillar protein loss rose to 12—13%, while after 28 days it was 25%. This means that as a consequence of disuse of the fast muscle for 4 weeks about one-quarter of the contractile proteins was degraded.

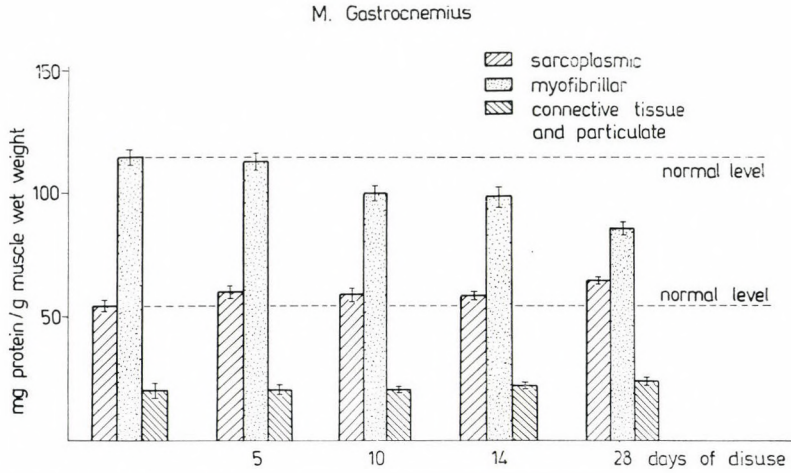


Fig. 1. Diagram of the protein distribution in cellular components during immobilization. Dashed lines represent the average values for intact gastrocnemius muscles

M. Gastrocnemius

Animals	Number analysed	S/M ratio	Myofibr.	Sarcoplasm.	Connective + part.	Total
Controls	14	0.47	60.63 ± 4.0*	28.74 ± 3.6	10.61 ± 2.9	100
5 days of disuse	6	0.52	58.56 ± 2.4	31.01 ± 2.66	10.42 ± 3.04	100
10 days of disuse	6	0.59	55.70 ± 3.3	33.00 ± 4.12	11.30 ± 3.35	100
14 days of disuse	12	0.59	55.10 ± 4.2	32.94 ± 2.15	12.14 ± 4.3	100
28 days of disuse	12	0.75	48.90 ± 2.8	35.50 ± 1.05	13.63 ± 1.2	100

*S.E. of the mean

Percentage distribution of the protein content in the cellular fractions, and alterations in the sarcoplasmic—myofibrillar ratio (S/M) of control and disused gastrocnemius muscles

Changes in the sarcoplasmic protein fraction

The total soluble protein content of the intact G was 54.15 ± 2.1 mg/g wet muscle weight (Fig. 1). The sarcoplasmic protein compartment of the immobilized muscle exhibited a significant increase of about 10% already on the 5th day (60 mg/g). During the subsequent 10 days, the picture did not vary essentially; if anything, there was a mild tendency to decrease. Later (by the end of the fourth week) there was a further increase of 10%. It may be concluded that while disuse of the muscle results in a continuous decrease in the specific (structural) proteins, and the mass of the muscle tissue diminishes with the destruction of the muscle cells, the sarcoplasmic protein content of the remaining cells is enhanced in both absolute and relative senses.

M. soleus

Changes in the myofibrillar fraction

In rabbit, the S is a typical slow tonic muscle. In intact animals the structural protein content of the S was the same as that of the G (114 mg/g) (Fig. 2). Early during immobilization (5th day) the contractile protein compartment tended to be decomposed, similarly as in the fast muscle: 4%. After 10 days, however, the values of the absolute and relative decreases already exhibited a significant difference: 16%. The protein loss slowed down thereafter. Yet, on the 14th day it was still almost double that for the G. On the 28th day the specific protein loss of the S amounted to 30%.

Changes in the sarcoplasmic protein fraction

The total sarcoplasmic protein content of the intact S was 53.5 mg/g. In the early stage of immobilization the change in the soluble compartment was not significant: +2.6%, considerably less than the 10% increase in the same experimental period for the G. Subsequently, the increase assumed a constant nature: by the 10th day it had caught up with the G value, and at the end of the 2nd week it was substantially larger than the G value. In the 4th week the relative increase was more than 25%.

The above results may be briefly summarized as follows. As a consequence of 28 days' disuse the sarcoplasmic and contractile proteins undergo significant changes. The quantity of structural proteins decreases continuously, while the absolute and relative amounts of the soluble compartment increase.

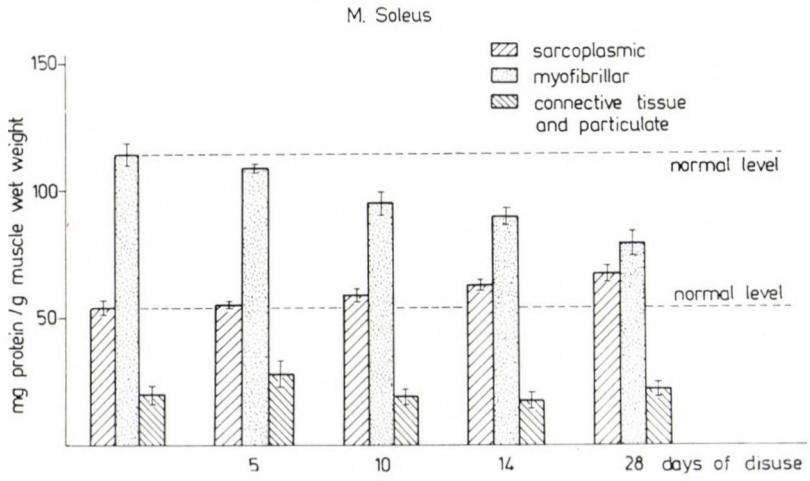


Fig. 2. Diagram of the protein distribution in cellular components during immobilization. Dashed lines represent the average values for intact soleus muscles

M. Soleus

Animals	Number analysed	S/M ratio	Myofibr.	Sarcoplasm.	Connective + part.	Total
Controls	14	0.46	60.08 ± 3.0*	28.53 ± 2.2	10.66 ± 3.2	100
5 days of disuse	6	0.50	56.80 ± 3.5	28.54 ± 1.1	14.64 ± 4.4	100
10 days of disuse	6	0.63	54.42 ± 2.5	34.33 ± 2.5	11.23 ± 2.44	100
14 days of disuse	12	0.69	53.10 ± 3.5	35.94 ± 1.8	10.00 ± 3.12	100
28 days of disuse	12	0.84	47.08 ± 4.8	37.53 ± 3.1	13.15 ± 2.7	100

*S.E. of the mean

Percentage distribution of the protein content in the cellular fractions, and alteration in the sarcoplasmic/myofibrillar ratio (S/M) of control and disused soleus muscles

The adaptations of the functionally different muscles, however, are of different nature in the state of inactivity. The slow or tonic muscles, which perform mainly an antigravitational function, undergo a more substantial alteration as regards the cell composition. In both muscles the S/M protein ratio for the intact muscle cell was 0.47, whereas after 4 weeks' immobilization it was 0.75 in the G, and 0.85 in the S.

Discussion

The recent literature contains much basic information on biochemical differences in the two muscles. For example, phasic and tonic muscles react differently to denervation [1]. In dystrophic mouse, the tonic muscle is less severely damaged than the tetanic muscle [2]. However, the biochemical basis of these differences has not been clarified.

As demonstrated in the preceding publication [6], the mass of the total muscle progressively decreased. On the other hand, the ultrastructural examinations have shown that the degeneration of muscle cells was not uniform; indeed, in adjacent fibrils we found every variant, from fully disorganized structures to regular ones [3].

A method was evolved for quantitative separation and analysis of the protein compartments of the muscle cells and the muscle tissue. The results obtained exhibit good agreement with the literature data which were generally based on the use of selective extraction methods. On summation of the partial results, the total protein contents were recovered in our experiments, and hence the total amount of protein could be accounted for.

On this basis it was established that the protein fractions of the muscle cell undergo characteristic changes during immobilization. The structural protein content of the cells decreases in parallel with the loss in mass of the muscle. This phenomenon can be conceived as the dedifferentiation of muscle cells. As a consequence of the disuse, there is no need for the contractile elements, and so these are gradually degraded by the proteolytic enzymes of the cell. The process of disorganization is faster and more pronounced in the tonic muscles, the muscles performing the antigravitational function. The difference was particularly striking in the first week of immobilization, but it remained significant up to the end of the experiment.

The dedifferentation was accompanied by an accumulation of non-specific (sarcoplasmic) proteins; that is, as the protein is lost there is a relative increase in the non-contractile protein content. The more extensive specific protein loss of the S can be conceived as a compensating mechanism: the relative increase in the soluble proteins is more marked, exceeding 25% by the end of the experiment, in contrast to the 10% increase observed in the G.

In the present experiments the "connective tissue and particle" fraction was classified and examined in one group, though, these are proteins with biologically completely different functions, for our aim was the estimation of the total protein content and, within this, the investigation of the ratio of the sarcoplasmic and soluble fractions.

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EXPERIMENTAL INVESTIGATIONS ON HYPOKINESIS OF SKELETAL MUSCLES WITH DIFFERENT FUNCTION IV.

CHANGES IN THE SARCOPLASMIC PROTEINS

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Abstract

The changes in the sarcoplasmic proteins of the m. gastrocnemius and m. soleus were examined by biochemical methods on the 5th, 7th, 14th and 28th days after plaster cast immobilization of the right hind limbs of adult rabbits. During 4 weeks the soluble/myofibrillar protein ratio increased from 0.47 to 0.75 in the m. gastrocnemius, and to 0.85 in the m. soleus. Evaluation of the relative quantities of the components identified after gel-electrophoresis separation led to the following results: (1) There was no, or no appreciable change in the glyceraldehyde-3-phosphate dehydrogenase, creatine kinase and enolase activities. (2) The enzymes lactate dehydrogenase, aldolase and the glycogenolytic enzymes showed a relative decrease in both muscles. (3) Phosphoglycerate kinase, phosphoglucose isomerase and pyruvate kinase increased in both muscles. (4) Changes of opposite directions were exhibited by myoglobin, myokinase and F-protein. These results provide new data on the biochemical characterization of these functionally different muscles, and on the mechanism of disuse atrophy.

Introduction

Intact motor and proprioceptive innervation and systematic functioning of the muscle fibres are necessary for the maintenance of the biological equilibrium of the skeletal musculature. Muscles which do not function actively undergo atrophy [3, 7, 13].

In earlier investigations, we found that considerable biochemical changes take place during immobilization for 1—4 weeks, primarily in the tonic muscle [20]. With biochemical methods, we demonstrated a decrease in the protein content, and an extensive damage of the contractile system. The muscular atrophy resulting from immobilization leads primarily to changes in the degradation and relative amounts of the low-molecular-weight peptides of myosin and the regulatory proteins [21].

In the present experimental series, we investigated the changes occurring in the components of the soluble compartment of rabbit muscles with different functions (m. gastrocnemius and m. soleus) during plaster cast immobilization of the hind limb.

Materials and methods

The techniques of immobilization and preparation of the muscles were the same as reported previously [18]. Animals were killed on the 5th, 7th, 14th and 28th days after immobilization. In each group there were 5 rabbits, 3 of which were immobilized, the remaining 2 served as controls. The 5- and 7-day experiments were performed in two, the 2,14 and 28-day experiments in three, groups. Thus, a total of 50 animals were subjected to a preparative procedure published previously [20].

After steps *9* or *10* of that scheme, the soluble protein fractions were obtained in a lyophilized state, After measurement of the weight of the dry protein powder, 10 mg samples were taken and dissolved in 2 ml of a 1% *(sodium dodecyl sulphate)* SDS + 1% 2-mercapto-ethanol + +10% sucrose solution by boiling at 100 °C for 1 min. In this way the quaternary structure of the individual proteins was lost, and their subunits were obtained. *Gel-electrophoresis:* the separation was performed with a slab-electrophoresis apparatus constructed in our laboratories [19]. Two 32×13 cm gel slabs, 3-mm thick, can be placed at one time in the bath, and a total of 48 samples can be run simultaneously. Identical quantities (50—100 μg) of the protein solutions were transferred to 7.5% polyacrylamide gel containing 0.1% SDS. Buffer and gel were prepared by the TALBOT and YPHANTIS modification [22] of the method of WEBER and OSBORN [23]. After fixation, the proteins were stained with Coomassie blue, and the individual fractions were then identified with reference proteins prepared from rabbit muscle (commercial products of Boehringer). The following compounds were used: myoglobin; myokinase (EC. 2.7.4.3); triosephosphate isomerase (TPI) (EC. 5.3.1.1); phosphoglycerate mutase (PGM) (EC. 2.7.5.3); α-glycerol phosphate dehydrogenase (GPDH) (EC. 1.1.99.5); lactate dehydrogenase (LDH) (EC. 1.1.1.27); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC. 1.2.1.12); aldolase (ALD) (EC. 4.1.2.8); creatine kinase (CK) (EC. 2.7.3.2); enolase (EN) (EC. 4.2.1.11); phosphoglycerate kinase (PGK) (EC. 2.7.2.3); phosphoglucose isomerase (PGI) (EC. 5.3.1.9); pyruvate kinase (PK) (EC. 2.7.1.40); adenosine deaminase (ADA) (EC. 3.5.4.2); phosphofructose kinase (PFK) (EC. 2.7.1.11); phosphorylase b kinase (EC. 2.7.1.38); and phosphorylase b (EC. 2.4.1.1). The proteinograms were evaluated quantitatively with a densitometer fitted with a Kipp and Zonen integrator. The relative amounts of the individual components were calculated from the density values.

Results

After separation in 7.5% acrylamide gel, the SDS-treated sarcoplasmic preparation of the m. gastrocnemius gave the picture shown in Fig. 1.

The proteinogram shows the presence of 22 fractions. The density data on the individual components are given in Table 1, which contains both the definitely identified sarcoplasmic components and those detected, but not yet identified, in order of increasing molecular weights.

Figure 2 shows a densitogram of the soluble proteins of the normal m. gastrocnemius.

Components were identified with reference enzymes on the basis of the coincidence of the distances of migration. The correlation between the logarithm of the molecular weight and the relative migration is shown in Fig. 3.

The total (preparative) molecular weights of the enzymes were calculated from the structures of the subunits, known from the literature.

It should be noted that the electrophoretic loading per sample was 100 μg, and that the threshold of detectabiling by staining was about 1 μg. Thus, the components present in less than 1% cannot be detected in the pro-

Table 1

No.	E.C. index	Trivial name	Abbreviation	Prep MW×10³	Number of subunits	Subunit MW×10³
1.	—	Myoglobin	—	18.0	1	18.0
2.	E.C.2.7.4.3.	Myokinase	—	21.5	1	21.5
3.	E.C.5.3.1.1.	Triosephosphate isomerase	(TPI)	54.0	2	27.0
4.	—	F protein	—	30.5	1	30.5
5.	E.C.2.7.5.3.	Phosphoglycerate mutase	(PGM)	66.0	2	33.0
6.	E.C.1.1.99.5.	α-glycerol phosphate dehydrogenase	(αGPDH)	67.0	2	33.5
7.	E.C.1.1.1.27.	Lactate dehydrogenase	(LDH)	140.0	4	34.5
8.	—	Unknown	—	142—144	?	35.0
9.	E.C.1.2.1.12.	Glyceraldehyde 3-phosphate dehydrogenase	(TPDH)	144.0	4	36.0
10.	E.C.3.1.2.8.	Aldolase	(ALD)	160.0	4	40.0
11.	E.C.2.7.3.2.	Creatine kinase	(CK)	82.0	2	41.0
12.	E.C.4.2.1.11.	Enolase	(EN)	83.0	2	41.5
13.	—	Unknown	—	—	?	45.0
14.	E.C.2.7.2.3.	Phosphoglycerate kinase	(PGK)	48.5	1	48.5
15.	E.C.5.3.1.9.	Phosphoglucose isomerase	(PGI)	108.0	2	54.0
16.	E.C.2.7.1.40.	Pyruvate kinase	(PK)	228.0	4	57.0
17.	—	Unknown	—	—	?	61.0
18.	—	Unknown	—	—	?	64.0
19.	E.C.3.5.4.2.	Adenosine deaminase	(AMDA)	270.0	4	67.0
20.	E.C.2.7.1.11.	Phosphofruktose kinase	(PFK)	295.0	4	74.0
21.	E.C.2.7.1.38.	Phosphorylase b kinase	—	1000.0	12	80.0
22.	E.C.2.4.1.1.	Phosphorylase b	—	185.0	2	94.0

Experimental data on the soluble proteins of normal muscle.
The components were identified by comparison with standard commercial preparations (Boehringer Mannheim GmbH) in SDS gels. Unidentified fractions were designated as "unknown"

teinogram. In addition, proteins with identical or very similar molecular weights cannot be separated by this method.

As to the soluble proteins of the intact m. gastrocnemius and m. soleus, there are striking differences between the two muscle types: *e.g.* in the myoglobin (peak 1), myokinase (peak 2), F protein (peak 4), PGM and GPDH (peaks 5 and 6), LDH (peak 7), CK and EN (peaks 11 and 12), PGI and PK (peaks 15 and 16), and the glycogenolytic enzymes (peaks 20—22). The changes resulting from immobilization in the two muscle types are indicated by small arrows in the Fig. 4. Arrows pointing upwards and downwards indicate

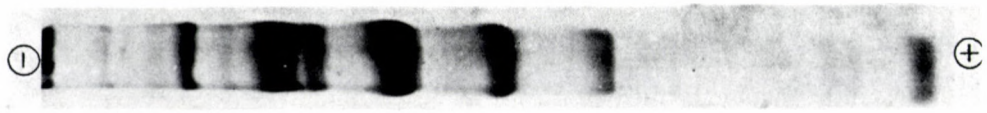

Fig. 1. Electrophoretic pattern of normal gastrocnemius muscle soluble proteins. Protein load: 100 μg. PAGE was performed in 7.5% gel according to TALBOT and YPHANTIS Ref. [22]

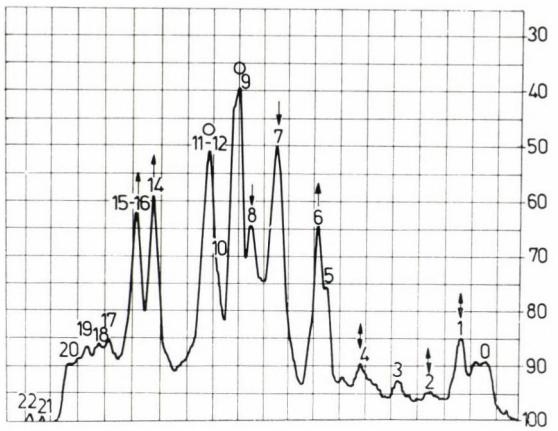

Fig. 2. Densitometric scan of a representative normal gastrocnemius soluble pattern. Arrows indicate the trends of changes. The numbers correspond to those in Table 1

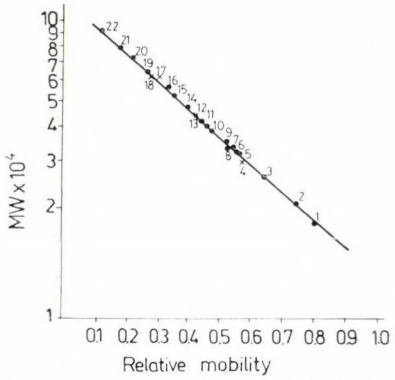

Fig. 3. Plot of logarithm of the molecular weight and relative migration of components resulting from SDS gel electrophoresis. Numbers as in Fig 2. Asterisks (x) indicate unknown proteins

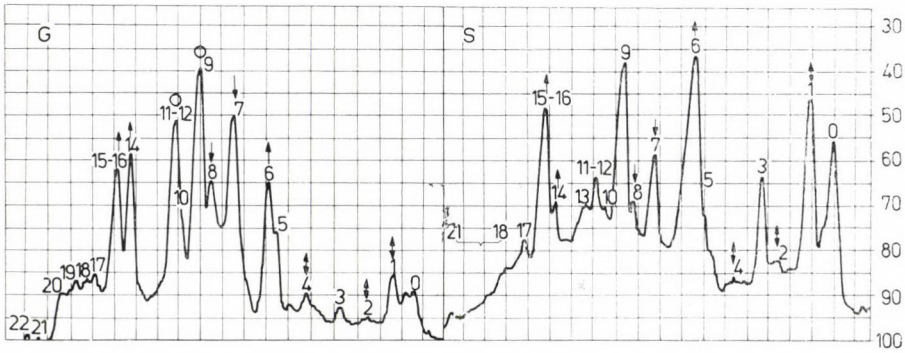

Fig. 4. Comparison of the densitogram of intact gastrocnemius and soleus muscle. Symbols as in Fig. 2

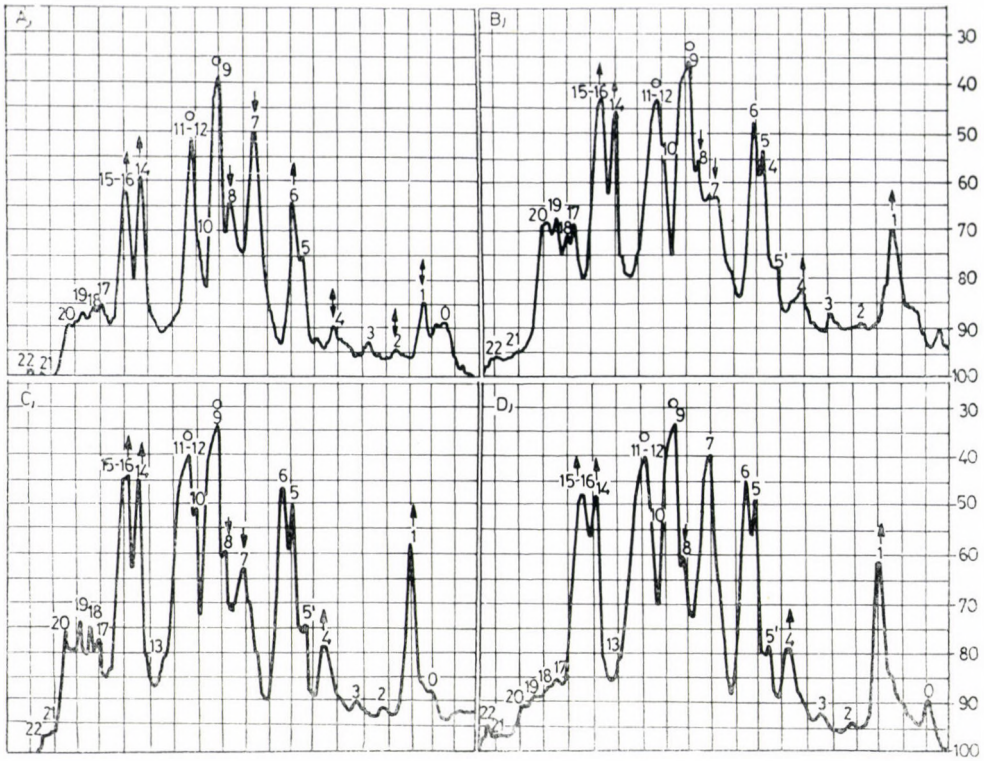

Fig. 5. Densitograms of the soluble proteins of gastrocnemius, demonstrating the changes during immobilization. Symbols: A, B, C, D indicate the samples originating from intact muscles (A), and muscles disused 7 (B); 14 (C) and 28 (D) days

increases and decreases, respectively; 0 exhibits practically no quantitative change.

Figure 5 presents densitograms of the soluble proteins of the immobilized m. gastrocnemius. The amount of myoglobin constantly increased during the experiment. F protein displayed a similarly increasing tendency. Peaks 5 and 6 (PGM and GPDH) increased slightly. The amount of LDH decreased continuosly. The quantities of GAPDH and of CK + EN remained approximately constant. The change in ALD (peak 10) cannot be measured exactly. The amounts of PGK, PGI and PK increased continuously. The high molecular weight components are represented by phosphorylase b kinase and phosphorylase b. Quantitative measurement of these is unreliable because of the unsatisfactory separation, but they exhibit a progressively decreasing tendency.

The noteworthy changes in the m. soleus are the decreasing tendencies of myoglobin and F protein (Fig. 6), in contrast to the tendencies in the m. gastrocnemius. An as yet unidentified protein (peak 8) with a molecular weight

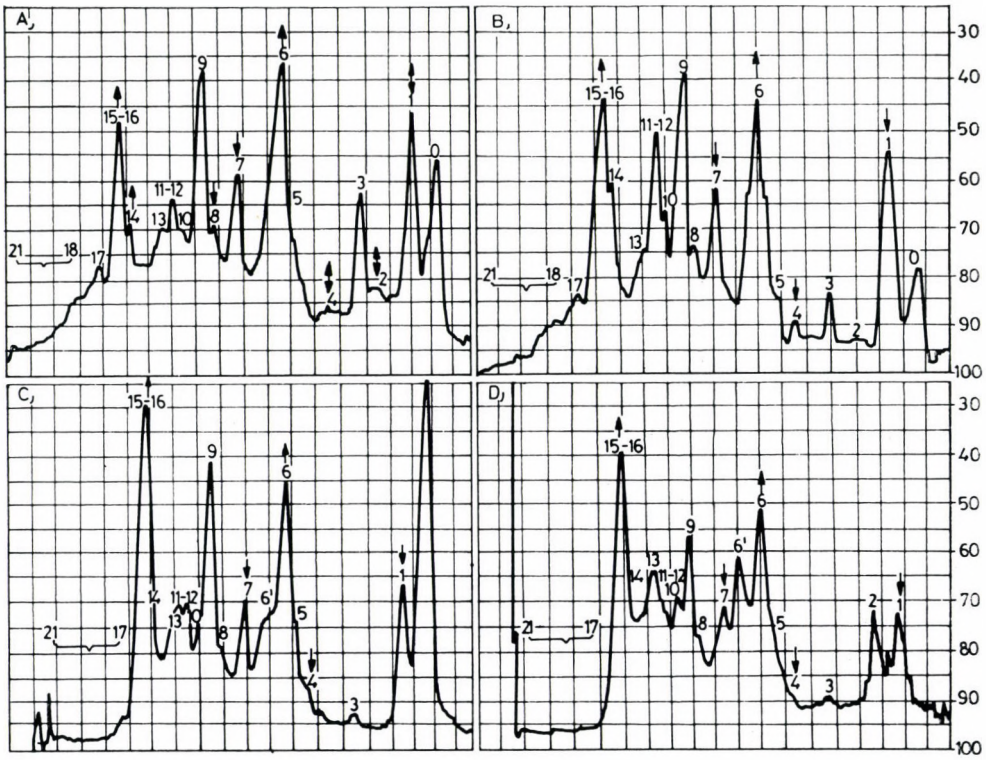

Fig. 6. Densitograms for the soluble proteins of soleus muscle during immobilization. Symbols as in Fig. 5

of 35 megadalton exhibits a decrease, in agreement with that described for the m. gastrocnemius. The decrease in ALD can also be well evaluated. As in the case of the m. gastrocnemius, the PGI and PK increase. The glycogenolytic enzyme cannot be determined exactly.

Tables 2 and 3 contain the integrated data relating to the above changes. The values are given as percentage distributions.

Discussion

We have demonstrated [20] that, as a result of immobilization for 5—28 days, sarcoplasmic proteins accumulate in muscles. This change leads to an increase in the sarcoplasmic/myofibrillar protein ratio. From a biological aspect, the phenomenon may be explained by the cessation of the specific function of the muscle (change of position, antigravitational function), which causes a dedifferentiation of the cells.

Table 2

M. gastrocnemius

No.	Protein	Normal	5 days	7 days	14 days	28 days
1.	Myoglobin	1.7	5.2	7.0	7.6	7.8
2.	Myokinase	3.1	3.7	1.8	1.2	1.2
3.	TPI	1.6	1.3	1.6	1.5	1.3
4.	"F" protein	2.9	2.4	4.4	5.1	4.8
5.	PGM	3.7	9.9	6.1	4.9	4.3
6.	α-GPDH	6.6		6.6	6.8	6.9
7.	LDH	13.5	16.6	3.4	7.0	13.8
8.	Unknown	8.3	8.3	4.2	3.4	4.0
9.	GAPDH	14.0	17.3	12.3	12.2	11.7
10.	ALD	15.1	14.9	2.4	2.3	16.4
11.	CK			12.8	14.5	
12.	EN					
13.	Unknown	—	—	—	—	—
14.	PGK	4.7	5.6	7.8	6.7	6.6
15—16.	PGI + PK	7.9	9.1	9.0	10.1	10.6
17.	Unknown	2.0	2.2	3.9	2.2	2.8
18.	Unknown	2.0		2.0	2.6	1.5
19.	AMPDA	2.8	1.7	2.9	3.4	1.1
20.	PFK	1.7	0.7	1.6	2.0	0.7
21.	Phosphorylase b kinase	1.4	1.0	0.8	0.8	
22.	Phosphorylase b				1.0	1.6

Percentage distribution of the components of intact and disused gastrocnemius soluble proteins. The values result from integrated data (Kipp and Zonen densitometer) Abbreviations: see Table 1

The proteins of the sarcoplasm perform many different functions and are extremely heterogenous biochemically. It is obvious, therefore, that disuse will exert different effects on the equilibrium of protein synthesis and decomposition in both tonic and tetanic muscles. In our best knowledge, publication referring to similar examinations have not been published yet.

BESTER and GEVERS [2] studied the synthesis of the myofibrillar and soluble proteins in the skeletal and cardiac muscles of neonate normal and polymyopathic hamsters. The soluble proteins were similarly separated by one-dimensional gel-electrophoresis. Their densitometric picture was similar to ours, but they did not evaluate the proteinogram qualitatively. BASS et al. [1] and GORI et al. [11] used the hormone-sensitive m. bulbocavernosus and m. levator

Table 3

M. soleus

No.	Protein	Normal	5 days	7 days	14 days	28 days
-	Unknown	7.9	8.4	3.7	18.7	16.1
1.	Myoglobin	7.7	7.6	7.5	5.9	3.2
2.	Myokinase	1.7	1.0	2.1	3.3	2.1
3.	TPI	4.8	4.5	2.4	3.9	3.1
4.	"F" protein	3.7	1.1	1.4	1.3	0.8
5.	PGM	1.0	1.0	0.8	0.7	—
6.	GPDH	17.7	20.4	12.0	10.9	6, 11, 84 6' 7, 3
7.	LDH	7.2	7.4	7.6	4.8	4.3
8.	Unknown	3.9	3.4	4.0	1.2	1.0
9.	GAPDH	8.3	11.4	11.0	13.0	9.1
10.	ALD	3.1	2.0	2.8	1.7	0.8
11.	CK	2.1	2.0	8.3	1.9	2.6
12.	EN					
13.	Unknown	4.7	4.8	1.2	0.7	4.1
14.	PGK	2.8	2.4	6.1	—	
15.	PGI	9.2	11.2	12.2	20.1	16.4
16.	PK					
17.	Unknown	3.1	3.0	3.2	—	—
18.	Unknown	2.6				
19.	AMPDA					
20.	PFK	8.4	8.3	12.5	12.1	12.9
21.	Phosphorylase b kinase					
22.	Phosphorylase b					

Percentage distribution of the components of intact and disused soleus soluble proteins.
Data as in Table 2

ani of the rat as models for disuse atrophy. As a result of castration, the sarcoplasmic and contractile proteins of the muscles decreased, though, ultrastructural examinations demonstrated the overproduction of the sarcoplasmic reticulum. The above authors also observed a general reduction in the energy providing enzymatic activity. We have found that immobilization leads to decreases in the ALD and LDH activities, and increases in the acid phosphatase and other hydrolytic enzyme activities [17].

The degradation of proteins of muscles with different functions is regulated by the activity of specific and non-specific proteases. In the normal,

functioning muscle, the mechanism regulating synthesis and degradation are in equilibrium. Numerous authors [5, 8, 9, 10, 12, 16] have investigated the turnover rates of enzymes and sarcoplasmic proteins. It was established by CITOLER et al. [4] and DÖLKEN and PETTE [6] that the higher rate constants of degradation of the individual enzymes, *e.g.*, phosphofructokinase, cannot be brought into correlation with the molecular weights of the subunits, for the enzymes with higher molecular weights may be degraded faster than those with lower molecular weights. PETTE et al. [14, 15] found interesting correlations regarding the properties of the soluble enzymes of muscles of different types. The enzymes of the constant-proportion group are characterized by the metabolic systems of the muscles with different functions. The proportions of these are more constant than the activity values for the individual enzymes. We consider it correct and constructive to study PETTE's constant-proportion group system and the turnover rates of the relevant enzymes.

Evaluating our experimental results, we may point to the interesting coincidence of opposite tendencies and great concentration differences in the two types of muscles in the case of several proteins. The highest differences were exhibited by myoglobin, F protein and myokinase. In the m. gastrocnemius the myoglobin and F protein levels are low and both increased progressively on immobilization. In the m. soleus, which is rich in myoglobin and F— protein, and disuse is followed by a continuous decrease in the relative amounts of both proteins.

We attribute greater importance to the proteins of this particular group. The proteins changing in the same direction in both muscles are mainly the glycolytic enzymes the biochemical functions of which are the same in both muscle types, although they may differ in amount. The opposite changes, reflecting metabolic differences between the two muscles, eventually lead to disappearance of the biochemical differences responsible for the tonic or tetanic character of muscles. Accordingly, the opposite tendencies indicate dedifferentiation of the muscle cells.

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MEMBRANE ALTERATIONS IN THE SEMINAL VESICLE CELLS OF THE MOUSE DURING COOLING IN VITRO

SHORT COMMUNICATION

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(Received 1976—11—11)

The binding of ribosomes to the membranes of the endoplasmic reticulum has been reported to change sensitively under conditions leading to cellular injury [3, 4]. In the course of *in vitro* experiments performed in our laboratory, it was noticed that cooling of the cells *per se*, without addition of any injurious agent to the incubation medium, induces morphologically detectable alteration of the rough-surfaced endoplasmic reticulum (RER). The results of an experiment on this phenomenon are presented here.

Slices of the seminal vesicles of male CFLP mice were incubated either in Krebs—Ringer bicarbonate solution or in medium 199, then fixed in 0.1 M cacodylate-buffered 5% glutaraldehyde, postfixed in buffered osmium tetroxide solution, and embedded in Araldite.

No significant differences were found in the fine structure between the cells incubated *in vitro* for 2 h at 22 °C and the controls fixed immediately after killing. Temperatures over 22 °C were not used in these experiments because autophagic vacuoles were occasionally detected in cells incubated at 26—37 °C.

Changes of the RER membranes were observed in the cells when freshly cut tissue pieces were placed into the medium at 22 °C which was then cooled to 6—8 °C within 20—25 min. and kept at this temperature for 95—100 min. The most frequently encountered alteration of the cooled cells was the absence of ribosomes in certain areas of membranes of RER cisternae. Sometimes, the ribosome-depleted areas emerged only on one leaflet of the membrane pair bordering the cistern. The smooth portions of RER cisternae often became curved, or showed a spiral pattern encircling a part of the cytoplasm (Fig. 1, 2). The composition of incubation media had no effect on these membrane alterations. They equally developed in cells incubated in Krebs—Ringer solution or in medium 199.

To determine the temperature limit below which the membrane changes occur we cooled separate tissue samples from 22 °C to 18, 17, 15, 13 or 12 °C. Membrane alterations of RER were observed in the cells cooled below 15 °C. When tissue samples cooled and kept at 6—8 °C for 120 min. were transferred

into a warmer medium the cold-induced smooth membrane areas disappeared within 5—8 min at temperatures higher than 17 °C.

It may be concluded that cooling of seminal vesicle cells *in vitro* induces detachment of ribosomes from certain areas of the RER and folding of the resulting smooth membranes around parts of the cytoplasm. The data available at present are not sufficient to explain the mechanism whereby cooling changes the membranes of RER. Some reports of the literature, however, may be re-

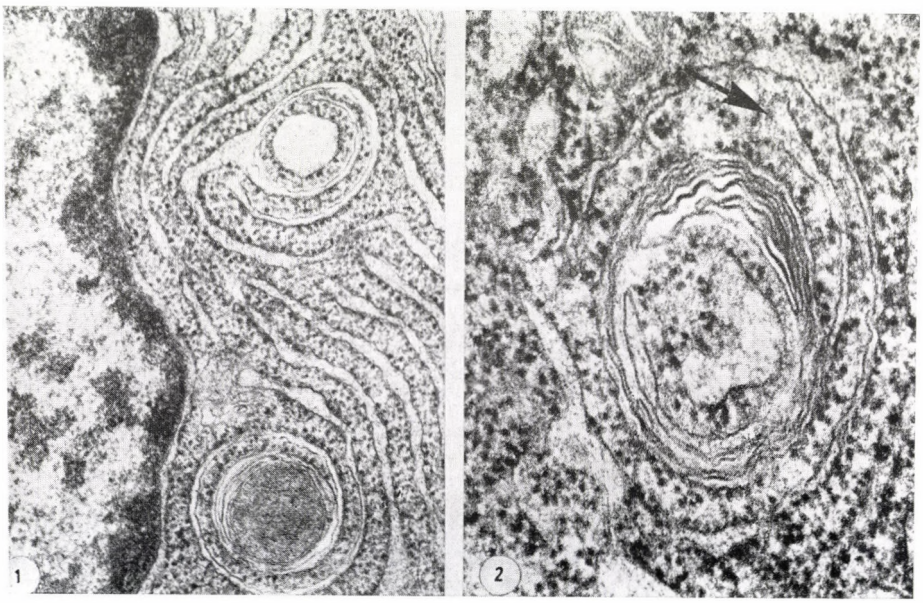

Fig. 1. Encircled portions of the cytoplasm. The bordering membranes are connected with cisternae of the RER. ×35,000

Fig. 2. Area of the cytoplasm surrounded by membranes derived from a cistern of the RER (arrow) ×35,000

levant to this point. Lipids of artificial and biomembranes have been shown to undergo phase changes from fluid to crystalline state during chilling [1, 2]. These temperature-dependent phase transitions were reported to take place in RER membranes at 16—22 °C [5, 8, 9]. Examination of freeze-etched tissues revealed the emergence of smooth areas, free of intramembranous particles, in the biomembranes of cooled cells which rapidly disappeared upon rewarming [6, 7, 8]. Alterations described here occurred in seminal vesicle cells within a range of temperature found characteristic of phase-transitions in other cell types. The present data suggest that the morphological changes observed may be related to the cold-induced phase-transition events in certain places of the membranes of the RER.

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SEPTAL PROJECTION TO THE ARCUATE NUCLEUS OF THE HYPOTHALAMUS*

SHORT COMMUNICATION

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The ventral part of the septum projects its fibres to the medial forebrain bundle which pass through the lateral preoptic area and reach the level of midbrain in the rat [4]. Using the electron microscope it was found that a bilateral cut along the arcuate nucleus produced degenerating axon terminals in the arcuate nucleus [1]. Furthermore, electrical stimulation of the medial forebrain bundle at the level of the lateral preoptic area suppressed both neural activity in the arcuate nucleus and LH release [2]. These facts suggest that neural fibres from the septum may pass through the medial forebrain bundle to reach the arcuate nucleus of the hypothalamus. Therefore, the following experiments were undertaken in order to examine the fibre connection between the septum and the arcuate nucleus.

Experiment 1. Electrolytic lesion (2mA—15 sec) was made unilaterally in the medial forebrain bundle at the level of the lateral preoptic area. Degenerating axon terminals were examined in two- or four-days-survival rats (3—3 animals).

Experiment 2. Electrolytic lesion (2mA—15 sec) was made unilaterally in the medial septum in two rats, in the dorsolateral septum in one rat, and in the dorso- and ventrolateral area of the septum in three rats. Degenerating axon terminals were examined in two- or four-days-survival animals.

The rats (female adult, Wistar Strain) were anesthetized with hexobarbital (VEB Arzneimittelwerk, Dresden, DDR, 100 mg pr kg body weight) two or four days after the operation and perfused with a fixative containing 1% paraformaldehyde, 1% glutaraldehyde, 1% polyvinylpyrrolidone in 0.1 *M* phosphate buffer (pH 7.4 at 20 °C). The brain was removed the following day, and the areas of the arcuate nucleus and median eminence were removed as small blocks. These blocks were washed with 0.1 *M* phosphate buffer and post-fixed with 1% phosphate buffered osmic acid solution. The blocks were

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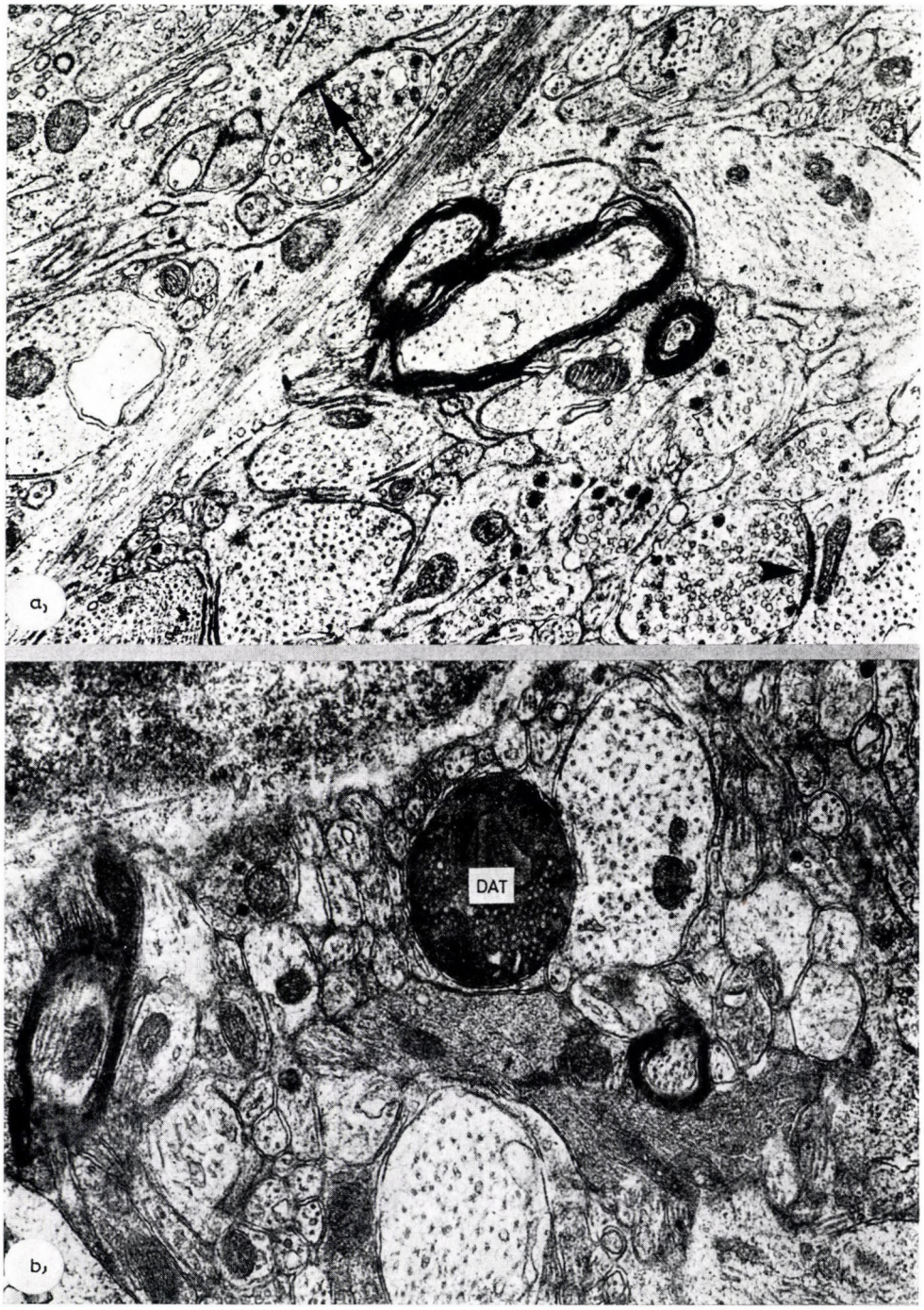

Fig. 1a Axo-somatic (arrow) and axo-dendritic (arrow head) synapses in the arcuate nucleus of the hypothalamus of the intact rat

Fig. 1b Degenerating axon terminal (DAT) in the arcuate nucleus of a four-days-survival rat with a unilateral electrolytic lesion in the medial forebrain bundle at the level of the lateral preoptic area

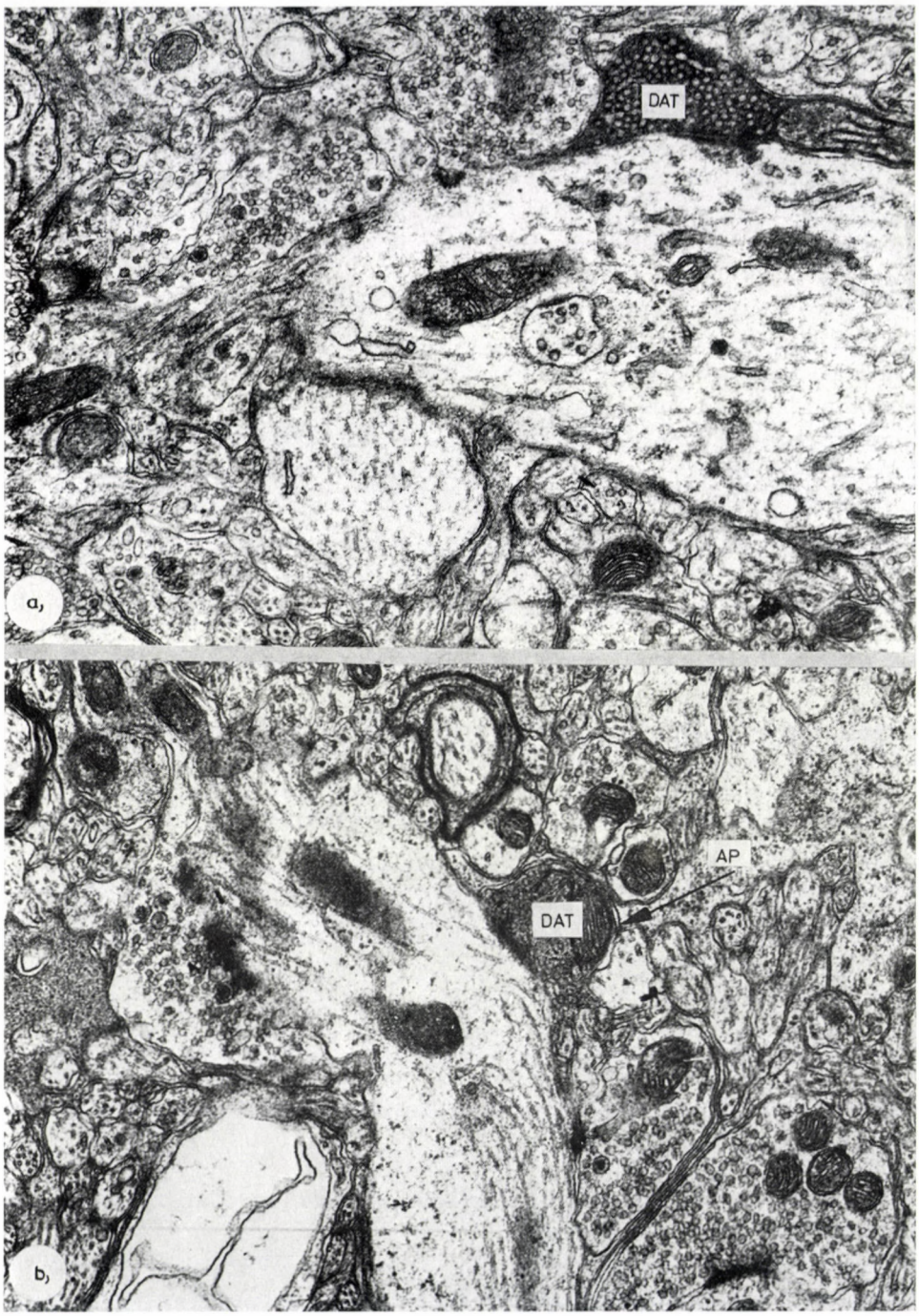

Fig. 2. Degenerating axon terminal (DAT) in the arcuate nucleus of two-days-survival rats having a unilateral electrolytic lesion in the ventrolateral *(a)* or in the dorsolateral *(b)* part of the septum. In *Fig. 2b*, an astroglial process (AP) attaches to the degenerating axon terminal

gradually dehydrated in alcohol and embedded in Durcupan (Fluka). Sections were made with the LKB Ultramicrotome III. The semithick sections were stained with toluidine blue for histological examination. The ultrathin sections were contrasted with uranyl acetate and lead citrate for examination of ultrastructure of the arcuate nucleus with the TESLA BS 242 electron microscope. After removal of the arcuate-median eminence region, brains were frozen; serial frontal sections were cut and stained with toluidine blue for histological localization of the lesions.

Two types of synapses can be distinguished in the arcuate nucleus of the hypothalamus in the intact rat (Figure 1*a*): one is the axo-somatic and the other is the axo-dendritic synapse. Degenerating axon terminals from the unilateral lesion of dorsolateral and/or ventrolateral parts of the septum (Fig. 2) appeared in the anterior and medial part of the arcuate nucleus bilaterally. Furthermore, unilateral lesion of the medial forebrain bundle at the level of the lateral preoptic area also produced degenerating axon terminals (Fig. 1*b*) in the entire area of the arcuate nucleus bilaterally. No degenerating axon terminals were found in the arcuate nucleus after unilateral lesion of the medial septum. The hippocampal projection to the medial forebrain bundle does not enter the arcuate nucleus [3], but only post-commissural fibres from the hippocampus terminate in this nucleus [5]. Therefore, the present findings together with those of RAISMAN [4] infer that nerve fibres which arise in the dorsolateral and ventrolateral parts of the septum enter the medial forebrain bundle and its fibres make a cross at the supra- and/or retro-chiasmatic area and reach bilateral sides of the arcuate nucleus of the hypothalamus. Considering the termination, this septal projection could be best named as septo-tuberal tract.

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RECENSIONES

The Pathology of Fishes

Ed. WILLIAM E. RIBELIN and GEORGE MIGAKI. The University of Wisconsin Press, 1975. pp. 1004.

Though studies on fish diseases have started already 70 years ago in Europe at different Veterinarian Highschools (München 1904, Budapest 1905), a really noteworthy development in this field has been reached only during the last two decades.

There has been since 1962 within the framework of the International Bureau of Animal Health a permanent Committee for fish pathology. This Committee has already organized several International Symposia. In 1953, there was in America the first Symposium on Fish Diseases.

Thereafter, a teamwork started with the participation of researchers of comparative pathology: physicians, veterinarians and biologists, to work up the most important questions of fish pathology. This tremendous work has been published in a large book entitled "The Pathology of Fishes" edited by W. E. RIBELIN and G. MIGAKI with the collaboration of 57 different authors.

This book contains several excellent photographs. Starting with a general presentation of comprehensive fish pathology, the bulk of the work can be divided into six main sections.

Part 1 presents specific fish diseases caused by bacteria, fungi and parasites. A special chapter is devoted to viral fish diseases from clinical, histopathological and comparative pathological viewpoints.

Part 2 contains the main diseases of important fish species of the States as Channel catfish and different salmonids.

In part 3, the diseases of organ systems: gills, heart, muscles, kidneys, eyes, skin, liver and spleen are considered besides the pathological importance of melanin formations.

In part 4, there is a discussion of the chemical and physical factors as radiation, heat, ammonia, pesticides, mercury, cadmium and drugs noxious to the health of fishes.

Part 5 is devoted to nutritional diseases caused by failures in feeding, starving, vitamin C deficiency, nutrition myopathy, visceral granuloma of the trout and the calcification of the kidneys.

In the last (sixth) section, there is a description of tumours, particularly concerning comparative pathology.

Besides the descriptive parts supplemented with an extensive list of literature, there is a very useful discussion of each topic explaining the opinions of well-known specialists. The work is completed by a list of the participants and of contents.

This excellent book, supplying a great want can be recommended to institutes and researchers in the field of comprehensive pathology.

L. BUZA (Budapest)

MACDONALD, A. G.: **Physiological Aspects of Deep-Sea Biology**

Cambridge University Press, Cambridge (1975), pp. 450, 212 figures, 86 tables.

This is the first book that demonstrates the physical and physiological properties as well as the adaptability of deep-sea animals in detail. The author writes about the special fauna of the most extensive biotope in this world in seven chapters. All the chapters contain the most recent results of researches.

Chapter 1 shows the deep sea as biotope and its characteristic animals, for example, Porifera living in a depth of 5378 m and fishes occurring even in 7000 m depth.

In Chapters 2 and 3 the adaptation of animal cells and tissues to high-pressure is discussed.

Chapter 4 treats of the physiology of deep-sea animals.

Chapter 5 demonstrates the function of the sense organs of various groups of animals and the different ways of their movement.

In Chapter 6 the nutrition of deep-sea animals is reviewed. There are some interesting data on the primary production. In the sea and oceans, owing to the absence of light, the primary production — the plant life — does not extent in general beyond 30—50 m depth, never beyond 100 m.

Chapter 7 describes the modern equipments suitable for collecting deep-sea animals and taking photographs of them at ocean deeps of several thousands metres as well as the equipments utilizable for the investigation of the physical, chemical and hydrobiological characteristics of sea-water. Further, this chapter refers to the man-controlled submarines, which can be used in deep-sea investigations.

At the end of the book, there is a comprehensive enumeration of references as well as an author and subject index.

This monograph can be recommended for everyone who intends to deal with the special biology of deep-sea animals.

B. PÉNZES (Budapest)

Marine mussels: their ecology and physiology

Ed. BAYNE, B. L. Cambridge University Press, London (1976), pp. 506, figs 142, tabl. 46, £ 22

This comprehensive review on marine mussels, mainly on *Mytilus* and closely-related species, was composed to the initiative of the Marine Productivity section of the International Biological Program. The book is divided into ten chapters discussing problems at rather different levels, written by specialists of the field.

The first chapter deals in short with The "mussel" form and habit (YONGE, C. M.). The parts Ecology (SEED, R.), The biology of mussel larvae (BAYNE, B. L.), Physiology of feeding, respiration, circulation, excretion and some other processes (BAYNE, B. L., THOMPSON, R. J., WIDDOWS, J.), Energy metabolism (GABBOTT, P. A.) and Population genetics (LEVINTON, J. S., KOEHN, R. K.) give detailed and critical information about recent results and earlier literature, especially of those which were published in English. The parts Mussels and pollution (ROBERTS, D.) and Cultivation (MARON, J.) review important practical questions both from the point of view of mussel growth, development and cultivation, and also of water purification. A more detailed description of this latter problem would have been significant, besides ecology and production, also for environmental control.

The book gives a good insight into the recent methods and results of many laboratories, especially on the topics of energetics and some parts, of physiology, and calls attention to a number of unsolved questions. Nevertheless, there is a feeling of deficiency because of the nearly total omission of neuromuscular physiology and neural regulation. This is the more surprising because the adductors play a key role in the functioning of the mussels and also neural mechanism cannot be restricted to the ciliary and heart regulation.

The presentation of the results is well documented, each chapter is based on detailed experimental background and high number of relevant references. The book can be recommended first of all for marine biologists but also for ecologists and comparative physiologists interested in fresh-water life, working either in basic research or in water management, or taking university courses.

J. SALÁNKI (Tihany)

PURCHON, R. D.: **The Biology of the Mollusca**

Pergamon Press, Oxford—New York—Toronto—Sydney—Paris—Frankfurt (1977)

International series in pure and applied biology: Zoology division; V. 57. pp. 560, figs 185, tables 21, $ 35.00

This second edition incorporates several new aspects and results achieved during the past 10 years. Based on discoveries of a half century research, it containes 8 reviews of selected topics of Molluscan Biology. These topics are: Form and function of the mantle cavity and

associated organs; Feeding methods and adaptive radiation in the *Gastropoda;* Feeding methods and evolution in the *Bivalvia;* Adaptive radiation in the pollysyringian bivalves; Digestion; Reproduction; Distribution of molluscs. The functions of the nervous systems in the dibranchiate *Cephalopoda.* In an Appendix of about 100 pages also the gross anatomy of all molluscan classes is desribed.

The papers are well concentrated, comprehensive reviews with strong comparative and evolutionary aspects, rendering primarily to understand not single processes but to emphasise the unity of diversities and variations for the same function in different species.

All the topics are based on a large number of descriptive and experimental reports (the references are given at the end of each chapter), and rich illustration is taken from the originals. However, as far as the reference literature is based nearly exclusively on publications appeared in English, a number of closely related and important earlier and recent results (and authors) are neglected.

The eight selected chapters cannot fulfil the requirement of a general and full-scale review on biology of the *Mollusca*, but everybody who is interested in the included topics will find this book as a source of valuable general information and exact data with a very good comparative zoological approach.

J. Salánki (Tihany)

Delucchi, V. L.: **Studies in biological control**

International Biological Programme 9.

Cambridge University Press, Cambridge—London—New York—Melbourne (1976), pp. 304. £ 13.00

This work has been done in the frame of an international programme in which hundreds of entomologists and more than thirty countries are collaborating. Some studies of the book resume in five programmes the results of 7—8 years' work. In the first part of the book we find a list of collaborators, followed by an introduction in which Delucchi gives a summary of the five themes and a chapter entitled "Definition and planning of the project" (Waterhouse).

The first project, Fruit flies, is coordinated by M. A. Bateman. He summarizes the results in a large chapter. Sub-titles: Life-table studies and pupal mortality. Significance of fly marking of oviposition site. Response to colour stimuli. Adults movements. Sexual behaviour of pest tephritids. Population and ecological genetics.

The second project is coordinated by M. Machauer and M. J. Way: *Myzus persicae* Sulz., an aphid of world importance. The results of the following subjects are discussed: General biology and population dynamics of *Myzus persicae.* Biological methods of aphid control. Integrated control of *Myzus persicae.*

The third project, Rice stem-borers is coordinated by K. Yasumatsu. Subchapters: The study area; Taxonomy and distribution of rice stem-borers and their natural enemies; The rice ecosystem; Ecology of rice stem-borers; Natural control of rice stem-borers; Feasibility of integrated control; Rice production and stem-borer damage.

The fourth project, Armoured scale insects is coordinated by P. de Bach and D. Rosen. Subchapters: Background; The natural enemies of armoured scale insects; Biological control attempts and their consequences; Exchange of natural enemies; World list.

The fift project: Spider mites. Coordinators: N. W. Hussey and C. B. Huffaker. Chapters: Biology of spider mites; Biology and ecology of natural enemies of spider mites; Effect of pesticides on spider mites and their natural enemies; Biological control of spider mites of various crops.

The title of the last chapter is Concluding remarks, written by M. J. Way.

The book beautifully demonstrates that the new system of defence needs the profound knowledge of the ecology, ethology and physiology of the species. In our days, successful research needs central international coordination.

The book closes with hundreds of literary references, index of animal names and subject index.

Gy. Sáringer (Keszthely)

KLAUS FUCHS-KITTOWSKI: **Probleme des Determinismus und der Kybernetik in der molekularen Biologie**

VEB Gustav Fischer Verlag Jena (1976), Second revised and enlarged edition, pp. 491, figures 31, DM 30,30.

The author has produced a pioneering work with the philosophical investigation of molecular biology, the newest, most rapidly developing field of biology. The book is divided into two parts. The first one involves the analysis of relationships between determinism, technical automations and living organisms. Part 2 analyses the cell as cybernetic system, investigates the problems of determinism, the ways of regulation in single cells and the evolution. On the basis of dialectic determinism with the help of cybernetics the author succeeds in finding new solution using the results of molecular biology and of such old questions as, *e.g.*, what is the real relationship between the machine and the living organism; what is the difference in the regulation of the work of biological and automatic machines; whether adaptation and the capacity of learning are excusively the living organisms' own, *etc.* As Professors L. RAPAPORT and H. LEY point out in the Preface here the task of the philosopher is to analyse and specify the problems and conceptions at the point of intersection of information theory, regulation technics, biology and psychology, and thus contribute to the development of both the gnoseology and the specialized sciences. Professor Fuchs-Kittowski follows a new path in many respects, provides solutions of complex problems and successfully serves both philosophy and natural science.

L. KOVÁCS (Debrecen)

AINSWORTH, G. C. : **Introduction to the history of Mycology**

Cambridge University Press, Cambridge—London—New York—Melbourne, pp. 359, with 106 figures and 1 coloured plate, £ 11.

Dr. AINSWORTH (Formerly Director of the Commonwealth Mycological Institute, Kew) gives a straightforward account of the main views held about fungi for the past three millennia and the development for the last 250 years.

Although much has been written during the past 200 years on the history of mycology in general and of diverse special aspects of the study of fungi, this literature is widely scattered and much of it difficult of access. An attempt is here made to bring together, for the first time in one volume, a documented outline of the development of the main branches of mycology, with emphasis on the solution of the major problems which have confronted students of fungi and novel discoveries which have given new insights. A number of important themes form the basis of the account, each one being traced from early times to the present days. With the main exception of the last few decades, which is treated more lightly, the development of mycology as reflected in the published record is covered as evenly as possible, and while a knowledge of the essentials of mycology is assumed, technicalities have been kept to the minimum because, besides mycologists, other biologists and historians of science will find interest in the history of the study of an important group of organisms. The themes are arranged in the chronology of their appearance in mycological studies. Most chapters are thus self-contained.

Chapters deal with the status of fungi, their morphology, nutrition and sexuality. These are follows by chapters covering the relationships of fungi to human affairs as pathogens and toxins for man, animals and plants and their use as food, and in medicine and industry. Finally, fungal classification and the organization for mycology are considered. There are an extensive chronological bibliography and names index.

G. BOHUS (Budapest)

FRANK FENNER: **Classification and Nomenclature of Viruses**

S. Krager., Basel (Switzerland), (1976), pp. 115. DM 15

This book brings up-to-date the second report of the International Committee on Taxonomy of Viruses (ICTV), one of the most important activities of the Virology Section of the International Association of Microbiological Societies. The book contains chapters dealing

with the list of officers and members of the ICTV, president's report and the rules of nomenclature of viruses. Of special interest are the chapters on several viruses, and the meanings of the symbols used in the virus cryptogram. The virus cryptogram consists of four sets of symbols with the following meanings:

1st pair: Type of nucleic acid/strandedness of nucleic acid

2nd pair: Molecular weight of nucleic acid (in millions)/percentage of nucleic acid in infective particles

3rd pair: Outline of particle, presence of envelope or occluding protein/outline of "nucleocapsid" (the nucleic acid plus the protein most closely in contact with it)

4th set (2 or 3 terms): Kind(s) of host infected/mode(s) of transmission/kind(s) of vector.

In this excellent book, there is a modification in both the third pair (or 3rd term), which is the inclusion (or omission) of the letter *e*, to indicate whether or not the virion is enveloped and the letter *e* to indicate virions occluded in a protein matrix; and the fourth set (or last term), by specifying whether the usual mode of transmission is congenital (C), by intestinal tract (I), by contact (O), by respiratory tract (R) or *via* an invertebrate vector (Ve). Cryptograms have been prepared, where possible, for each type species and for the higher taxa. The book contains the taxonomic status, vernacular name, approved name, cryptogram and the main characteristic of the viruses of vertebrates, invertebrates, bacteria and plants, with the main references and with the subject index (families, genera and groups).

This excellent book is of an invaluable help to virologists.

J. Horváth (Budapest)

Mclaren, Anne: **Mammalian Chimaeras**

Developmental and Cell Biology 4. Cambridge University Press, Cambridge (1976), pp. 154, £ 8.00.

In the past few years some exciting new areas of the modern biology have been expanding very rapidly. One of them is the analysis of chimaeric animals. Chimaeras can be formed by aggregating or combining cells from different embryos at a very early stage of development, or can arise spontaneously at fertilization.

The book is mainly concerned with two types of experimental study for which chimaeras provide uniquely suitable material. The first belongs to the field of experimental embriology, making use of the two cell populations to trace the origin and fate of tissues and cell lineages in development. What is a chimaera? (Chap. 1), Techniques (Chap. 2), Early development (Chap. 3), Sexual development (Chap. 4).

The second is an aspect of developmental genetics and seeks to analyze how genetically different cells collaborate to form an adult animal. Pigment patterns (Chapt. 5), Other morphological characters (Chap. 6), Immunology and blood (Chap. 7), Tumours (Chap. 8), Chimaeras versus mosaics (Chap. 9), Distribution of cell populations (Chap. 10), Spontaneous chimaeras (Chap. 11), Perspectives (Chap. 12).

Quotation from the Preface: "This book is on a very specialized topic. The few dozen people in the world who have worked with experimental chimaeras will share my enthusiasm for their beauty, their unexpectedness, the insight that they provide into old questions, and above all for new questions that they continually raise, questions that one never dreamt existed in the days when an individual had two parents only. If this book communicates some part of this fascination to a few other people interested in mammalian development (and who isn't?), I shall be satisfied."

You may, Dr. Mclaren.

V. Csányi (Budapest)

Tribe, M. A., Tallan, I., Eraut, M. T. and Snook, R. K.: **Basic Biology Course, Vol. 3**

Cambridge University Press, Cambridge (1976), pp. 119, figs. 41

This volume is the third part of the Biological Course, which is completely programmed like the earlier ones, and is complemented with four films.

The cytological part is closely reasoned: after the student has got acquainted with the light and electron microscopic methods and with the general cellular and subcellular structure

(Vols 1 and 2), the dynamics of cells using the methods of tissue cultivation and phase-contrast microscopy are to be learned. Here the authors mainly treat of the processes which utilize the whole cell, *e.g.*, motion, contact inhibition, mitosis and cell cycle. The process of fertilization and the meiosis as well as the genetical consequences of the latter process, which supplies the modern explanation of Mendel's laws, cytogenetics, and also its methods, each are separately discussed.

Of course, the volume gives preference to the cell division and genetics, for these problems will not be touched upon in the following volumes.

The unusually great number of schematic figures, the transmission — and electron microscopic photos help on understanding the very difficult subject and learning it even by oneself.

G. CSABA (Budapest)

Human Malformations

British Medical Bulletin, Vol. 32/1, London (1976)

In spite of the great actuality of the problem of human malformations, the solution remains still to be found. The real number of human malformations is unknown, as the probably numerous aborted cases are not even taken into account. This volume involves the discussion of the most timely problems of this subject.

Antenatal detection of fetal abnormality — physical methods: JOHN MAC VICAR (Leicester). This paper evaluates the methods of antenatal diagnostics (radiology, sonar and fetoscopy). Author confirms the need of new techniques as even the combined use of today's methods is unsatisfactory.

Prenatal diagnosis of chromosome disorders: K. M. LAURENCE and P. GREGORY (Cardiff). Authors describe their own cytogenetic method applied in amniotic cell cultures in early pregnancy. The method has proved particularly successful in cases of Down's syndrome.

Prenatal diagnosis — chemical methods: D. J. H. BROCK (Edinburgh). The paper discusses and evaluates chemical methods used for examining amniotic fluid punctates in cases of spina bifida and anencephaly, these are completed by the determination of the fetoprotein content of maternal serum.

Genetics of common single malformations: C. O. CARTER (London). Based on family studies in England, the sex distribution of some malformations, such as cleft lip and palate, pylorustenosis, neural tube malformations, congenital dislocation of the hip and cardiac developmental abnormalities. These studies indicated a polygenic aetiology of these common single malformations.

Environmental teratogens of man: R. W. SMITHELLS (Leed). Steroid hormones, alcohol, spasmolytic drugs and certain activities related to the operating theatre and anaesthesia might exert teratogenic effects, but in contrast to literary data, the author attributes no teratogenic effects to sex hormones, smutty potatoes and drinking-water factors.

Some remaining problems in the reproductive. Toxicity testing of drugs: C. L. BERRY (London). Recent developments in the testing of drugs for teratogenic effect are surveyed. The importance of precise selection of animal species and statistical survey need emphasizing. Genetically determined factors play a greater role than environmental factors.

Surveillance of malformations: JOSEPHINE A. C. WEATHERALL and J. C. HASKEY (London). For finding the factors involved in malformations and for evaluation of all these, malformations should be recognized already at the time of birth, including cases of stilbirth. The importance and the possible modes of the precise registration is emphasized.

Descriptive epidemiology of common malformations (excluding central nervous system defects): IAN LECK (Manchester). Common malformations are discussed in two groups, namely, (1) those caused by chemical, physical or biological factors and (2) those designated as demographic varieties, characterized by time, place, sex, ethnic group and familial properties including age and social class of parents.

Model systems in teratology: FELIX BECK (Leicester). Author describes 3 groups of models used in teratology. In the first type the blastocyst is exposed to teratogenic factors before implantation and its development is observed thereafter. The second type involves an exposure of the fetus after implantation. In these the mother, fetus or placenta may be exposed, or the embryo is explanted and examined in vitro. The third group involves investigations on non-mammalian embryos, *e.g.*, bird eggs. The results of these studies may be utilized in relation to human beings.

Mechanisms and pathogenesis of malformation: DANID POSWILLO (Kent). The causal genesis, the site affected by the teratogenic factor, the further consequences of this effect on the cells, tissues and developing organs, furtheron, the possibilities of inhibiting this process and the possible therapeutic means are discussed.

Mechanism of limb development and malformation: L. WOLPERT (London). In experimental embryology, the development of limbs has often been a subject of investigation. Limb regeneration, and the related cytodifferentiation display the basic events leading to a better understanding of limb malformation. Polydactylia and clubfoot may be caused by many different agents.

Congenital postural deformities: P. M. DUNN (Bristol). Congenital malformations may be divided into two large groups, the first results from some sort of affect during embryogenesis — a result of error in morphogenesis, the second is the result of a failure occurring in the intact structure, developing in a later period of intrauterine development. In the majority of the latter group, the musculosceletal system is affected (so-called postural deformities). The significance of intraamniotic factors and fetal movement is also discussed.

Infective causes of human malformations: J. A. DUDGEON (London); *Comparative aspects of infective malformations:* CEDRIC MIMS (London). The first paper reviews the diagnostics and pathogenesis of virus infections, rubella, cytomegaly, herpes simplex, varicella-zoster, influenza and mumps, those of enteric infections and toxoplasmosis. The second paper discusses the differences among infection affecting the mother and those affecting the placenta or the fetus. Authors draw attention to the fact that after recovery from an infectious disease no sign indicating the infective origin of the malformation remains demonstrable.

Legal responses and the right to compensation: GODFREY CARTER (London). Numerous legal problems have arisen in relation to the thalidomide tragedy. The paper is involved with some of the legal aspects of these problems.

The many-sided discussion of human malformations will be of interest for embryologists, cytologists, biologists, pharmaceutists, paediatricians, obstetricians and, in my opinion, to everybody interested in embryology and the factors affecting embryonic development.

I. Törő (Budapest)

British Medical Bulletin,

Vol. 32 (3).

Ed. J. C. WEATHERALL. London (1976).

This issue deals with the structure, function and synthesis of haemoglobin.

In the first paper by M. F. PERUTZ, the stereochemical structure and function of the haemoglobin molecule are analyzed with exact thermodynamic methods on the basis of many years' works of the Cambridge Institute of Molecular Biology. The ligand-bond of the monomer, dimer and tetramer haemoglobins and the importance of their conformation are separately discussed.

The oxygen-binding capacity of haemoglobin as well as the way how it is influenced by 2,3 - DPG, CO_2 and H^+ are reviewed by J. V. KILMARTIN, while a cooperative model of oxygen-binding is presented by J. M. BALDWIN. The above two articles give a detailed elaboration and application of PERUTZ's theory.

The following three publications treat of a highly significant group of the pathological haemoglobins each. J. M. WHITE gives a short review of the literature of labile haemoglobins, A. MAY and E. R. HUEHNS deal with the HbS disease, the development of sickle cells, the mechanism of polymerization and its inhibition in chemical ways. The authors summarize the data relating to this subject in clinical and therapeutical respects. A. J. BELLINGHAM reviews the pathological haemoglobins with altered affinity to oxygen. Haemoglobins having subnormal affinity to oxygen and the HbM variants are not dealt with in detail.

The genetics of human haemoglobins is reviewed by A. LANG and P. A. LORKIN, while the direct measurement of the number of globin genes is given by R. WILLIAMSON. This most important and interesting new subject of research has resulted in numerous new findings concerning both the inheritance of haemoglobinopathies and the general genetics.

The nucleotide sequence of the globin messenger RNA is dealt with by N. J. PROUDFOOT and G. G. BROWNLEE. Their up-to-date investigations enable the genes coding for the globin molecule and the non-coder nucleotide sequences to be identified and their function to be clarified.

A review on molecular interpretation of the development of thalassaemia is presented by J. B. CLEGG and D. J. WEATHERALL. The pathology, therapy and prevention of thalassaemia is dealt with by B. MODELL.

The last two reviews of the volume treat of the questions relating to the synthesis of haemoglobin and cell differentiation (by J. PAUL) and the problems of the synthesis of haemoglobin during the fetal development.

The articles have been selected with care, they summarize the most recent results of the corresponding research fields very well. The most useful issue has a great importance not only for haematologists and specialists in haemoglobin researches, but also for anyone interested in the protein synthesis, genetics as well as in the questions concerning the relationships between structure and function.

JUDIT SZELÉNYI (Budapest)

Kompendium der Allgemeinen Biologie

(Compendium of General Biology)

Ed. E. LIBBERT. VEB Gustav Fischer Verlag, Jena (1976), pp. 475, figs 179, tables 12, M 18.

The authors considered to be very important a compendious book of general biology to be issued, as it can be used by biologists, biochemists, physicians, pharmacists, veterinary surgeons and agronomists alike for their introductory studies. As a university professor I welcome this idea.

The book consists of 12 chapters: Definition of living systems (H. PENZLIN); Chemical construction (E. LIBBERT); Cell structure (E. LIBBERT); Substance and energy metabolism at cellular level (E. LIBBERT; Genetic information (E. GÜNTHER); Replication and segregation (E. GÜNTHER); Multicellular organisms (E. LIBBERT and H. PENZLIN); Metagenezis (H. PENZLIN); Stimulus, movement and behaviour (H. PENZLIN); Inheritable alterations (E. GÜNTHER); Evolution (L. KAMPFE and E. GÜNTHER); Environmental factors and their interrelationships (H. J. MÜLLER).

The most important literature is referred to in each chapter. The composition of the book is excellent, clear sticking to essentials with demonstrative figures and tables. The modern work of high level presents a review most useful for anyone interested in biology. The detailed subject index makes the book easy to use.

I. KÁRPÁTI (Keszthely)

M. Kedves:

Paleogene Fossil Sporomorphs of the Bakony Mountains Part III.

(Studia Biologica Academiae Scientiarum Hungaricae 15)

The present volume is the final part of a systematic elaboration of extinct plant spores and pollens recovered from the Lower Tertiary deposits of the Bakony Mountains. The systematic part deals with the pollens of Longaxones and those of tetrads and polyads. The botanical relations of the individual pollen grains and their distribution in the Bakony Mountains are discussed. The work includes the treatment of 33 genera, of which four are new to science. Within the genera several new species are described. The descriptions are based on the exine structure revealed by electron microscopy.

In English — Approx. 130 pages — 47 figures — 554 photographs — 17 × 25 cm — Cloth

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Abstract (not exceeding 200 words)

Introduction

Material and method

Results

Discussion

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3. UMBREIT, W. E., BURRIS, R. H., STAUFFER, I. F. (1957) Manometric techniques. Burgess Publishing Co., Minneapolis.

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IN VITRO CARCINOGENESIS STUDIES ON MOUSE FIBROBLAST CELLS

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(Received 1976—10—06)

Abstract

Fibroblast cell lines were established from pulmonary explants derived from inbred CBA T_6T_6 mouse embryos. Cell lines controlled for the absence of spontaneous transformation were treated with 20=methylcholenthrene (0.1 μg/ml). The altered biological characteristics were studied during the process of the malignant transformation by the comparison of the untreated and 20-methylcholanthrene pretreated cell populations: the loss of contact inhibition and the connection between the malignant transformation and the aryl hydrocarbon hydroxylase enzyme activity were investigated. No changes in the cell proliferation rate could be found following malignant transformation, but an increased resistance against altered circumstances was observed. In the course of passages, a gradual decreases in aryl hydrocarbon hydroxylase activity of the untreated line was seen, which disappeared or significantly decreased following 20-methylcholanthrene treatment, compared to the controls.

Introduction

Malignant transformation of tissue culture cells can be induced with various carcinogenic substances [1, 2]. For carcinogenesis studies the polycyclic hydrocarbon methylcholanthrene (MC), 3-MC and 3-MC 11 or 12-oxide, are usually applied for *in vitro* investigations. The administered concentrations are high in most studies, 0.5—10 μg/ml [3]. The effect of the carcinogenic compounds varies by the route of administration (*in vivo* or *in vitro*) and by the experimental animal or cell type. The transformation of the studied substance into an active form is needed for its carcinogenic effect. For this process a sufficient quantity of the microsomal aryl hydrocarbon hydroxylase (AHH) enzyme is essential. The enzym is present in mammalian livers, but it may be absent in various other tissues (*e.g.*, human embryonic fibroblasts) [7]. Another important factor for the malignant transformation induced by the chemical carcinogens is the presence of the cytoplasmic H-protein or ligandin which mediates the interaction between the active compound and DNA [6]. The evaluation of the *in vitro* obtained results is made difficult by the fact that a spontaneous malignant transformation of cultured cells — derived mostly from mice — can be observed after several passages [9]. The *in vitro* methods that have been suggested for proving the malignant transformation are not sufficient

[10]; the tumour-forming capacity of the replanted cells is only accepted as evidence [4].

In our studies the malignant transformation of the CBA T_6T_6 mouse embryo fibroblast cells was attempted with relatively low doses of the infrequently used carcinogen 20-MC. Comparing the treated and untreated cultures we tried to obtain data (1) on their altered growth characters, (2) on the correlation of the malignant transformation with the AHH activity of the cells, (3) on the transformation possibilities of the isolated CBA T_6T_6 mouse embryo cells and (4) on the alterations of the biological characteristics accompanying and indicating the transformation, with special regard to the AHH activity of the cells.

Material and methods

Establishment of tissue cultures

To establish cell lines 20 days old embryos of inbred CBA T_6T_6 mice were used. The lungs of the embryos were sliced with scissors under sterile conditions, then washed in PBS, and digested with 0.25% trypsin solution for 20 min. Following filtration and separation by centrifugation at 1000 r.p.m., the cells were incubated in 10 ml medium (Parker's 199 + 10% calf serum) in Falcon plastic flasks at 37 °C.

Sustenance of the cultures

For the passage of the cultures, 0.25% trypsin solution, for their sustaining Parker's 199 containing 10% calf serum was used. On the first occasions, the passages were carried out at long intervals, alternately with or without medium change, later, stabilized lines were transferred, generally, on every fifth day (500,000 cells/10 ml).

Selection of lines for use

No stabilized cell lines could be established from a great part of the started cultures. Some of the stabilized lines showed signs of malignant transformation before the 20th passage.

Cell lines showing no evidence of malignant transformation even with reimplantation into animals following the 20th passage of untreated cultures were selected for our studies. No biological or morphological evidence of malignant transformation could be proved in this cell line.

Treatments

For treatment with 20-MC 10,000 cells/flask were plated, then 20-MC dissolved in DMSO was added to the medium of the cells up to the final DMSO concentration of 0.05%. The MC-containing medium was kept on the cells for 14 days without change. Untreated cultures and those with 0.05% DMSO in medium were used as controls.

Determination of cell density

Treated and untreated cells were plated in a concentration of 0.5 million cells/flask. The total cell number and the cell count for 1 cm^2 flask surface were daily determined in a Buerker chamber during the culture period. On the 5th day the medium was changed, and in this way the cell density could be followed up to the 9th day.

Study of serum concentration dependence

The medium containing 10% calf serum was diluted to 5, 3 and 1% serum concentrations. At passages low numbers of cells were plated. In this way the colonies formed from single cells could be studied separately. After the incubation time (5th and 6th day), the cell numbers for each colony were determined.

Study of the colony-forming capacity in soft agar medium

Five % agar-agar (DIFCO) solution, kept in hot bath, was gradually added to the cell suspensions to reach the final concentration of 0.4%. Following this, 5 ml volumes of the suspensions were distributed in Falcon plastic Petri dishes of 5 cm diameter and were incubated for 8 days in a thermostat at 37 °C perfused with a mixture of 5% CO_2 and 95% of air. After the incubation period the mean cell numbers for colonies were calculated.

Transplantation

The cells were removed from the bottom of the flask with trypsin, then centrifuged (1000 r.p.m.), three times washed with saline and resuspended in saline, so that the final suspension contained 4×10^6 cells in 0.1 ml. The cells were injected into the backs of newborn CBA T_6T_6 mice subcutaneously (0.1 ml). The injected mice were observed for 6 months if no tumours developed. In case of tumour formation, the tumours could be palpated generally on the 10th day after the injection, and on the 20th day the tumours reached a diameter of 1—1.5 cm. Then the animals were killed, the tumours were prepared for morphological studies and the cells were used for further *in vitro* cultures.

Biochemical studies

Cultures of 48 h were used. Each sample contained 2×10^6 cells. The AHH activity [5] was measured according to the method described by NEBERT and GELBOIN [8] and expressed in fluorescence intensity related to 1 mg cellular protein. The fluorescence of the metabolic product from benzpyrene (BP) was compared with that of 3-OH-BP (kindly supplied by Dr. H. W. GELBOIN, National Cancer Institute, Bethesda, MD.)

Morphological studies

The monolayer cultures were fixed in ethanol–acetic acid and stained with Giemsa solution. Histological preparations were fixed in 4% formaldehyde, embedded in paraffin and stained with haematoxylin–eosin.

Results

Effect of methylcholenthrene treatment on growth characteristics of the fibroblast cell lines

The selected cell line was stabilized following the 6th passage with no signs of malignant transformation, thus, it was acceptable for treatment with the carcinogenic substance. The toxicity of 20-MC on the multiplication of the cell line was tested at the 8th passage. Cells were incubated for 5 days in the presence of 0.1 μg/ml 20-MC (Table 1). This concentration, causing successful malignant transformation, had no toxic effect on the cells. The treatment of

Table 1

The toxicity of 20-methylcholanthrene on CBA T_6T_6 mouse fibroblast cells

Concentration of 20-methylcholanthrene μg/ml	Cell count/flask ($\times 10^3$)
0.01	310
0.1	320
1	310
10	270
50	180
Control	320
DMSO control	310

Original cell number: 10^4 cells/flask
Treatment period: 5 days
Passage number: 8

the culture was performed following the 8th passage. After 14-day treatment with 0.1 μg/ml 20-MC, significant morphological differences were found between treated and untreated cells (Figs 1 and 2). No contact inhibition was seen in the treated cultures: besides multidirectional growth, more than one cell layers were formed. Both the treated and the untreated cultures were then subjected

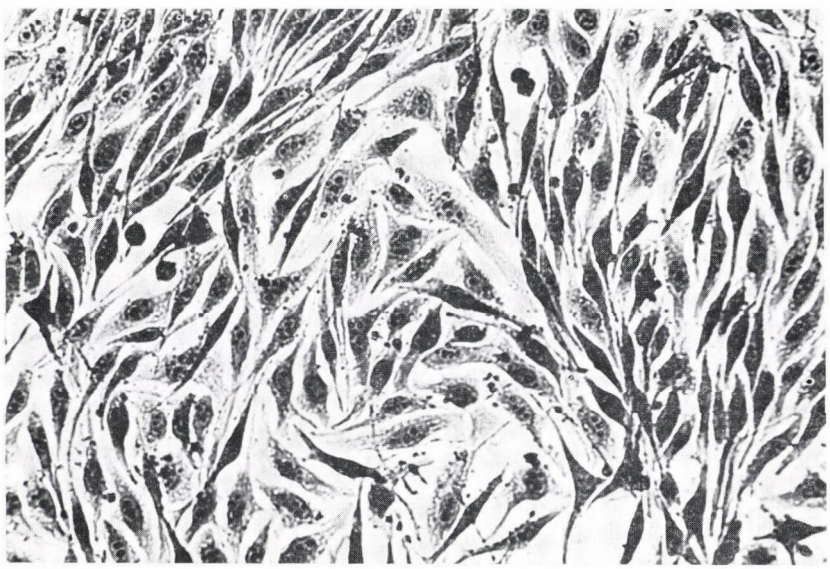

Fig. 1. Untreated CBA T_6T_6 mouse lung fibroblasts, 13th passage. Well-defined contact inhibition: only one layer is formed by the cells.
(Giemsa, ×200)

Fig. 2. MC-treated (0.1 μg/ml) CBA T_6T_6 mouse lung fibroblast cell line; 3rd passage following treatment. Suspended contact inhibition: the cells are growing at some sites in several layers. (Giemsa, ×200)

Table 2

Cell counts of the untreated and 20-methylcholanthrene treated cell lines in the course of passages

Days	Control		MC-treated	
	Passage No.	Cell count/flask ($\times 10^6$)	Passage no.	Cell count/flask ($\times 10^6$)
0		0.6		0.6
1		0.6		0.6
2		0.9		1.0
	13		3	
3		1.6		1.6
4		2.1		2.3
5		2.2		2.3
0		0.5		0.5
1		0.4		0.4
2		0.7		0.8
	18		8	
3		1.1		1.1
4		1.5		1.6
5		2.1		2.1
6		2.3		2.3

to several passages and the biological characteristics of the cells were compared again. No differences in the proliferation characteristics of cell populations could be detected, while the treated and the untreated lines were further passaged under the usual conditions without changing the medium during the incubation of the cultures (Table 2).

Following a change of the medium after the monolayer had developed, the cell densities were compared. Remarkable differences were found concerning the total cell numbers in the various flasks, and between cell numbers per square unit. After change of the medium on the 5th day, the cell number per cm^2 exceeded 1×10^4 only in the MC-transformed culture (Table 3).

Table 3

The cell density and total cell count of untreated and 20-methylcholanthrene treated cell lines before and after change of the medium

Days	Control passage No. 15		MC-treated passage No. 5	
	Cell count ($\times 10^6$)	Cell/cm^2 ($\times 10^4$)	Cell-count ($\times 10^6$)	Cell/cm^2 ($\times 10^4$)
0	0.5	0.2	0.5	0.2
1	0.4	0.16	0.45	0.18
2	0.7	0.28	0.8	0.32
3	1.1	0.44	1.3	0.52
4	1.8	0.72	1.8	0.72
5	2.2	0.88	2.3	0.92
6	2.4	0.96	2.7	1.08
7	2.3	0.92	3.2	1.28
9	1.8	0.72	2.4	0.96

Cell count at the start of experiment: 0.5 million (flask/10 ml). Change of the medium on the 5th day

The effect of the reduced serum concentration on the cell proliferation was studied after the 14th (4th for the MC-treated cells) passage. As it is indicated in Table 4 the mean cell number per colony showed the greatest differences in the presence of 3% serum, *i.e.*, the untreated cells were not capable of dividing whereas the cells from the MC-treated populations formed colonies of 15 cells on the average.

Although in small percentage, only the cells from the MC-treated populations were able to form colonies in soft agar (Table 5). Owing to the low number of cells per colony, this study can be regarded as an exploratory one.

Table 4

Serum concentration dependence of the untreated and 20-methylcholanthrene treated cell lines

Serum concentration, %	Control		MC-treated	
	mean cell count/colony		mean cell count/colony	
	5th day	6th day	5th day	6th day
1	0	0	3	3
3	1.5	0	9.5	15
5	8.2	14	9.7	17
10	9.8	16	10	17

Cell count at the start of experiment: 5000 (flask/10 ml)
Passage number: Control 14
MC-treated 4

Table 5

Colony forming capacity of untreated and 20-methylcholanthrene treated cells in soft agar medium

Cell line	Passage No.	2 cells pro colony	3–6 cells pro colony	7–10 cells pro colony
Control	18	10%	—	—
MC-treated	8	12%	8%	2%

Agar concentration: 0.4%
Incubation period: 8 days
Cell count at the start of experiment: 100/Petri dish/8 ml

Metabolic transformation of benzpyrene

The microsomal AHH enzyme activity was studied at the 13th, 17th and 26th passages of the control cells and at the 3rd, 7th, 16th passages following the MC-treatment. The fluorescence characteristic of 3-OH-BP could be detected in the case of the untreated population at the 13th and 17th passages; in the case of the transformed line at the 3rd passage in a low intensity. Simultaneously, a substance with new fluorescence characterstics (excitation maximum at 370 nm, fluorescence maximum at 480 nm) was found in the NaOH-extractable phase. Fluorescence like this could not be detected either in the dissolved BP or in the soluble phase of the hexane or in the NaOH-soluble phase formed by the microsomal enzyme of the liver, thus, it is postulated that a BP transformation product, unknown to us, was formed.

The differences between the two lines were the following: in the 1st and 2nd passages of the normal lines the fluorescence of both the 3-OH-BP and the new product could be found; in the 3rd passage of the transformed line the fluorescence was weaker than in the normal. The fluorescence of the new

product appeared in the 7th passage of the malignant line with a high intensity, compared to the normal cells; simultaneously, the fluorescence characteristic of 3-OH-BP disappeared. During the 16th passage, the formation of the new substance decreased below the quantity formed by the normal line.

Implantation of control and 20-methylcholanthrene treated cells into newborn mice

At each passage the cells of the untreated and treated lines were implanted into newborn CBA T_6T_6 mice. The results are shown in Table 6. The reimplantations have shown that the cell line under study could not be regarded as a transformed one until the 21st passage. Following this passage, a spontaneous transformation of the line ensued. The process advanced relatively quickly, and the cells caused tumours in the animals, similarly to the MC-treated ones. Following the 21st passage, the cells of the untreated population lost their contact inhibition capacity and also other biological characteristics showed the signs of malignant transformation.

Morphologically, in the subcutaneous connective tissue, the tumours were firm, well circumscribed nodules weighing 1—4.2 g each. Histology revealed spindle cell sarcoma in all cases (Fig. 3). The cells of the developed tumour were digested with trypsin and explanted *in vitro*. Culturing was successful in

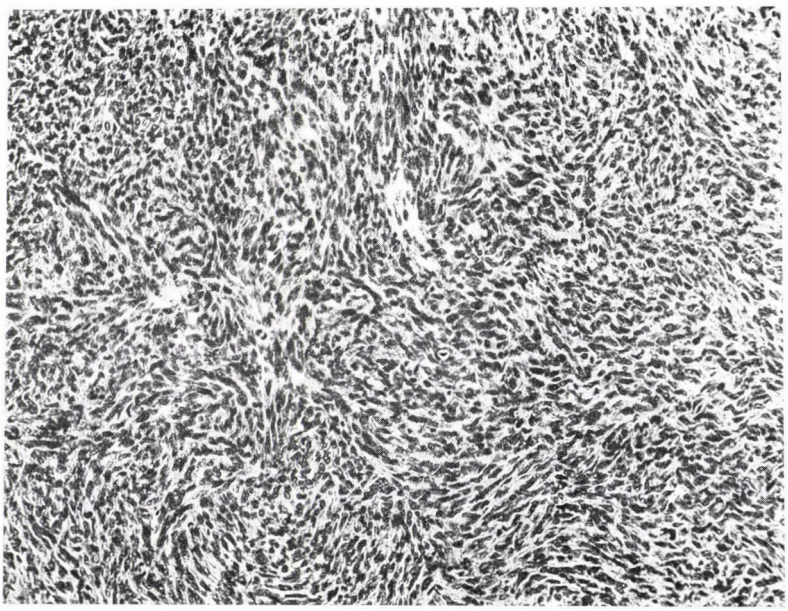

Fig. 3. Light micrograph of a tumour of a mouse inoculated with MC-treated fibroblast cells (6th passage following treatment). Spindle-cell sarcoma. (Haematoxylin–eosin, ×200)

Table 6

Replantation of untreated and 20-methylcholanthrene treated cells into newborn CBA T_6T_6 mice

Cell lines	Passage No.	Body weight (g)	Tumour weight (g)	Proliferation of tumour cells again in tissue culture
Control	9			
		9	—	—
		10	—	—
		9	—	—
	13	8	—	—
		8	—	—
		9	—	—
	16	11	—	—
	18	7	—	—
	18	10	—	—
		11	—	—
	19	10	—	—
		8	—	—
		9	—	—
	21	7	1	+
	26	8	1.2	+
	30	7	2	+
		8	2.5	+
MC-treated	3	7.5	3.6	+
		8	3.2	+
		9	4	+
	6	8	3.2	+
		9	4	+
		6.5	3.2	+
	7	7.2	3.1	+
		10	4.2	+
	9	8	2	+
		7.9	3.8	+
		8.5	3.5	+
		8	3	+

Cell count: 4×10^6/animal, subcutaneously
Evaluation on the 20th day following implantation

all cases. The light microscopic morphology and *in vitro* proliferation characteristics of the cell cultures started from the explanted tumours, derived of MC-treated cells, were similar to those of the MC-pretreated and *in vitro* passaged populations.

Discussion

Malignant transformation of the CBA T_6T_6 mouse embryo fibroblast line, a line established by us, can be induced with a relatively low, 0.1 μg/ml concentration of 20-MC. Several other fibroblast cell lines obtained from the same mouse strain could not be regarded as well defined nontransformed or malignantly transformed lines. In some cases, the various signs of malignant transformation appeared gradually, independently of each other, in other cultures a sudden spontaneous malignant transformation was observed. The speed of the malignant transformation can depend on individual characteristics of the donor animals. According to our investigations the spontaneous malignant transformation of the cell line used in the described study developed in a relatively short period. The slow decrease in the AHH activity indicates an alteration of the cell metabolism which had started before any measurable sign of malignant transformation could be detected. By a correct choice of the adequate cell line it can be achieved that the biological characteristics change in one step following treatment with the carcinogens. The direct effect of MC on the morphology of single colonies has not been studied, since no separate colonies were formed by the chosen line at the time of the 8th passage.

It can be concluded from our results that no change in the proliferation rate of the cell population occurred while the viability and resistance of the cells increased. This is indicated by the retained growth capacity under altered circumstances (low concentration of serum, soft agar). The loss of the contact inhibition capacity can be proved by the higher cell density following the change of medium. The study on the microsomal oxidase activity revealed qualitative and quantitative differences in the metabolic transformation of BP during the course of passages, and also between the normal and MC-transformed lines. The most frequently measured, and from literature the best known transformation product of the BP, the 3-OH-BP could only be detected in early passages of both lines (normal, 13, 17; transformed, 3). Simultaneously, fluorescence of a still unknown product could be found (A) 370(480) which was, similarly to OH-BP, produced in smaller quantities during further passages. In the MC-treated and transformed cell line less 3-OH-BP was produced and later this production ceased. Simultaneously, the fluorescence of the new product was seen, and the intensity of this fluorescence quickly decreased in the course of the subsequent passages. After 10 passages, the quantity of the substance produced in time unit had decreased to 1/20 part, while one-third

of the previous activity of the normal line could be detected. Accordingly microsomal oxidase activity required for malignant transformation is decreasing during the process of malignant transformation, which phenomenon could be considered as an indication of the reduction in the nonreproductive function of malignant cells.

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ULTRASTRUCTURAL EFFECTS OF 6-HYDROXY-DOPAMINE AND 5,6-DIHYDROXYTRYPTAMINE ON THE CENTRAL NERVOUS SYSTEM OF FRESH-WATER MUSSEL, *ANODONTA CYGNEA* L.

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Abstract

Ultrastructural effects of 6-hydroxydopamine and 5,6-dihydroxytryptamine treatments were investigated in the central nervous system of fresh-water mussel. Two days after the treatments, the following characteristic fine-structural alterations could be observed in the neuropil of the ganglia: frequent occurrence of multilamellar bodies, lysosomatic structures and elongated tubular forms; shrinking of varicose axon profiles with an enhancement of the density of the axoplasm and clumping of its content; abnormal swelling of certain axons in the neuropil. This degenerative process was accompanied by an intense phagocytosis. The damages evoked by the employed "false transmitters" in the mussel ganglia were, in general, simular to those found in vertebrates. Statistical analysis of the vesicle population of ganglia suggests the intragranular uptake of 6-hydroxydopamine and 5,6-dihydroxytryptamine and, in addition, the role of dense-core vesicles of different types in the storage of both serotonin and catecholamines. Perikarya composing the cortical layer of the ganglia were not affected by the "false transmitters". This shows that different parts of a mussel neuron are differently sensitive to 6-hydroxydopamine and 5,6-dihydroxytryptamine.

Introduction

Fine structural aspects of the degenerative effects of 6-hydroxydopamine (6-OHDA) and 5,6-dihydroxytryptamine (5,6-DHT) have been studied in detail on the monoaminergic neurons of vertebrates [2, 3, 4, 7, 8, 17, 19, 26]. It has been shown that catecholaminergic and serotoninergic terminals are damaged by these pharmacons, previously taken up as "false transmitters" into the terminals. Fine-structural investigations dealing with the *in vivo* effects of 6-OHDA and 5,6-DHT in the central nervous system (CNS) of invertebrates have not yet been carried out. *In vitro* effects of 6-OHDA on an identified dopamine containing neuron of *Planorbis corneus* (Gastropoda, Mollusca) have been studied by electron microscopical, fluorescence-histochemical and electrophysiological methods [5]. It was established that 6-OHDA is taken up by the nerve cell and the density of the dense-core vesicles is enhanced in certain axons of the neuropil. Some axon profiles showed degenerative phenomena. In addition, peripheral degenerative effects of 5,6-DHT [24] and 6-

OHDA) were observed in the *Venus* heart and in the anterior byssus retractor muscle of *Mytilus edulis* (Pelecypoda, Mollusca), respectively.

The main purpose of our present work was to clear up in detail the ultrastructural effects of the two "false transmitters" in the CNS of fresh-water mussel, with particular reference to the occurrence of the monoamines in high amounts in the ganglia, which has been proved by both biochemical [12, 13] and fluorescence-histochemical [28] investigations.

Material and methods

6-OHDA and 5,6-DHT were administered by injecting the substances diluted in *Anodonta* physiological saline [20], containing 0.1 mg/ml ascorbic acid, into the foot of experimental animals. 6-OHDA was partly injected as a single dose of 25 mg/kg, while in other cases, repeated doses (1×10 mg/kg $+ 2 \times 7.5$ mg/kg) were applied at two-day intervals. Ganglia were prepared for electron microscopy 2, 4, 12, 24, 48 h, and 5, 10 days after the last injection. 5,6-DHT was injected in 3×10 mg/kg doses at two-day intervals. Ganglia were prepared 4, 10, 20 and 30 days after the last injection. In addition, the fine structure of the ganglia was studied 5h after injecting 1×10 mg/kg 5,6-DHT and 2 days after injecting 2×10 mg/kg 5,6-DHT. Biochemical and behavioural investigations [15, 16] were the basis for the choice of these time intervals.

Ganglia were fixed in 3% glutaraldehyde diluted in *Anodonta* physiological saline [20] for 2 h at room temperature. After short washing, ganglia were postfixed in 2% OsO_4 buffered with s-collidine for 30 min at 4 °C. After dehydration, ganglia were embedded in Araldite (Durcupan, ACM, Fluka). Sections were cut on an LKB Ultrotome III ultramicrotome and investigated in TESLA BS 413A and TESLA BS 500 electron microscopes. Sections were stained with uranyl acetate and lead citrate.

In order to analyze the general structural appearance of the ganglia after the treatments, light microscopic investigations were also carried out. For this, 1—2 μm thick sections were cut and stained with 1% toluidine blue.

To decide whether there is any change in the rate of the granular and clear vesicles, we made in each treated and control animal countings in 100 different axon profiles chosen randomly in ganglia. For analyzing the further alterations possibly ensuing in the dense-core vesicles, frequency distribution histograms of the diameters of the dense-core vesicles were calculated. The differences between distribution histograms (T) of control and treated animals were determined at each diameter value and represented in a system of co-ordinates. For the calculation of the distribution histograms, a final enlargement of 42,000 was used.

Results

Two to five hours after 6-OHDA and 5,6-DHT administration, respectively, no fine-structural changes were found in the neuropil of the ganglia, compared to the control (Fig. 1). Ultrastructural changes appearing consistently in the axons of the neuropil were observed 1—2 days after 6-OHDA treatments and 2 days after an injection of 2×10 mg/kg 5,6-DHT. Similar degenerative phenomena were seen after the administration of the two pharmacons and they can be summed up as follows.

(1) Frequent occurrence of multilamellar bodies, membrane-bound electron-dense structures in a number of axon profiles of the neuropil, accompanied by the appearance of elongated cisterns, sometimes very abundant

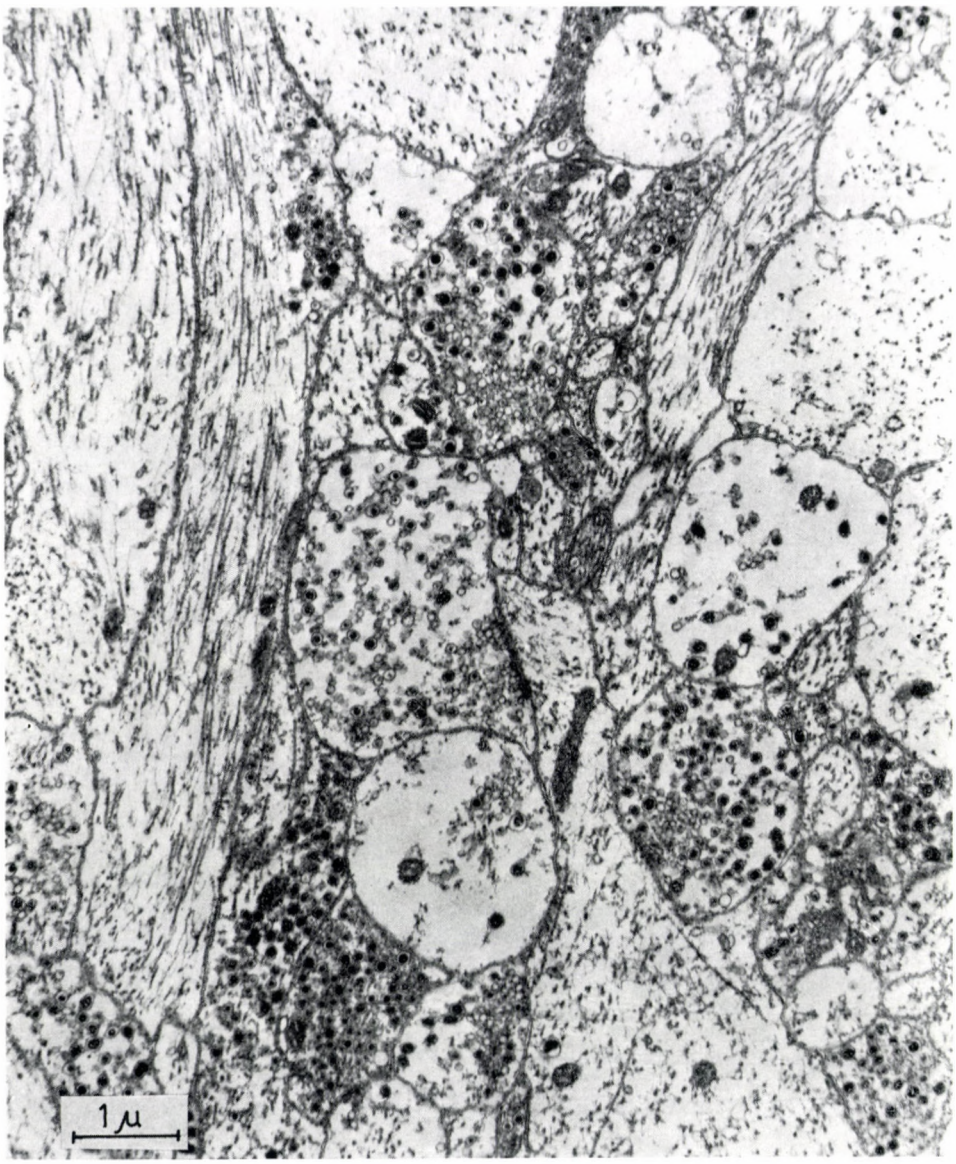


Fig. 1. Detail from the neuropil of the pedal ganglion of a control animal injected 2 days before treatment with 1 ml *Anodonta* saline containing 0.1 mg ascorbic acid. No sign of ultrastructural damage. × 7000

(Fig. 2). Similar structures were sometimes seen in controls but much less frequently than in certain areas of the neuropil after the administration of 6-OHDA or 5,6-DHT.

(2) Appearance of dense bodies in presynaptic terminals (Fig. 3). These dense bodies were generally surrounded by unit membrane, sometimes by

double-membrane. Microvesicles were frequently seen in the finely granulated matrix of the dense bodies.

(3) The most characteristic, most frequent and most final form of the whole degenerative process ensuing in the *Anodonta* ganglia after 6-OHDA and 5,6-DHT treatments is the axoplasmic shrinking of varicosities in the neuropil (Fig. 4a, b and c). The shrunken structures are characterized by a matrix of high electron density and a great number of vesicular elements embedded in the matrix. In addition, the whole structure is frequently vacuolized.

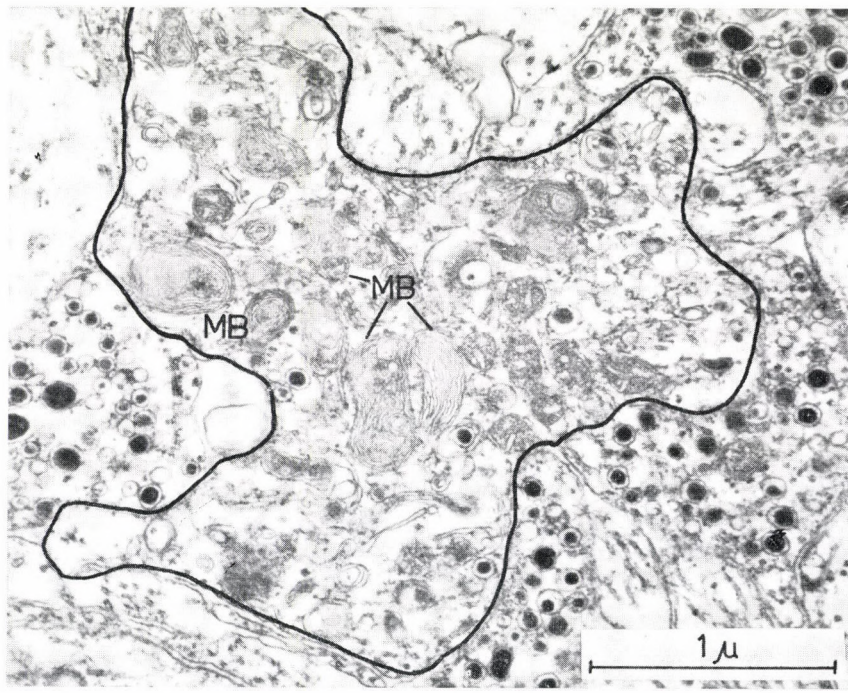


Fig. 2. Degenerating axon profile (outlined) 24 h after 1 × 25 mg 6-OHDA administration. The abundance of tubular forms and multilamellar bodies (MB) is conspicuous. × 32,000

From the vesicle types described earlier in the CNS of fresh-water mussel, *Anodonta* [10] preferably small and large dense-core as well as eccentric dense-core vesicles occurred in the degenerated axon profiles. The shrunken axoplasm is often separated from the axolemma by an electron-transparent halo. In other cases, however, more degenerated profiles were seen in the extracellular space, being already engulfed by glial processes (Fig. 4c and 5).

(4) Axon dilatations (Fig. 6) were found in the neuropil after both treatments. Compared to the size of the surrounding axons, the injured axon is abnormally dilated, and the considerable abundance of different cellular compartments (mitochondria, dense-core vesicles, multivesicular bodies) as well as the

frequent occurrence of digestive vacuoles and lysosomes were characteristic. Dense-core vesicles occurring in the dilated axons often showed an enhanced density.

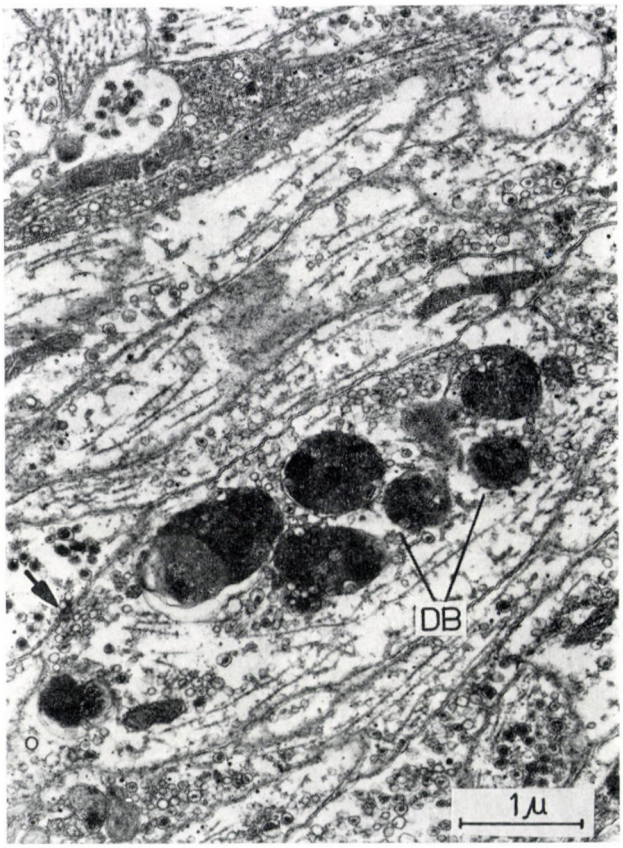


Fig. 3. Dense-bodies (DB) surrounded by membranes and containing vesicular elements are shown in an axon terminal. (Arrow indicates the site of the synaptic connection.) 10 days after 3×10 mg/kg 5,6-DHT administration. ×16,000

(5) Degenerative processes were accompanied by an intense glial digestion and appeared mainly after prolonged treatment (2, 4, 5, 10 and 20 days) with 6-OHDA or 5,6-DHT. The whole process of the phagocytosis can well be traced, since both degenerated axon profiles already engulfed by glial processes in the neuropil and degenerated axons being already in the cytoplasm of the glia cell could be observed (Fig. 4c and 7). Gliosomes in rather great number appeared in the glia cells near the degenerated axons (Fig. 7).

The time curves of the degenerative processes evoked by 6-OHDA and 5,6-DHT, were not quite similar. While on the 10th day after injecting 6-OHDA, degenerated axon profiles were rarely found in the neuropil, in the case of

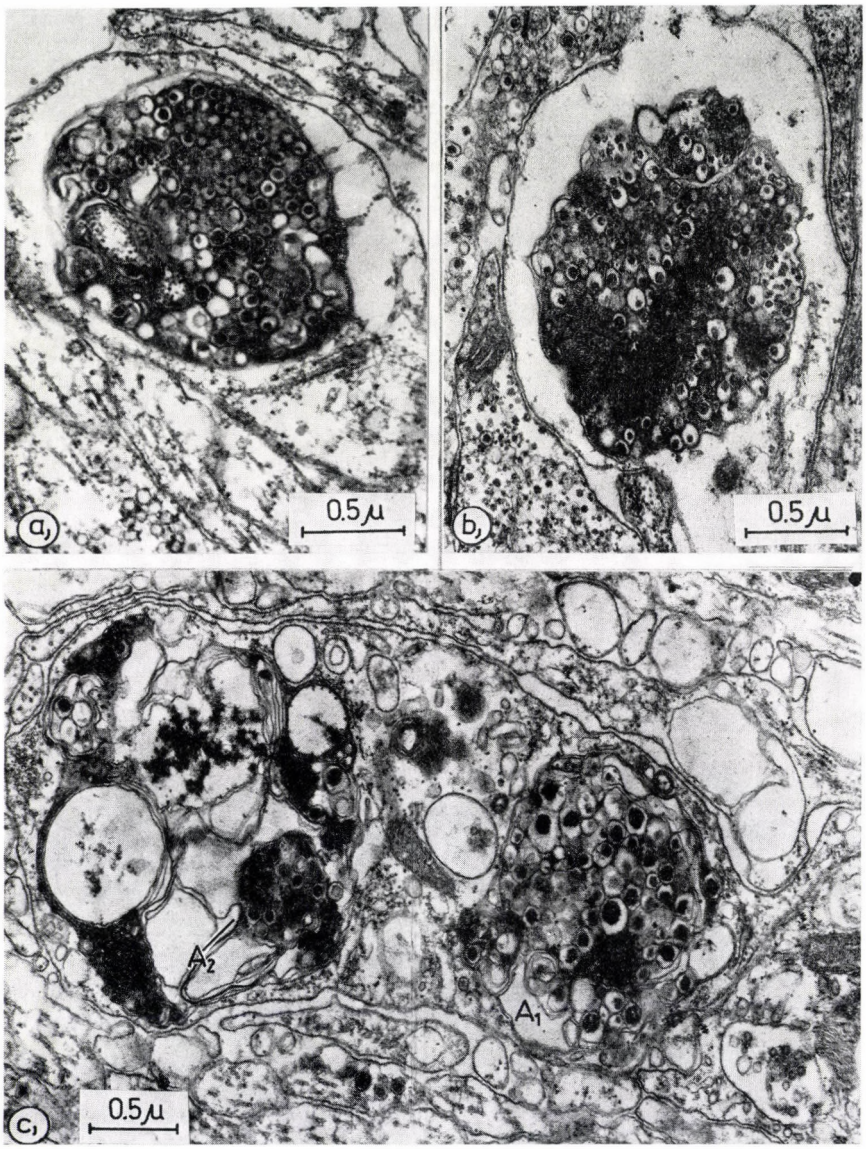
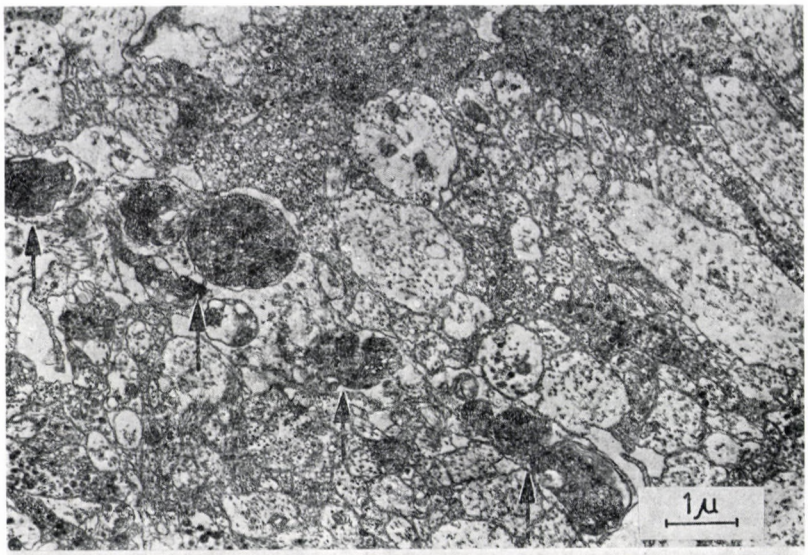


Fig. 4. a) Electron microscopic picture of a degenerated axon profile in the neuropil of the pedal ganglion 5 days after 1×25 mg/kg 6-OHDA. The shrunken axoplasm with high electron density contains, first of all, middle-sized (700—960 Å) dense-core vesicles. $\times 28{,}000$

b) Degenerated axon in the neuropil of the pedal ganglion 20 days after 3×10 mg/kg 5,6-DHT injection. Eccentric dense-core vesicles with wide range in size occur exclusively. $\times 28{,}000$

c) Group of degenerated axon being already totally engulfed by glial processes (GP) in the neuropil of the visceral ganglion 2 days after 2×10 mg/kg 5,6-DHT administration. One of the degenerated axon profiles (A_1) contains only eccentric dense-core vesicles with larger diameter, while in another (A_2) middle-sized normal dense-core vesicles are present. $\times 24{,}500$



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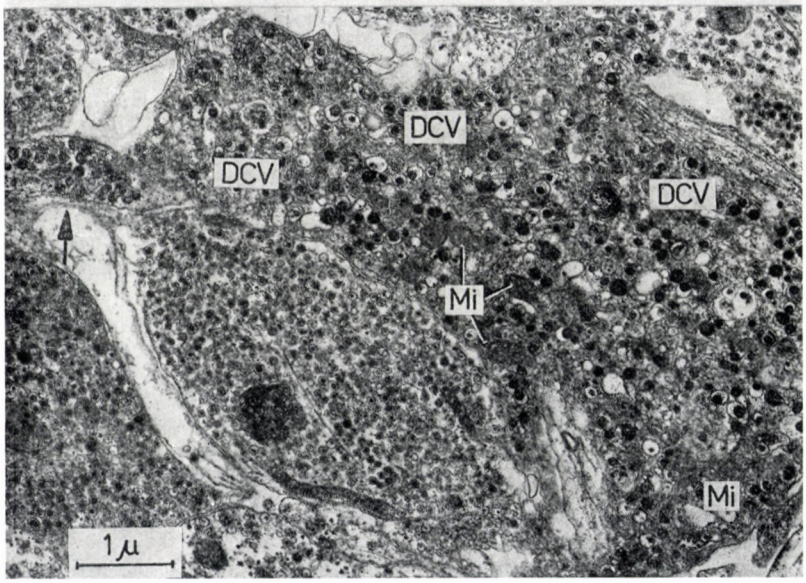
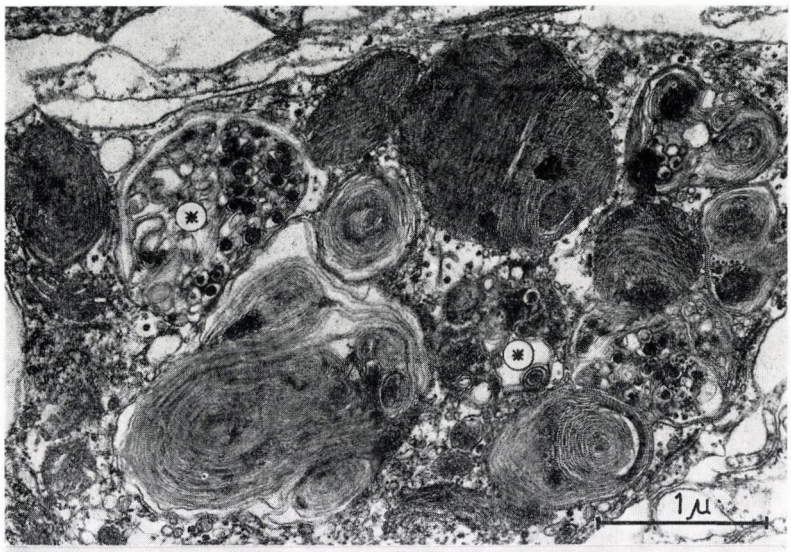


6.

Fig. 5. Electron micrograph from the pedal ganglion 10 days after 3×10 mg/kg 5,6-DHT treatment. Note the degenerated axons (arrows) in this area of the neuropil. ×9600

Fig. 6. Abnormally dilated axon profile with a number of mitochondria (Mi) and dense-core (mainly eccentric dense-core) vesicles (DCV) showing high electron density. Arrow indicates a neighbouring axon in which dense-core vesicles with conspicuous high density and filling out is also well seen, compared to the vesicular elements of other axons of the picture. 10 days after 3×10 mg/kg 5,6-DHT treatment. ×13,000



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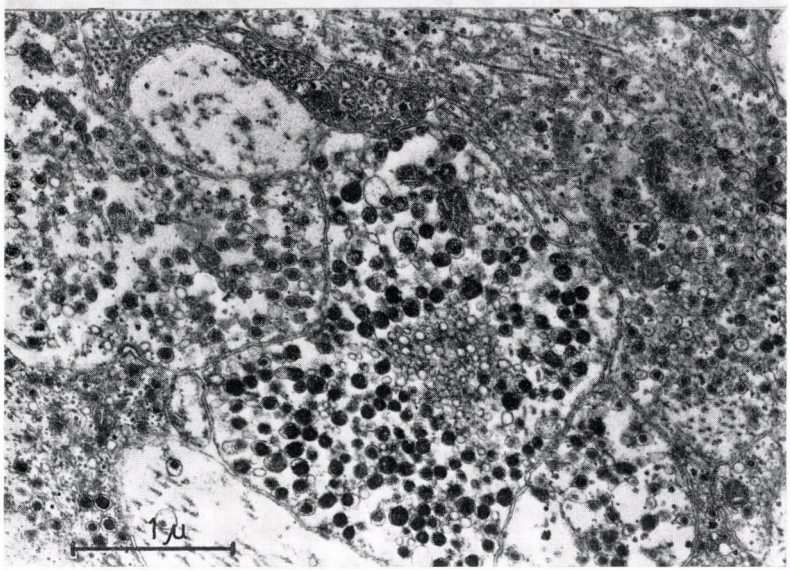

8.

Fig. 7. Detail from a glia cell 20 days after injecting 3×10 mg/kg 5,6-DHT. Note the abundance of gliosomes and the axon profiles (asterisks) which had degenerated and then phagocytosed by the glia cell. ×22,500

Fig. 8. Detail from the neuropil of the cerebral ganglion 10 days after injecting 3×10 mg/kg 5,6-DHT. Note the axon terminal in the centre containing dense-core vesicles with enhanced density and filling out. In spite of the total filling out, the eccentric localization of the dense-core is well seen in some vesicles. ×22,500

5,6-DHT administration damaged terminals were frequently seen even after 20 days. However, comparing to the total number of the axons viewed in the sections, the occurrence of degenerative phenomena does not show a high frequency after any treatment.

We failed to observe general increase in the density of the dense-core vesicles described as "short-term effect" of the false transmitters in vertebrate [4, 9] and invertebrate [24] peripheral nerves 2, 4 and 5 after the treatments. On the other hand, 1—2 days after 6-OHDA administration intact axons

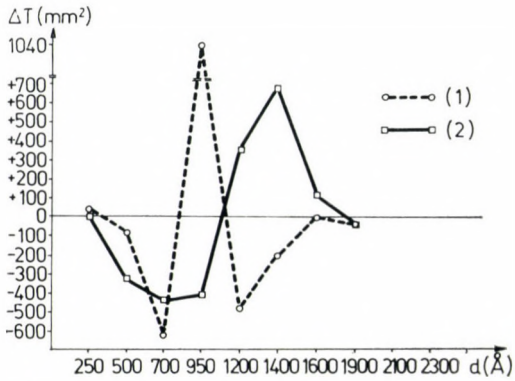

Fig. 9. Frequency distribution histograms obtained after different 6-OHDA treatments on the basis of the diameters of the dense-core of vesicles, compared to the control (0 point on the ordinate); (1) 4 h after 1×10 mg/kg 6-OHDA administration, (2) 2 days after 1×25 mg/kg 6-OHDA.

containing dense-core vesicles with increased electron density were found in the vicinity of the degenerated profiles. Four to ten days after injecting 3×10 mg/kg 5,6-DHT, eccentric dense-core vesicles being much more filled out than usual and showing extremely high electron density could be seen in certain terminals of the neuropil (Fig. 8).

Statistical analysis of the alterations ensuing in the vesicle population after the treatments did not reveal any change in the number of granular (dense-core) and clear vesicles. After both short and long-term treatments of 6-OHDA and 5,6-DHT, the percentage distribution of granular and clear vesicles showed a value of 70—75/30—25, being also characteristic of the control. However, alterations were observed in the diameter of the dense-cores of the vesicles 4 and 48 h after injecting 25 mg/kg 6-OHDA and 5 and 48 h and 10 days after injecting three different doses of 5,6-DHT (1×10 mg/kg, 2×10 mg/kg, 3×10 mg/kg) (Figs 9 and 10). Obviously the size of dense-cores of the vesicles is influenced by 6-OHDA and 5,6-DHT already within the short intervals following the injection(s). Four hours after injecting 25 mg/kg 6-OHDA into the foot, both the small (700 Å) and large (1200—1400 Å) dense-cores decreased, and at the same time dense-cores with 1000 Å diameter

increased in number (Fig. 9). Nevertheless, the former alteration showing a double character in shifting direction had disappeared by the 48 h after the administration and the long lasting effect of 6-OHDA on the dense-cores is reflected by a massive decrease in small dense-cores (500 —900 Å) and by a simultaneous considerable increase in the large ones (1200 —1400 Å) (Fig. 9). As compared with the control, histograms obtained after 5,6-DHT treatments showed shifting in only one direction in the co-ordinate system either at earlier (5 h) or later (2 and 10 days) points of time (Fig. 10). Five hours after

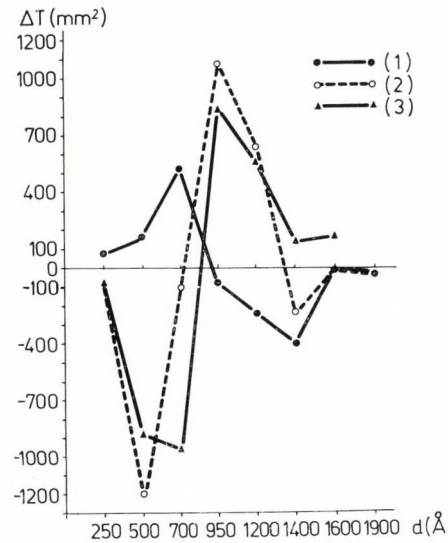

Fig. 10. Frequency distribution histograms obtained after different 5,6-DHT treatments on the basis of the diameters of the dense-core of vesicles, compared to the control (0 point on the ordinate); (1) 5 h after 1×10 mg/kg 5,6-DHT administration, (2) 2 days after 2×10 mg/kg 5,6-DHT, (3) 10 days after 3×10 mg/kg 5,6-DHT

injecting 1×10 mg/kg 5,6-DHT, the number of large dense-cores (1200 —1400 Å) decreased, while later (2 days after 2×10 mg/kg and 10 days after 3×10 mg/kg 5,6-DHT) a considerable decrease in the small (500 —700 Å) dense-cores is characteristic.

In the perikarya composing the cortical layer of the ganglia, neither structural nor fine structural changes were detected after the treatments.

Discussion

On the basis of our results, it may be stated that fine structural changes in the neuropil of the ganglia of fresh-water mussel are evoked by both 6-OHDA and 5,6-DHT. On the one hand, degenerative phenomena appear

in the axons, on the other, alterations take place in the size of the dense-cores of the granular vesicles. Following 6-OHDA and 5,6-DHT treatments, early effects of the "false transmitters" (loading of vesicles, signs of degeneration) were absent. This partly corresponds to that found in the CNS of vertebrates after chemical sympathectomy [22], however, ultrastructural damage of central monoaminergic neurons of vertebrates was already seen as early as a few hours after 6-OHDA [7] or 5,6-DHT [6] administration.

The ultrastructural appearance of the degenerative phenomena evoked by 6-OHDA and 5,6-DHT did not differ from each other and this is in agreement with other results, *viz.*, similar degenerative phenomena had been caused by 5,6-DHT in the anterior byssus retractor muscle [24] and by 6-OHDA in *Venus* heart [25]. In general, the degenerative phenomena observed by us in *Anodonta* ganglia resembled those described in vertebrates [2, 3, 7, 17, 22]. Axon profiles dilated abnormally, as it had been suggested by BARTHOLINI et al. [1], RICHARDS [22] and HÖKFELT and UNGERSTEDT [17], correspond to the ultrastructural picture observed at axonal stumps after cutting of sympathetic nerves [18], and, presumably, points to the stop of the centrifugal axoplasmic flow. Axons showing similar ultrastructure were described in *Anodonta* after cutting of the cerebrovisceral connective [8].

The relative rare occurrence of degenerative phenomena found after either 6-OHDA or 5,6-DHT treatments is in good agreement with findings in vertebrates [17, 22]. The intense glial phagocytosis, observed during the investigations and appearing almost simultaneously with the degenerating processes in the ganglia, could partially explain the rare occurrence of degenerated profiles. The quick disappearance of degenerated structures in the rat CNS was also explained by an intense phagocytosis after 6-OHDA treatment [7, 17].

Present results show that dense-core vesicles are responsible for the storage of both catecholamines and serotonin in the *Anodonta* ganglia, supporting former density-gradient centrifugation results [14], versus earlier electron microscopic findings [28] suggesting that dense-core vesicles would play a role only in the storage of dopamine.

According to biochemical analysis [15, 16], catecholamine as well as serotonin content of the *Anodonta* ganglia were selectively reduced by 6-OHDA and 5,6-DHT. We failed to differentiate between serotonin- and catecholamine-containing terminals as well as between serotonin- and catecholamine-containing dense-core vesicles on the basis of axon profiles being already degenerated or showing early signs of degeneration. After both treatments, the damage of similar terminals containing similar types of vesicles were seen. This finding suggests two possibilities. First, after a commercial glutaraldehyde-osmium double fixation serotoninergic and catecholaminergic terminals and axons do not appear to be different. Second, the storage of a certain transmitter

could be connected to axons with vesicle population differing morphologically from each other. The occurrence of different dense-core vesicles in different axon profiles as well as their presence in one axon profile have already been described in fresh water mussel ganglia [11].

The effect of 6-OHDA and 5,6-DHT on the whole vesicle population of dense-core vesicles seems to be proved by the semiquantitative measurements. A few hours (4—5) after the treatments with the "false transmitters", the changes of the histograms, compared to the control, point to an intense mobilization of the monoamine-storing structures. This fast transmitter mobilization could, in both cases, well be connected to the increased activity appearing in the behaviour (rhythmic activity) of the experimental animals [15, 16]. In spite of this, the increase of the diameters of dense-cores observed after 2—10 days is in good agreement with the considerable and long-lasting depletion of catecholamine and serotonin levels of the ganglia [15, 16]. Presumably, this diameter increase reflects an intragranular uptake of these "false transmitters" being already general at these points of time. This seems to be also supported by the electron microscopic observation, *viz.*, dense-core vesicles with enhanced density appeared in certain axon profiles only later after treatment. It is remarkable that eccentric dense-core vesicles showed enhanced electron density and filling out 4—10 days after 5,6-DHT injection. Occurrence of eccentric dense-core vesicles in serotonin-containing neurons of invertebrates has been described [10, 21, 22, 27].

In the course of our investigations, we failed to find any light or electron microscopic alterations in the perikarya of nerve cells of the ganglia either after 6-OHDA or 5,6-DHT administration, although dense-core vesicles occurred frequently in them. The same was found by Berry et al. [5] in the dopamine-containing giant neuron of *Planorbis corneus* after *in vitro* incubation with 6-OHDA. In our case, this fact could be explained so that different parts of a mussel neuron are differently sensitive to 6-OHDA and 5,6-DHT. Fluorescence-histochemical investigations are in preparation to check the validity of this postulation.

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CONDITIONS OF TRANSFORMATION BY DNA OF *NEUROSPORA CRASSA*

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Abstract

The DNA uptake and transformation of inositol-requiring recipient *Neurospora* strains were investigated. Exponentially growing cultures can accumulate 5—10-fold quantities of donor DNA than older ones. The rate of DNA uptake depends on the physiological state of the recipient cell, and on the molecular weight of donor DNA. The exocellular DNase activity of the recipient culture may influence the DNA uptake and the transformation process. "Young" inositol-requiring *Neurospora crassa* cultures can be transformed by wild type DNA reproducibly, but with low efficiency.

Introduction

The possibility of the transformation of biochemically mutant *Neurospora* strains by DNA or RNA preparations from wild type cultures has been dealt with by many authors [6, 13, 14, 15, 20, 21]. Despite positive results, the conditions of reproducibility of the transformation experiments have not been determined yet.

In the present paper, the conditions of DNA uptake by *Neurospora crassa*, the determination and a maintenance of competence-like physiological state of the recipient culture, the reversion rate of inositol locus after treatment with DNA from wild type (allo-DNA) and DNA from mutant strain (iso-DNA) under optimum experimental conditions were investigated.

Material and methods

Strains

Wild type (RL-3—8 A) and inositol-requiring (inl^-), colonially growing (rg^-) aconidial (R 2506—5—101 a, and R 2506—8—12 A) mutant *N. crassa* strains obtained from the Rockefeller collection were used. The conditions of cultivation [22] and the genetic characteristics of the strains have been published [4, 5, 16]. The inositol locus was chosen to be investigated because of its low reversion rate [8], whereas strains carrying multiple mutations were applied to exclude contamination. *Neurospora* strains were maintained and incubated on VOGEL's medium [24] with and without inositol supplement (100 μg/ml).

Preparation of DNAs

^{32}P-labelled and unlabelled donor DNAs were prepared from 24-h submerged culture of wild type and 56—60-h inositol-requiring mycelia. To determine dry weight, definite volumes of cultures were washed five times with d H_2O, dried at 105 °C for 18 h, then the weight was measured. Hyphae were disintegrated by X-press (Biotech. Inc., USA) and DNA was extracted by MARMUR's method [12]. The DNA preparations were fibrous, but polydisperse in size. To study the effect of molecular weight of donor ^{32}P-DNA on their own uptake, we used DNA preparations of different molecular weights *viz.* $5—10 \times 10^6$ and 1×10^6 daltons, and DNase-digested DNA samples. For transformation experiments, we applied DNA samples collected from the excluding [17] volume of Sepharose-2 B column to reduce their polydispersity [2]. Molecular weight was determined by density-gradient centrifugation (in 5—20% sucrose and by gel electrophoresis (in 0.7% agarose (1)). Lamda b_2 phage genome was used as reference. The DNA content and RNA contamination were determined by diphenylamine [7] and orcinol [19] reactions, respectively. The quantity of protein was measured by LOWRY's method [11].

The DNA preparations from wild type *N. crassa* strains and those from inl$^-$ mutant strains have been designated as allo-DNA, and iso-DNA, respectively.

Cultivation and treatment of recipient cultures

For DNA uptake [22] and transformation experiments, recipient colonies were grown in a rotatory shaking machine (New Brunswick) at 27 °C at 240 rev/min. After a suitable incubation time, the cultures were harvested and washed immediately with VOGEL's medium by centrifugation at 5000 *g* for 5 min. We applied DNase-treated DNA controls in order to check the specificity of high-molecular-weight DNA. The DNA samples were digested with pancreatic DNase (Worthington) of 30 μg/ml in 5×10^{-3} *M* $MgCl_2$ at 37 °C for 30 min. The morphological characteristics of the cultures were examined by phase-contrast microscopy.

The activity of extracellular nucleases of the cultures was determined with denatured DNA as substrate, by the UV absorption changes of the acid-soluble fraction [10]. Transformation experiments were carried out under conditions appropriate for DNA uptake [22]. Colonies (18—22-h) with characteristic morphology were washed in Vogel's medium at 27 °C, *i. e.*, at the optimum temperature. The recipient colonies were resuspended in inositol-supplemented medium of the starting volume, then were treated with 10 μg/ml native or DNase-digested DNA preparations at 27 °C. The samples were incubated at 110 rev/min shaking rate for 60 min. The unadsorbed DNA molecules were digested by DNase treatment and, after washing twice, the colonies were cut into short fragments in a Waring Blendor [23]. The number of hyphae was determined in a hemocytometer and by their viable count. The number of nuclei in hypha fragments was calculated from their DNA content.

The colony-forming capacity of hypha suspensions was determined in Petri dishes on solid VOGEL's medium supplemented with inositol. The number and appearance of revertants to wild type were determined on days 5, 7 and 10 of incubation after plating on minimal medium. Revertants were transferred to minimal medium for biochemical and genetic studies.

Results and discussion

Figure 1 shows the changes in dry weight and DNA content as well as the DNA uptake in various periods of the vegetative growth cycle in submerged culture of *Neurospora crassa* (rg$^-$). It is shown that the DNA uptake of "young" 18—22-h cultures is 5—10-fold higher than that of the older cultures. The rate of growth of recipient cultures of different ages was determined by measuring the concentration of DNA and the dry weight of mycelia, whereas the uptake of exogenous DNA was determined by measuring the quantity of ^{32}P-labelled DNA accumulated during 60 min of incubation. The DNA uptake of 18—22-h cultures suggested a reciprocal relation between the molecular weight of the donor DNA and the length of the time of the uptake.

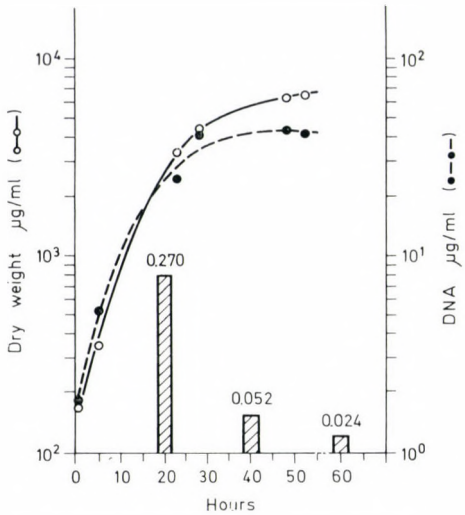

Fig. 1. The dry weight, DNA content and the DNA uptake in submerged cultures of *Neurospora crassa* (rg^-), in various periods of the vegetative growth-cycle. Columns represent the quantity of DNA taken up by the hyphae, given in μg per mg dry weight

As shown in Fig. 2 the uptake of larger molecular weight DNA preparation requires less time than of the same amount of DNA of lower molecular weight. Preparations exposed to DNase digestion lost the ability to penetrate the cells. The DNA uptake was highly influenced even by slight changes in the physiological state of cells.

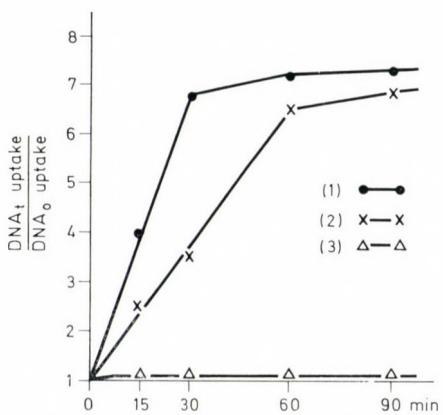

Fig. 2. Effect of the molecular weight of the donor DNA samples on their time-dependent uptake. Symbols: (1) native ^{32}P DNA (M.w. about 5×10^6 daltons); (2) low mol weight ^{32}P DNA (M. w. about 1×10^6 daltons); (3) digested ^{32}P DNA. DNA_t= DNA uptake at different time intervals (min); DNA_0= DNA uptake at "0" time (min)

The rate of DNA uptake can be diminished by lowering the temperature and changing the pH or oxygen supply [22]. It is therefore suggested that the competence-like state of *Neurospora* cells may be due to a sensitive "endocytotic" activity depending on the life cycle. Colonially-growing *Neurospora* strains possess a large number of hypha branches. The metabolic activity of the hyphae is most pronounced on the tips. Microscopic studies have revealed that organelle accumulation is marked by an increased refraction in the zone behind growing tips, whereas excessive vesiculation of the cytoplasm refers to the secretion of enzymes necessary to cell wall synthesis and degradation. Slight environmental effects result in the structural changes of hypha tips [3] and the loss of ability of DNA uptake. A life cycle dependent nuclease activity can also be demonstrated [9, 10, 18].

The failure of transformation in some experiments may be due to the high DNase activity as well. Nucleases had to be removed or inactivated to ensure the integrity of DNA samples during incubation with the recipient cells. According to results shown in Fig. 3, the DNase activity of the medium was significantly decreased in the presence of protamine sulphate, and the continuing uptake of DNA increased by about 100% (not shown in the Figure). DNase activity can be decreased by repeated washing, too, but the DNA uptake of hyphae is reduced by this procedure significantly. The advantage of the use of protamine sulphate, besides its DNase-inhibitory effect, is that in its presence it is enough to wash the mycelia once.

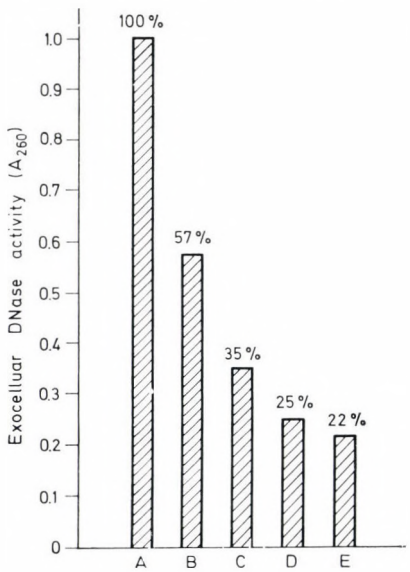

Fig. 3. DNase activity of the 22-h culture medium (A), of the first (B), the second (C) and the third (D) washing medium, and of a first washing medium (E) containing 25 μg per ml protamine sulphate

Table 1

Effect of native and DNase-digested DNA on the reversion of inl⁻ locus in N. crassa

	Number of experiments	Treatment	Number of inl+ revertants $\times 10^{-7}$ viable count		Rate of increase*
			native DNA	DNase treated DNA	
A.	1	allo-DNA (inl+)	5	1	5
	2	allo-DNA (inl+)	12	1	12
	3	allo-DNA (inl+)	8	1	8
B.	1	iso-DNA (inl⁻)	3	3	—
	2	iso-DNA (inl⁻)	1	2	—
	3	iso-DNA inl⁻)	0	1	—

$$\text{* Rate of increase} \sim \frac{\text{No. of revertants after allo-DNA treatment}}{\text{No. of spontaneous revertants}}$$

The optimum conditions for DNA uptake were used in studying the reversion rate of the recipient strain. Ten μg/ml allo-DNA of $2\text{-}3 \times 10^7$ daltons molecular weight increased the inl+ reversion rate by about an order of magnitude in a culture deficient in myoinositol-1-P-synthetase enzyme. The results of the transformation experiments are shown in Table 1. These data suggest that the treatment with native allo-DNA results in a reproducible transformation effect compared to the digested DNA preparation, but the rate of the DNA-induced reversion of the inl⁻ locus is very low. The native iso-DNA does not influence the inl+ reversion rate of the inositol-requiring strain. This indicates that DNA molecules by themselves do not cause a nonspecific increase of the reversion in this part of the genome (Table 1B). Colonies that grew out after DNase-digested allo-DNA or after native iso-DNA and digested iso-DNA treatment are looked upon as spontaneous revertants.

Under our experimental conditions, the frequency of spontaneous reversion belongs to the 10^{-7} order of magnitude, related to the viable count. But calculated from the number of nuclei, the spontaneous reversion rate may be estimated at 10^{-10}—10^{-9}, which is exceedingly low in the case of the inl⁻ locus [8].

Conclusions

We have presented data about the conditions of DNA uptake and allo-DNA-mediated genetic change in *N. crassa*. Further examination of transformation by DNA, particularly the detailed genetic analysis that is possible in

this eukaryotic microorganism, would be valuable in elucidating the mechanisms of integration and expression of genetic information carried by the allo-DNA. It seems probable that information obtained in a relatively simple organism, will be pertinent to similar studies with more complex eukaryotic cells and organisms.

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INVESTIGATIONS ON MYO-INOSITOL-1-PHOSPHATE SYNTHASE FROM THE WILD TYPE AND THE INOSITOL-DEPENDENT MUTANT OF *NEUROSPORA CRASSA*

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Abstract

The inositol-dependent mutant of *Neurospora crassa* lacks inositol-1-phosphate synthetase activity. This defect can be revorted by the addition of high-molecular DNA isolated from the wild type. To elucidate the biochemical background of inositol dependence, inositol-1-phosphate synthetase was studied. A method has been developed for the isolation of the enzyme from the wild type strain in 10 mg scale by salt fractionation, gel filtration and ion-exchange chromatography. The specific activity of the purified enzyme is 4750 U/mg protein and its purity has increased about 100-fold. Polyacrylamide gel electrophoresis indicated that, in addition to the main enzymatically active band, several accompanying proteins occur in very small amount. The molecular weight of the enzyme is 225,000 daltons. Probably it consists of four subunits, two with a molecular weight of 64,000 daltons and another two of 50,000 daltons.

An enzymatically inactive protein has been isolated from the mutant with the same procedure as that of the enzyme; it migrated at gel electrophoresis similarly to the enzyme. It may be supposed that the isolated protein is the defective enzyme molecule.

Introduction

An inositol-dependent mutant of *Neurospora crassa* has been applied as recipient to study the conditions of transformation in eukaryotes. It has been found that the DNA extracted carefully from the culture of the wild type increased significantly the reversion rate of the inositol-dependent mutant [12, 21].

The wild type strain synthesizes myo-inositol from glucose-6-phosphate by the enzyme myo-inositol-1-phosphate synthase (MIPS; [E.C. 5.5.1.4.]), while the mutant strain requires added inositol for its growth. In order to understand the process of reversion, we have studied the characteristics of the enzyme from the wild type strain and compared them to the defective protein of the inositolless mutant, if it has any.

MIPS had been found in several biological objects [8]. The properties of the *N. crassa* enzyme were described by Pina and Tatum [18] and others [4, 13, 15, 16, 17].

In this paper, we describe a procedure for the purification of MIPS from the wild type strain in a quantity of about 10 mg and characterize its molecular properties. In addition, applying the same procedure, we made efforts to demonstrate whether a homologous, but defective protein is synthesized by the mutant and whether it can be isolated by the same method.

Material and methods

Materials

Glucose-6-phosphate-Na_2, dithioerythrol, phenylmethyl-sulphonylfluoride, bovine serum albumin (BSA), ovalbumin and protamine sulphate were purchased from SERVA (Heidelberg, GFR) and immunoglobulin G (IgG) from HUMÁN (Budapest, Hungary). Immunoglobulin A (IgA) was isolated from the serum of a patient with multiple myeloma. BSA, ovalbumin, IgG and IgA were purified by chromatography to homogeneity. The other reagents were of analytical grade.

Neurospora crassa strains and cultivation

The RL-3—8 A wild type strain and the R 2506—8—12 A inositol-dependent mutant were cultivated and maintained on VOGEL's minimal medium with and without inositol supplement (100 μg/ml), in a shaking apparatus at 27 °C. After 24 h incubation, the wild type culture was harvested and the mycelia were washed three times with 0.15 *M* NaCl, 0.001 *M* EDTA (pH 8.0). A 48 h preinoculum was prepared with the inositolless mutant and the inoculated cultures were incubated for 55 to 60 h.

Isolation of the MIPS enzyme

(1) *Preparation of crude mycelial extract*

Mass of cells of about 20 g wet weight was suspended in 40 ml 0.1 *M* Tris-HCl, 5 m*M* EDTA buffer (pH 7.7), and it was passed through an X-press, at —30 °C. Then 40 ml buffer and phenylmethylsulphonylfluoride to 1 m*M* concentration were added. The suspension was homogenized, and centrifuged for 20 min at 5000 *g*.

(2) *100,000* g *supernatant*

The crude extract was centrifuged for 1 h at 2 °C in a Beckman preparative ultracentrifuge, and the supernatant was dialysed twice for 3 h against 3000 ml 25 m*M* Tris-HCl, 5 m*M* EDTA, 2 m*M* mercapto-ethanol buffer (pH 7.7).

(3) *Protamine sulphate fractionation*

To the dialysed supernatant a 2% protamine sulphate solution was added to give the ratio of nucleic acid to protamine sulphate equal to 1 (w/w). The pH was adjusted to 7.7 with 4 *N* NH_4OH and the mixture was allowed to stand for 10 min at 0 °C, then was centrifuged for 20 min at 5000 *g*.

(4) *Ammonium sulphate fractionation*

The protein concentration of the supernatant was diluted to 15 mg/ml and solid ammonium sulphate was added to 50% saturation at 0 °C (31.0 g to 100 ml); the pH was adjusted to 7.7. The suspension was allowed to stand for 15 min and was centrifuged for 20 min at 5000 *g*. To the supernatant, ammonium sulphate was added up to 67% saturation (11.5 g to 100 ml) and the pH was corrected to 7.7 After standing for 30 min at 0 °C the precipitate was centrifuged and dissolved in 3—4 ml 50 m*M* Tris-HCl, 0.25 m*M* EDTA, 5 m*M* mercapto-ethanol buffer (pH 7.7). The solution was dialysed overnight against 500 ml of the same buffer. The buffer was changed once, after 2 h dialysis.

(5) *Sephadex G-200 chromatography*

The dialysed solution (6—7 ml, 55—60 mg protein/ml) was applied upon a 60×2.5 cm column and was eluted with Tris-HCl buffer of the same composition as used for the dialysis. Fractions of 5 ml were collected at a flow rate of 0.13 ml/min. Protein content and enzyme activity were determined and the fractions containing the enzyme were combined.

(6) *DEAE-Sephadex A-25 chromatography*

The combined active fractions were applied upon a 25×1.2 cm column equilibrated with 25 m*M* Tris-HCl, 0.25 m*M* EDTA, 5 m*M* mercapto-ethanol buffer, pH 7.7, and it was washed with the same buffer until the A_{280} of the eluate was less than 0.05. The MIPS was eluted with a linear concentration gradient of 0.05 and 0.5 *M* Tris-HCl buffer (150 ml 50 m*M* Tris-HCl + 150 ml 0.5 *M* Tris-HCl pH 7.7, both solutions contained 0.25 m*M* EDTA and 5 m*M* mercaptoethanol). Fractions of 5 ml were collected at 0.25 ml/min flow rate. The active fractions were combined and concentrated by vacuum dialysis to 2 mg/ml protein concentration and stored at —30 °C or at —70 °C. All procedure was carried out at 0—5 °C, if not indicated otherwise.

Enzyme activity was determined by measuring P_i released by periodate oxidation of inositol-1-phosphate [2]. One ml reaction mixture prepared according to PINA and TATUM [18] contained 1 m*M* dithio-erythrol to protect the sulfhydril groups of the enzyme [14]. After one-hour incubation at 30 °C, 0.3 ml of 20% trichloroacetic acid was added, then the precipitate was removed by centrifugation and P_i was measured [7] in 0.5 ml aliquots directly and after periodate oxidation. One unit of activity is expressed as 1 nmol P_i released per one hour incubation.

Protein content was determined by the biuret method as well as by measuring the absorbancy at 280 nm. BSA was used as reference.

Polyacrylamide gel electrophoresis was carried out in 7.5% acrylamide gel (0.6×10 cm tubes) at 5 °C according to MAURER's [11] gel system No. 1. The samples (50 μg protein/0.05 ml) were applied directly to the gel surface. An initial current of 2 mA/tube was maintained for 30 min, followed by 4 mA/tube for 150 min. Protein bands were stained with Amido Black 10 B. Regions corresponding to the stained areas were recovered from parallel, unstained, gels and homogenized gently in 1.2 ml 50 m*M* Tris-HCl buffer, pH 7.7, using a glass homogenizer. The suspension was centrifuged, and the supernatant was tested for enzyme activity.

Determination of molecular weight was performed on Sephadex G-200 column (60×2.5 cm) by gel filtration, according to ANDREWS [1]. Ribonuclease I, ovalbumin, BSA, IgG and IgA were used as reference proteins.

Sedimentation constant was determined with an enzyme solution of 2 mg protein/ml in MOM G 120 analytical ultracentrifuge (42,800 r.p.m., at 20 °C), applying Philpot-Svensson optics.

Subunit analysis was performed by treating the enzyme with sodium dodecylsulphate, and the subunits were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate as described by WEBER and OSBORN [22]. BSA, IgG and ovalbumin were used as reference proteins.

Results and discussion

Isolation of inositol-1-phosphate synthase from wild type strain

The characteristics of the MIPS enzyme preparations are shown in Table 1. Starting from mycelia of 20 g wet weight, the crude extract contained 4200 mg protein. From the 186,000 enzyme units measured in the 100,000 *g* supernatant after centrifugation, 57,000 units, *i.e.* about 30%, was found in 12 mg final product, the specific activity of which is about 4800 units/mg protein, *i.e.* nearly 80 fold the activity of the 100,000 *g* supernatant. PINA and

Table 1

Purification of inositol-1-phosphate synthetase from wild type Neurospora crassa

Purification, steps	Total protein (mg)	Total* activity, (U)	Specific activity (U/mg protein)	Recovery, per cent	Purification** degree
(1) Crude extract	4200				
(2) 100,000 $\times g$ supernant	3040	186,000	60	100	1
(3) Protamine sulphate supernatant	2630	200,000	76	108	1.3
(4) Ammonium sulphate fraction (0.5 to 0.67 saturation)	370	99,000	268	53	4.5
(5) Sephadex G-200 chromatography (fractions 23—28)	110	85,000	773	46	13
(6) DEAE-Sephadex A-25 chromatography (fractions 19—23)	13	59,500	4580	32	77
(7) Final product after concentration and dialysis	12	57,000	4750	30	80

* 1 activity unit (U): 1 nmol P_i released per hour per ml assay mixture. Activity was calculated by measuring the amount of inorganic phosphate released from inositol-1-phosphate after periodate oxidation (2)

** It is expressed on the basis of increase in specific activity as compared to the 100,000 g supernatant

TATUM [18] obtained 5.5% of the total activity in the final product, but their data on specific activity appear to be surprisingly high (1,195,000 units/mg protein). Our results accord well with the data obtained with the enzyme from yeast [5, 6] (specific activity 1600 units/mg protein) and with an enzyme of plant origin [9, 10].

In accordance with LOEWUS and LOEWUS [9] we observed that in the course of salt fractionation, redissolving and dialysis, 50% of the total activity was lost. The bulk of the activity can be found in the fraction obtained at 50 to 67% ammonium sulphate saturation. The loss of activity may partly be due to inactivation. In the subsequent steps, however, the loss was negligible, whereas the specific activity increased significantly. The enzyme assays gave reproducible results. In the crude extract, however, this method could not be applied because of the high inorganic phosphate concentration. In steps 2, 3 and 4 of the purification procedure 5 m*M* EDTA was added to the reaction mixture, that almost completely inhibited phosphatases. EDTA at this concentration has hardly any influence on MIPS activity, whereas its higher concentrations are strongly inhibitory [13].

Gel filtration on Sephadex G-200 column subsequent to salt fractionation appeared to be very suitable to remove phosphatases (Fig. 1). The MIPS activity can be found in fractions eluted immediately after the exclusion volume (fractions 23 to 29), whereas phosphatases are found in the following

fractions. MIPS extracted from yeast could not be well separated from phosphatases by gel filtration [6].

The eluted fractions which contained most of the enzyme activity were combined and were purified further by ion-exchange chromatography on DEAE-Sephadex column, applying linear gradient elution (Fig. 2). The

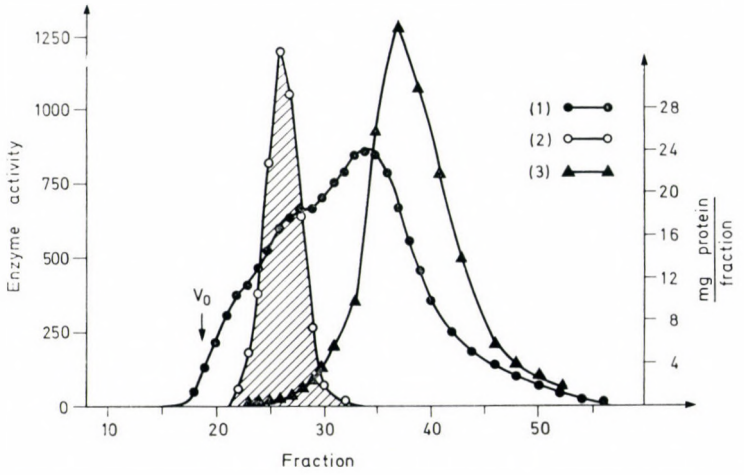

Fig. 1. Gel filtration on Sephadex G-200 column of protein fraction precipitated by ammonium sulphate from the extract of wild type *Neurospora crassa*. (Column size: 2.5×60 cm, fraction volume: 5 ml, flow rate: 0.13 ml/min, elution: 50 m*M* Tris-HCl — 0.25 m*M* EDTA — 5 m*M* mercapto-ethanol, pH 7.7.) (1): mg protein/fraction; (2): inositol-1-phosphate synthetase activity (μg P_i/fraction), (3): phosphatase activity (μg P_i/fraction)

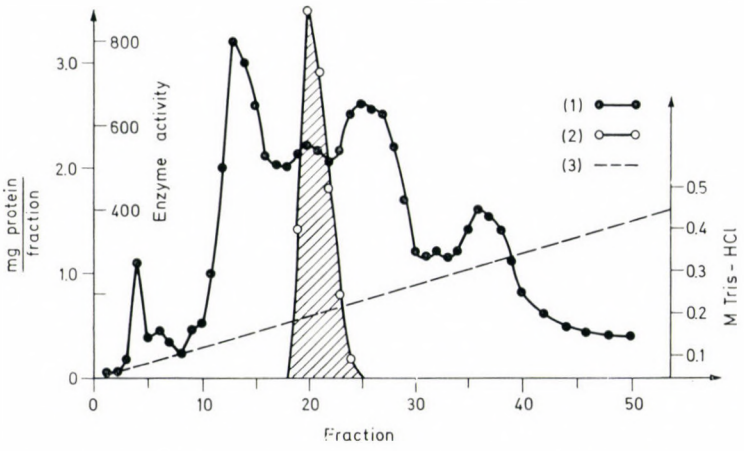

Fig. 2. Fractionation of inositol-1-phosphate synthetase activity obtained by gel filtration on DEAE-Sephadex A-25 column by linear gradient elution (pH 7.7). (Column size: 1.2×25 cm, fraction volume: 5 ml, flow rate: 0.25 ml/min, gradient elution: 150 ml 50 m*M* Tris-HCl +150 ml 0.5 *M* Tris-HCl, pH, 7.7, both solutions contained 0.25 m*M* EDTA and 5 m*M* mercapto-ethanol.) (1): mg protein/fraction; (2): enzyme activity (μg P_i/fraction); (3): buffer concentration

MIPS was recovered in the third peak, between 0.15 and 0.25 *M* Tris-HCl concentration. Similar conditions were reported by PINA and TATUM [18] and LOEWUS and LOEWUS [9], who had eluted the synthetase from DEAE-cellulose column by 0.15 and 0.125 *M* NaCl solutions, respectively. CHEN and CHARALAMPOUS [6] applied DEAE-Sephadex column and used Tris-HCl buffer as eluent. Their enzyme was eluted with 0.4—0.5 *M* Tris-HCl.

Molecular characteristics of the inositol-1-phosphate synthase

The molecular characteristics of the MIPS are listed in Table 2. According to the data of PINA et al. [17] MIPS has a molecular weight of 150,000. The somewhat higher value 225,000, obtained by us by gel filtration (1) is supported by the simultaneous determination of the sedimentation constant (8.0 S) and by the subunit analysis.

Table 2

Molecular characteristics of purified myo-inositol-1-phosphate synthase

Sedimentation coefficient	8.0 S
Molecular weight	225,000 daltons ± 5%
Subunit composition	2×64,000 daltons
	2×50,000 daltons
Specific activity	4750 U/mg protein
Michaelis constant (K_M)	1.82 m*M* (for glucose-6-phosphate)

The high molecular weight and the molecular structure of MIPS obtained from other sources [19, 20] suggested that the *N. crassa* enzyme is of oligomeric structure. The purified enzyme was treated with dodecyl sulphate and was subjected to gel electrophoresis [22]. Two protein bands were obtained, with molecular weights of 64,000 and 50,000, respectively. Since the molecular weight of the native enzyme was found to be 225,000, it seems probable that it consists of four subunits, and they are composed of two identical pairs.

The molecular weight of the enzyme isolated from rat testis [19] was found to be 215,000, in accordance with BARNETT et al. [3]. It consists of two identical pairs of subunits. Recently it has been demonstrated that the enzyme isolated from bull testis [20] is an oligomer, too, and it consists of four identical subunits and its molecular weight is 218,000. The molecular weight of MIPS obtained from other sources can be either higher or lower. For example, LOEWUS and LOEWUS [9] isolated an enzyme from plants of 150,000. On the other hand, NACCARATO et al. [14] found that the enzyme from rat mammary glands is of 290,000 molecular weight. The subunit composition of these enzymes has not yet been investigated.

Attempts to isolate inositol-1-phosphate synthase from the inositol-dependent mutant

With the method applied with the wild type strain, we obtained a protein fraction from the inositol-dependent mutant. This behaved identically with the active enzyme during fractionation though, showed no enzyme activity. Care was taken of the identity in every step of the isolation procedure. By ion-exchange chromatography, only one dominant protein peak with a shoulder on the descending side was obtained (Fig. 3). The protein shoulder coincided

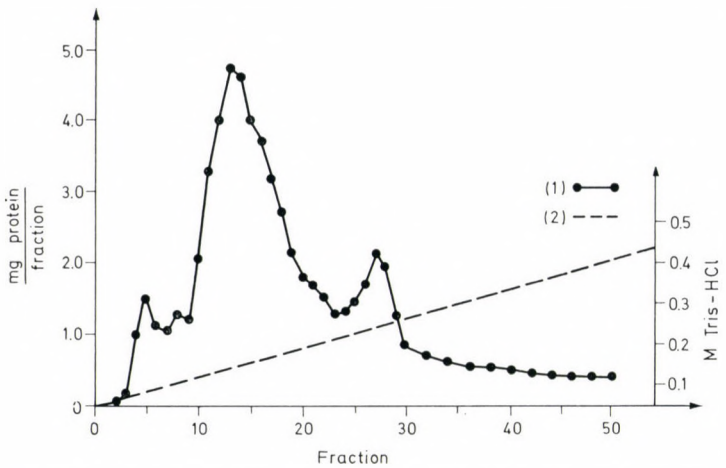

Fig. 3. Purification of protein fractions (23—28, see Fig. 1) obtained from the inositol-dependent mutant by gel filtration on DEAE-Sephadex A-25 column, applying linear gradient elution (pH 7.7). (Experimental conditions as in Figure 2.) (1): mg protein/fraction; (2): buffer concentration

with the fractions in which the active enzyme was detected with the wild type strain. These fractions were analyzed by gel electrophoresis, simultaneously with the wild type MIPS (Fig. 4).

The purified MIPS obtained from the wild type is not completely homogeneous (gels 1 and 12). Apart from the sharply separated main band, it contains several proteins in very small amounts. Only the intensively stained third band has proved to have enzyme activity. More than 90% of the activity applied to the gels was regained in this band. LOEWUS and LOEWUS [10] demonstrated 8 to 10 protein bands in MIPS preparations obtained from plants. They found two enzymatically active protein bands, one of them in a similar position on the gel as our enzyme.

Proteins isolated from the inositol-dependent mutant (gels 2 to 11 in Fig. 4) were also composed of several, well-defined, bands in gel electrophoresis. In some of the fractions eluted at the same salt concentrations as the enzyme

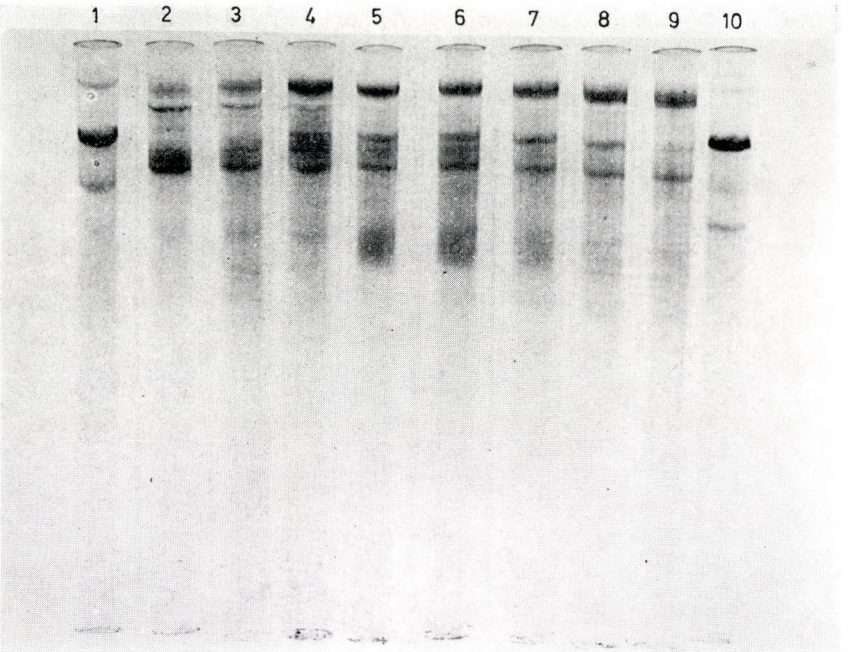

Fig 4. Gel electrophoretic analysis of purified inositol-1-phosphate synthetase and of protein fractions obtained in the same way from the inositol-dependent mutant. (These fractions were eluted from DEAE-Sephadex A-25 column.) (Run on 7.5% polyacrylamide gel, at pH 8.9 and 5 °C, initial current 2 mA/tube for 30 min, followed by 4 mA/tube for 150 min; staining with Amido Black 10 B; sample: 50—100 μg protein per tube.) No. 1 Purified synthetase enzyme from wild type *Neurospora crassa*; No. 2 DEAE-Sephadex fraction 14 from inositol-dependent mutant; Nos 3—9 DEAE-Sephadex fractions 16—22 from inositol-dependent mutant; No. 10 purified synthetase enzyme (another preparation then shown by No. 1)

a pale band appeared at the same position on the gel as the active enzyme. This protein band, stained somewhat stronger than the others in fraction 20, could consistently be demonstrated in several experiments. Its identical position with that of the MIPS can be seen on densitograms from other experiments, too (Fig. 5). Assuming that there is no significant difference in charge and molecular size, we expect that this is the defective enzyme protein.

In further experiments, we want to go on fractionating these proteins for analysis. The identification of the supposed defective enzyme protein requires complex studies, however, in preliminary experiments some of the protein fractions isolated from the inositol-dependent mutant resulted in a positive reaction with immune sera produced against the purified MIPS.

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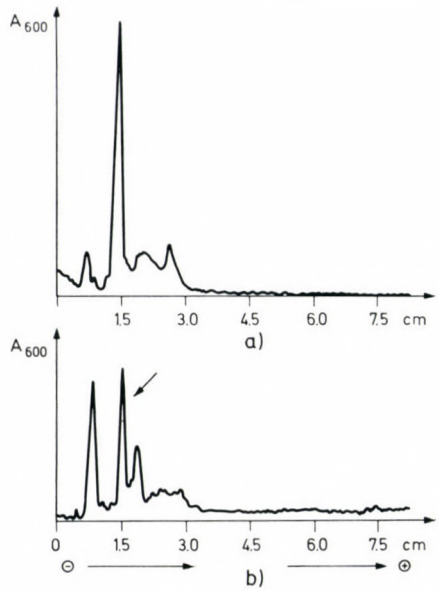

Fig. 5. Densitograms of proteins separated by gel electrophoresis. (Experimental conditions see in Fig. 4.) a) Purified synthetase enzyme from wild type *Neurospora crassa* 1; b) protein fraction obtained in the same way from inositol-dependent mutant (eluted from DEAE-Sephadex column in the same position)

Abbreviations

DEAE: Diethyl aminoethyl
DNA: Deoxyribonucleic acid
EDTA: Ethylene diamine tetraacetic acid, Na_2 salt
MIPS: myo-inositol-1-phosphate synthase
Tris: Tris-(hydroxymethyl) aminomethane
BSA: Bovine serum albumine
IgG: immune globulin G
IgA: immune globulin A

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A DIRECT LEAD TECHNIQUE FOR HISTOCHEMICAL DEMONSTRATION OF LEUCOCYTE ALKALINE PHOSPHATASE ACTIVITY IN BLOOD SMEARS

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Abstract

A histochemical technique for demonstrating leucocyte alkaline phosphatase activity (LAP), based on direct precipitation of lead phosphate at pH 9.5, is described. The effect of fixative, temperature and stability of the medium on the activity of the enzyme and stability of the colour reaction was thoroughly studied. Peripheral blood smears obtained from both normal humans and pathological cases were studied and the results were compared with these obtained by the azo-dye method.

Introduction

The cytochemical techniques for visualization of leucocyte alkaline phosphatase (LAP) in current use are modifications of either the original GOMORI–TAKAMATSU method [4, 15], which is based on indirect precipitation of heavy metals, or the azo-dye coupling technique described by KAPLOW [7, 8] and HAYHOE and QUAGLINO [5].

The principle on which the GOMORI–TAKAMATSU technique was based is to incubate fixed smears in buffered medium containing β-glycerophosphate in presence of calcium chloride and Mg^{2+}; the latter act as activator. The liberated phosphate ions are then precipitated as calcium phosphate, which can be converted through a soluble cobalt salt into the corresponding insoluble cobalt phosphate. The latter is then converted to dark brown cobalt sulphate by adding ammonium sulphide.

However, many investigators [2, 10, 11, 12, 13, 14, 16] have shown that this procedure may produce false staining of nuclei and other structures and the long incubation period that is usually required results in an unfavourable diffusion pattern of staining.

In the present paper, we describe the application of a simple and rapid lead method developed by EL-AASER and HASSANEIN [3] for demonstrating the LAP activity. The method is based on direct precipitation of lead phos-

phate at pH 9.5, the optimum pH for the enzyme activity, then converting it to dark brown precipitate of lead sulphide.

Material and methods

Peripheral blood smears were obtained from 40 healthy controls (12 females and 28 males) and 17 patients with various pathological disorders.

The smears obtained from each subject were submitted to the present lead method and the azo-dye method of HAYHOE and QUAGLINO [5], latter slightly modified.

The effect of fixative, temperature and stability of the medium on the activity of the enzyme, in addition to the stability of the colour stain, was thoroughly studied.

The lead method

The lead method developed by EL-AASER and HASSANEIN [3] for tissue sections was used. It is based on using tris as buffer, L-tartarate as chelating agent for lead ion and β-glycerophosphate as substrate.

Stock buffer substrate solution

It consists of 150 ml of 0.2 *M* tris buffer; 25 ml of 0.05 *M* sodium β-glycerophosphate; 50 ml of distilled water and 6 ml of 1% L-tartaric acid. This solution has a pH of 9.5 and is stable at 4 °C for months.

Working substrate solution

The medium for immediate use was prepared by adding 4.5—5 ml 1% lead nitrate dropwise, while stirred, to 45 ml of the stock buffer substrate solution; then 2.0 ml of 0.1 *M* magnesium chloride was added to serve as activator. Filtration was carried out when necessary. To check for the presence of enough lead in solution we added several drops of diluted solution of disodium hydrogen phosphate to about one ml of the medium. Immediate appearance of white turbidity indicates the presence of sufficient amount of lead in the medium.

Technique

Fresh dried smears were fixed according to KAPLOV and BURSTONE [9] at room temperature in acetone-citrate fixative for one min, washed with distilled water, and air-dried. The dried slides were incubated in the working substrate solution at 37 °C for 30 min. After incubation, the smears were washed with distilled water, then immersed for 1 min in freshly-prepared weakly alkaline ammonium sulphide solution. Slides were then washed in distilled water and counterstained in 2% methyl green for 10 min. Lead phosphate produced by the reaction was visualized as dark brown lead sulphide granules. In control smears either heated in boiling water for five min or incubated in substrate-free media, no staining was observed after incubation.

The azo-dye method

The incubation medium a modification of HAYHOE and QUAGLINO [5]. It consisted of

Solution A:

40 mg sodium alpha-naphthyl-phosphate dissolved in 60 ml of 0.5 *M* 2-amino-2-methylpropane 1,3 diol (pH 9.7) to which 2 ml of 0.1 *M* $MgCl_2$ solution was added.

Solution B:

40 mg of fast blue BB salt dissolved in 20 ml distilled water. For immediate use solution A and B were mixed in the ratio of 3 : 1.

Technique: Fixed smears in acetone-citrate were incubated in the medium for 20 min, then counterstained with 2% methyl green.

Scoring of stained smears

Slides stained by either technique were scored in the same way as described by KAPLOW [7] and HAYHOE et al. [6].

Results and discussion

In the present investigation the lead method developed by EL-AASER and HASSANEIN [3] on tissue sections was used for evaluating the validity of the method in demonstrating leucocyte alkaline phosphatase in blood smears. Comparable studies were carried out by the azo-dye method. The effect of fixative, magnesium ions and temperature on the activity of the enzyme as well as the stability of incubation medium and colour stain were investigated. Results expressed in both methods as total score are presented in Tables 1—5.

Studies on the choice of fixative showed that acetone buffered with citrate (pH 4.2) was a better fixative than formalin vapour or formo-methanol. Better morphological details with high level of enzyme activity were obtained.

Table 1 presents the total score obtained from reactions carried out with incubation media prepared 1/2, 1, 2, 14 and 48 h before use. In the azo-dye method, no reaction could be obtained except with freshly prepared medium. On the other hand, with the lead method, though there was a partial drop in the reaction after 1/2 h and over, a reaction could be obtained even with the medium prepared 24 h before use.

Mg^{2+}, 5 m*M*, was found to activate the enzyme by about 60% in either of the methods in smears obtained from male or female subjects.

Table 2 presents scores obtained from a reaction carried out at both 20 °C and 37 °C for the same smears, by using the lead method. It is obvious that incubation at 37 °C gave higher scores than when the reaction was per-

Table 1

Stability of the incubation medium

Method	No. of cases	Total score					
		used immediately	Age of the medium before use (hours)				
			½	1	2	24	48
Lead	2	140	132	125	123	92	0
		(135—145	130—130)	(118—128)	(118—128)	(78—106)	0
Azo-dye	2	98.5	0	0	0	0	0
		(98—99)	0	0	0	0	0

formed at room temperature. Total scores obtained at 37 °C were by 51% to 71% higher than those obtained at 20 °C.

The stability of the colour stain obtained by both methods expressed as total scores is presented in Table 3. Scoring was carried out immediately,

Table 2

Effect of temperature on total score of LAP. Lead method

No. of cases	20 °C (room temperature)		37°C	
	total score	per cent	total score	per cent
3	60 (21—109)	100	94 (36—165)	156 (151—171)

Table 3

Colour stability of LAP stain in lead and azo-dye methods

Method	Storage in	No. of cases	Period of storage			
			0 score	2 days score	4 days score	6 days score
Lead	dark	2	134.5 (84—185)	133.5 (83—184)	132.5 (83—182)	133.0 (83—183)
	light	2	134.5 (84—185)	133.5 (83—184)	132.5 (82—183)	133.0 (83—183)
Azo-dye	dark	2	155.3 (68—163)	95.0 (58—132)	49.0 (32—60)	21.0 (8—34)
	light	2	155.5 (68—163)	62.5 (36—89)	23.0 (22—24)	6.0 (2—10)

and after 2, 4 and 6 days of storage. The effect of dark and light on the total scores is also presented. Using the azo-dye method, scores rapidly diminished when smears were kept in light. When stained smears were stored in the dark, the loss of colour stain was less marked. With the lead method however, scores remained almost the same throughout and scoring could be carried out after any period of time. In the latter method, the colour stain was equally stable in both light and dark.

Table 4 presents data obtained from 12 normal healthy female and 28 male subjects between 20 and 50 years of age. Scores for the normal female subjects ranged between the 45 and 100 with an average of 73.5 when the lead method was used. With the azo-dye method, the scores for the same subjects ranged between 36 and 80 with an average of 61.3.

The scores for the normal healthy males ranged between 32 and 114 with an average of 58.3 when the lead method was used and between 20 and 96 with an average of 43.1 with the azo-dye method.

Scoring data obtained by both lead and azo-dye method on 18 cases with various pathological disorders are presented in Table 5. The highest score was obtained for a patient with chronic lymphatic leukaemia, *viz.*, 228 with the lead method and 196 with the azo-dye method. The lowest scores were obtained in two cases of chronic granulocytic leukaemia. The first case gave score of 6 when the lead method was used and zero with the azo-dye method; the second case gave a total score of 40 with the lead method and 3 with the azo-dye method. We have shown that one of the disadvantages

Table 4

Scoring data for LAP. Blood smears from normal healthy subjects

Sex	Age, years	No. of cases	Total count ×1000	Method used	Grade of score (percentage of neutrophils)				Total score
					0	1	2	3	
Female	20—50	12	4.8—7.5	Lead	35.75	61.66	5.91	0	73.5
					(10—60)	(34—36)	(0—10)		(45—100)
				Azo-dye	41.25	56.16	2.58	0	61.3
					(20—60)	(28—85)	(0—5)		(36—80)
Male	20—50	28	4.0—100	Lead	52.5	38.75	7.46	1.28	58.39
					(24—72)	(21—76)	(0—22)	(0—10)	(32—114)
				Azo-dye	58.7	39.07	1.5	0.35	43.14
					(8—80)	(20—88)	(0—10)	(0—6)	(20—96)

of the azo-dye method is the instability of the medium and instability of the colour reaction and, unless the smears are stained immediately after preparation of the incubation medium and scoring carried out immediately after incubation, the results obtained cannot be considered reliable. Furthermore, using this method for routine investigation may expose the person to the hazards of a possible carcinogenic effect of substances as naphthol and diazonium salts.

The main problem in using lead for direct visualization of alkaline phosphatase in tissues is the difficulty in keeping it in solution at pH 9.5, the pH optimum of the enzyme activity. According to BARKA and ANDERSON [1], the maleate compounds allows a sufficient amount of lead ions to remain in solution. However, at pH 9.0 lead hydroxide is formed which has relatively low solubility.

Table 5

Scoring data for LAP on blood smears from patients with various diseases

Diagnosis	No.of cases	Method	Total score
		Lead	72.7 (60—104)
Cancer-Bladder	3	Azo-dye	71.3 (54—100)
		Lead	123.0 (92—140)
Lymphosarcoma	3	Azo-dye	102.0
	3	Azo-dye	(84—118)
Reticulum cell sarcoma	4	Lead	110.75 (88—170)
		Azo-dye	88.25 (65—136)
Hodgkin's lymphoma	1	Lead	98.0
		Azo-dye	70.0
Acute lymphatic leukaemia	1	Lead	170.0
		Azo-dye	125.0
Chronic lymphatic leukaemia	1	Lead	228.0
		Azo-dye	196.0
Monocytic leukaemia	1	Lead	170.0
		Azo-dye	158.0
Chronic granulocytic leukaemia	2	Lead	23.0 (6—40)
		Azo-dye	1.5 (0—3)
Ulcer of the foot with infection	1	Lead	131.0
		Azo-dye	110.0

El-Aaser and Hassanein [3] found tartarate to be more efficient than maleate as a chelating agent for lead. This makes it possible to work at pH optimum of the enzyme (pH 9.5), avoiding the precipitation of lead from the medium. Using a substrate other than β-glycerophosphate gave less stable medium.

Pitfalls caused by the transformation of calcium phosphate into cobalt or lead phosphate are avoided by the direct method. Since the reaction is carried out at optimum pH, the possibility of false localization as caused by the activity of other enzymes is reduced.

One of the advantages of the present technique is the stability of the incubation medium, which can be stored in stock solution ready for use for many months. Moreover, the stability of the colour stain permits the possibility of carrying out scoring after any period of time. Neither light nor dark had any effect on the final results. The present lead method gave a well-localized

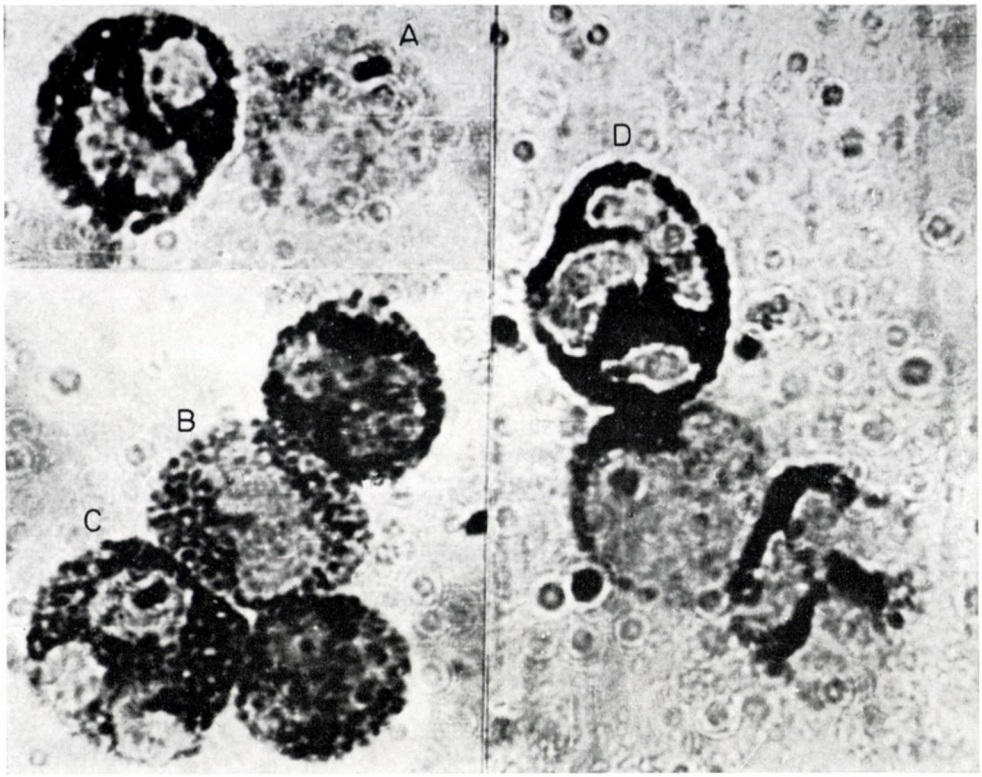

Fig. 1. Neutrophil alkaline phosphatase reaction demonstrated by the lead method from a patient with lymphosarcoma. Note the variation in the intensity of the stain which is well localized in the cytoplasmic granules of neutrophils; nuclear stain is absent. ×2100

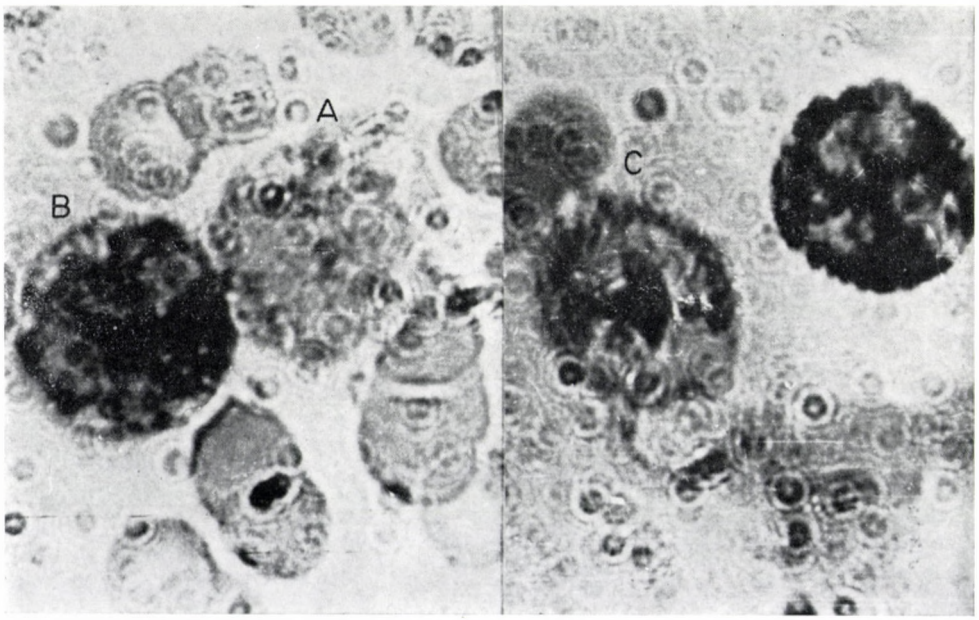

Fig. 2. Neutrophil alkaline phosphatase reaction demonstrated by the azo-dye method from the same patient as in Fig. 1 ×2100

stain in the cytoplasmic granules with different degrees of intensity and the nuclear stain is completely absent as shown in Fig. 1 (compare with azo-dye method Fig. 2).

It is of importance that in all cases, both normal and pathological, the lead method resulted in higher scores than the azo-dye method, thus, confirming the sufficient sensitivity of the lead method. Moreover, the adaptation of the lead method for electron microscope studies can be achieved.

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HISTOCHEMICAL OBSERVATIONS ON THE GIANT NEUROSECRETORY CELLS OF THE THORACIC GANGLION OF THE ADULT AND JUVENILE CRABS, *POTAMON MAGNUM MAGNUM* (PRETZMAN)

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Abstract

The giant neurosecretory cells in the thoracic ganglion of the adult and juvenile crab, *Potamon magnum magnum* (Pretzman) were histochemically investigated. The secretion is mainly proteinaceous in nature, containing considerable amounts of acid mucosubstances, sulphate esters, lipids and a little carbohydrate but no glycogen. The detailed nature of the proteinaceous neurosecretory material in the adult crab was further tested. It appears that the neurosecretory material of these cells contains moderate amounts of sulfhydryl groups and few of disulphide bonds. No trace of tyrosine could be observed. The neurosecretory granules were associated with considerable amounts of cytoplasmic RNA. In general, stronger reactions were obtained in summer and winter than in other seasons.

Introduction

In decapod *Crustacea*, the phenomenon of neurosecretion has been widely studied; both morphological and physiological investigations have been carried out on the neurosecretory systems of these arthropods. The morphology of the neurosecretory systems of *Decapoda* has been described [3, 4, 17, 25, 26], however, few histochemical studies have been made on their neurosecretory material [13, 14, 21, 22, 26, 27, 28, 29, 36, 37, 38, 40].

The morphology of the giant neurosecretory cells in the thoracic ganglion of *Potamon magnum magnum* (Pretzman), a fresh-water crab, has been described by BAID et al. [3, 4]. These cells, designated as A-type cell, occurred with and without axons. We undertook the present work to reveal, firstly, the histochemical nature of the neurosecretory material in juvenile crabs and secondly, the detailed histochemical nature of the protein representing the major part of the secretory material in the adult crabs.

Material and methods

Juvenile and adult crabs, *Potamon magnum magnum* (Pretzman) were collected from the suburbs of Mosul, a town in Northern Iraq. The thoracic ganglia were excised and fixed in appropriate fixatives. Horizontal paraffin sections of 8-μm thickness were obtained. All

the histochemical tests were performed as described in the monographs of PEARSE [33] and GURR [18].

The following staining reactions and histochemical techniques were employed for the juvenile crabs:

(1) CAMERON and STELEE's [18] modification of GABE's aldehyde fuchsin (AF) technique.

(2) BARGMANN's [5] modification of GOMORI's chromalum haematoxylin–phloxine (CHP) technique.

(3) MALLORY–HEIDENHAIN stain [18].

(4) Acrolein-Schiff technique for protein having NH, NH_2, SH and imidazole reactive groups (VAN DUIJN, [35]).

(5) Alloxan-Schiff technique for protein-bound amino groups [33].

(6) Performic acid-alcian blue (PFAAB) technique for disulfide groups (ADAMS and SLOPER) [35].

(7) Methyl green–pyronine technique for RNA (BRACHET [35]).

[8] Periodic acid-Schiff (PAS) technique for hexose-containing mucosubstances (MCMANUS, [35]). Two series of serial sections were treated, one for each of the following tests:

a) the standard method for PAS reaction;

b) diastase digestion (30 min at 32 °C).

(9) Alcian blue technique (AB) for acid mucopolysaccharides or acid mucosubstances (STEEDMAN, [35]).

(10) Acriflavine technique for sulphate esters (LILLE, [35]).

[11] Sudan black B (SBB) technique for lipids (MCMANUS, [35]).

Most of the sections obtained from adult crabs were examined by histochemical techniques similar to those carried out for the juvenile crabs. Tests Nos 4, 5, 6 and 7 are common for both stages. In addition, the following three tests were carried out in adult crabs:

1.) DDD technique for sulfhydril and disulphide groups (BARRNETT and SELIGMAN, [35]).

2.) Modified gallocyanine chromalum technique for nucleic acids (BERUBE et al., [35]).

3.) Millon's reaction for tyrosine (BENSLEY and GERSH, [35]).

The reading of the tests was subjective, and the responses were scored on a scale ranging rom 0 (negative) through + to ++ (positive) to +++ (strongly positive).

Results

The results for juvenile crabs are summarized in Table 1, those for adult crabs in Tables 2 and 3, in which are indicated the responses of both types of the giant neurosecretory cells to these techniques.

The staining reactions and histochemical tests for juvenile and adult crabs

(1) Aldehyde fuchsin (AF) technique. Both types of the neurosecretory cells, with and without axon, showed deep purple granules while the cytoplasm, nucleus and nucleoli remained unstained. The cytoplasm was light green-positive, while the nucleoli were orange G-positive.

(2) Chrom-haematoxylin–phloxine (CHP) technique. The neurosecretory granules in both cell types stain dark or deep purple. In the faintly stained nucleus, the nucleoli appeared darker.

(3) Mallory's triple stain. The neurosecretory granules in both cell types stain purple, the cytoplasm reddish-brown. The nucleus appeared pale purple, the nucleoli red.

(4) Acrolein-Schiff technique. The neurosecretory granules in both cell types stain magenta, indicating the presence of significant amounts of

Table 1

Staining reaction and histochemical tests in the neurosecretory material of the giant neurosecretory cells of the juvenile crab, Potamon magnum magnum *(Pretzman)*

Technique used	Fixative	Giant neuro-secretory cells with axon	Giant neuro-secretory cells without axon	Interpretation
AF	Bouin	deep purple	deep purple	Neurosecretion
CHP	Bouin	deep purple	deep purple	Neurosecretion
Mallory triple stain	Zenker	purple	purple	Histology
Acrolein-Schiff	Ca-formol, Bouin	+++	+++	Protein with, NH, NH_2, SH and imid-azole-reactive groups
Alloxan-Schiff	Ca-formol, Bouin	+++	++	Protein-bound NH_2
Performic acid–alcian blue	Ca-formol, Bouin	+	+	Disulphide bonds
Methyl green–pyronine	Formol (10%)	+++	+++	RNA
Periodic acid-Schiff (PAS)	Helly, Ca-formol	++	++	Polysaccharides
(PAS diastase)	Helly, Ca-formol	++	++	Glycogen (control)
Alcian blue (AB)	Helly, Ca-formol	+++	++	Acid mucosub-stances
Acriflavine	Helly, Ca-formol	+++	+++	Sulphate esters
Sudan black B (SBB)	Ca-formol	+++	+++	Lipids

Scoring: 0 = negative; + to ++ = positive and +++ = strongly positive, reaction

Table 2

Histochemical reactions of the neurosecretory material in the giant neurosecretory cells of the adult crab, Potamon magnum magnum *(Pretzman)*

Technique used	Fixatives	Reactions in giant cells with axons summer	autumn	winter	spring	Interpretation
Acrolein-Schiff	Ca-formol, Bouin	++	+++	+++	++	Protein having NH, NH_2, SH and imid-azole reactive groups
DDD	Ca-formol, ethanol	++	+	+	+	Sulfhydryl groups
DDD (N-ethyl maleimide)	Ca-formol, ethanol	+	+	+	+	Disulphide bonds
Ninhydrin-Schiff	Ca-formol, Bouin	+	+	+++	+	Protein-bound NH_2
PFAAB	Ca-formol, Bouin	+	+	+	+	Disulphide bonds
Millon's reaction	Ca-formol, Bouin	0	0	0	0	Tyrosine
Gallocyanin–chromalum	Formol (10%)	++	++	+++	+	Nucleic acids
Methyl green–pyronine	Formol (10%)	++	+++	++	++	RNA

See footnote of Table 1

Table 3

Histochemical reactions of the neurosecretory material in the giant neurosecretory cells of the adult crab, Potamon magnum magnum *(Pretzman)*

Technique used	Fixatives	Reactions in giant cells without axons summer	autumn	winter	spring	Interpretation
Acrolein-Schiff	Ca-formol, Bouin	+++	++	+++	++	Protein having NH, NH_2,SH and imidazole reactive groups
DDD	Ca-formol, ethanol	++	+	+	+	Sulfhydryl groups
DDD (N-ethyl maleimide)	Ca-formol, ethanol	+	+	+	+	Disulphide groups
Ninhydrin-Schiff	Ca-formol, Bouin	+	+	+++	+	Protein-bound NH_2
PFAAB	Ca-formol, Bouin	+	+	+	+	Disulphide groups
Millon's reaction	Ca-formol, Bouin	0	0	0	0	Tyrosine
Gallocyanin–chromalum	Formol (10%)	++	+	+++	++	Nucleic acids
Methyl green–pyronine	Formol (10%)	++	+++	++	++	RNA

See footnote of Table 1

protein with NH, NH_2, SH and imidazole reactive groups. The secretory granules were scattered throughout the perikaryon and showed large aggregations at the perinuclear zone and the cell periphery (Fig. 1). In adult crabs, the neurosecretory granules, in both cell types, were accumulated around the nucleus, towards the periphery and, as dense masses, around the vacuoles. In summer, autumn and winter, these cells showed more pronounced positive reaction than in spring (Figs 4—11).

(5) Alloxan-Schiff. This is a general protein stain. The secretory granules in both cell types stain faint pink to red, indicating the presence of little to moderate amounts of protein-bound amino groups. In adult crab, the neurosecretory granules in both cells produced faint pink to red colour indicating the presence of limited amount of protein-bound amino groups.

In winter, significant amounts of protein-bound amino groups were found to be associated with the neurosecretory material in both types of cells, whereas, in spring and summer, the amount of protein-bound amino groups considerably decreased. In autumn, the cells were positive for protein-bound amino groups. In the giant cells, the concentration of reactive groups was high during winter, progressively declined in spring and summer, but again showed an upward trend in autumn. The protein-bound amino groups of the neurosecretory material were thus found to undergo seasonal changes.

(6) Performic acid–alcian blue (PFAAB) technique for disulphide bonds. In both juvenile and adult crabs, the neurosecretory granules of both cell

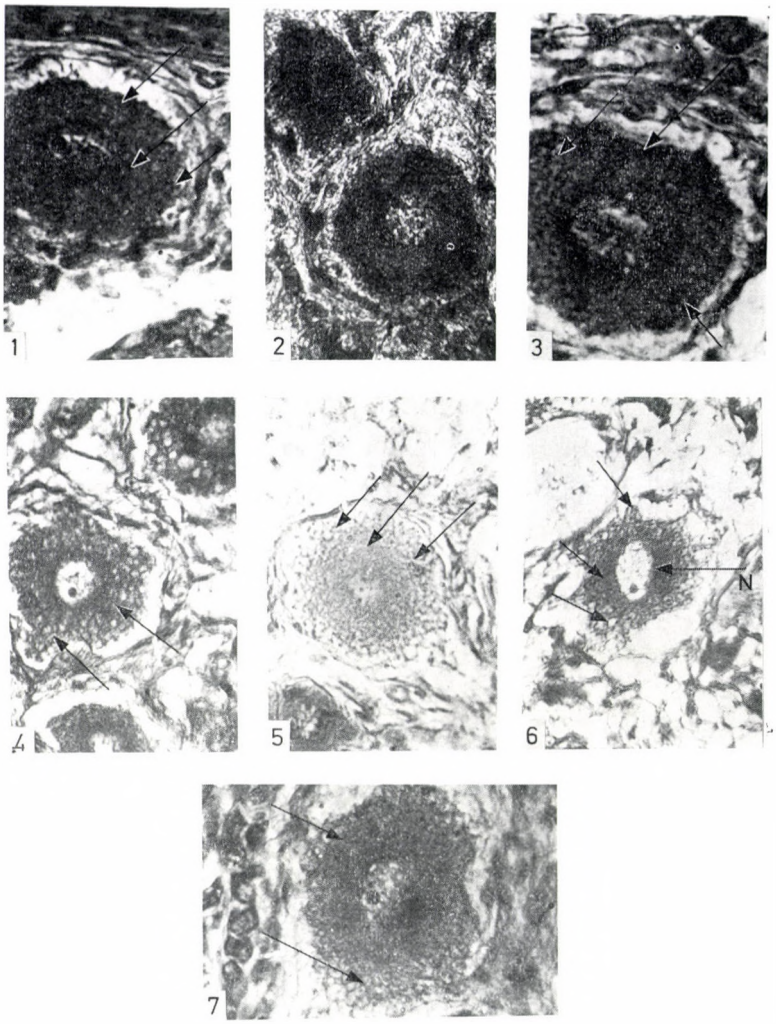
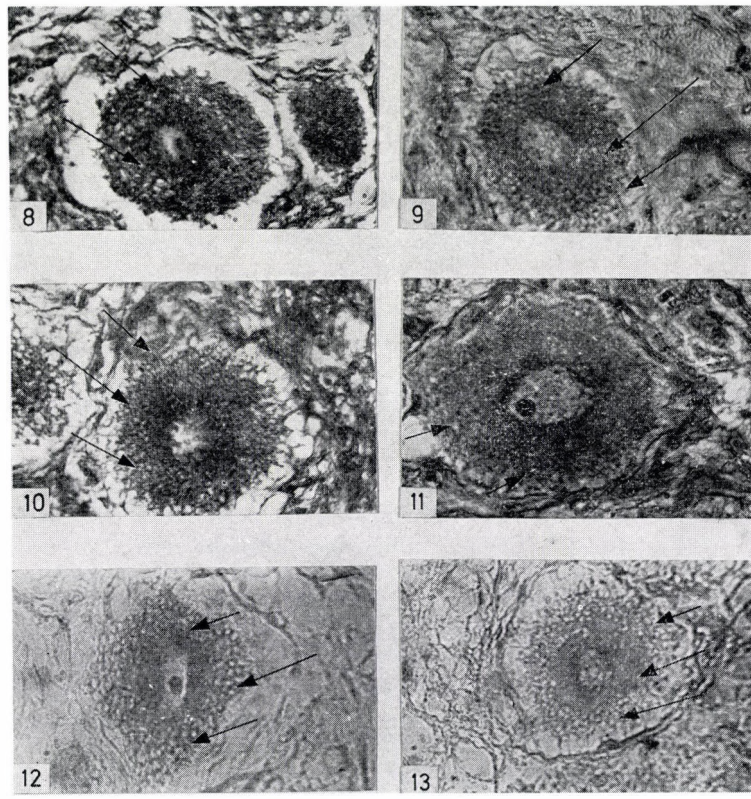


Fig. 1. Horizontal section through the thoracic ganglion of a juvenile crab, *Potamon magnum magnum*, showing a giant neurosecretory cell without axon. Arrows indicate Acrolein-Schiff-positive neurosecretory material. Acrolein-Schiff ×444

Fig. 2. Horizontal section through the thoracic ganglion of a juvenile crab, *Potamon magnum magnum*, showing a giant neurosecretory cell without axon. Note the fine granular sudanophilic neurosecretory material throughout the perikaryon. Sudan black B ×444

Fig. 3. Horizontal section through the thoracic ganglion of a juvenile crab, *Potamon magnum magnum*, showing a giant neurosecretory cell without axon. Arrows indicate alcian bluepositive neurosecretory material. Alcian blue ×444

Figs 4—7. Horizontal sections through the thoracic ganglion of adult crabs, *Potamon magnum magnum*, showing giant neurosecretory cells with axon. N = nucleus. Specimens collected in summer *(Fig. 4)*, autumn *(Fig. 5)*, winter *(Fig. 6)* and spring *(Fig. 7)*

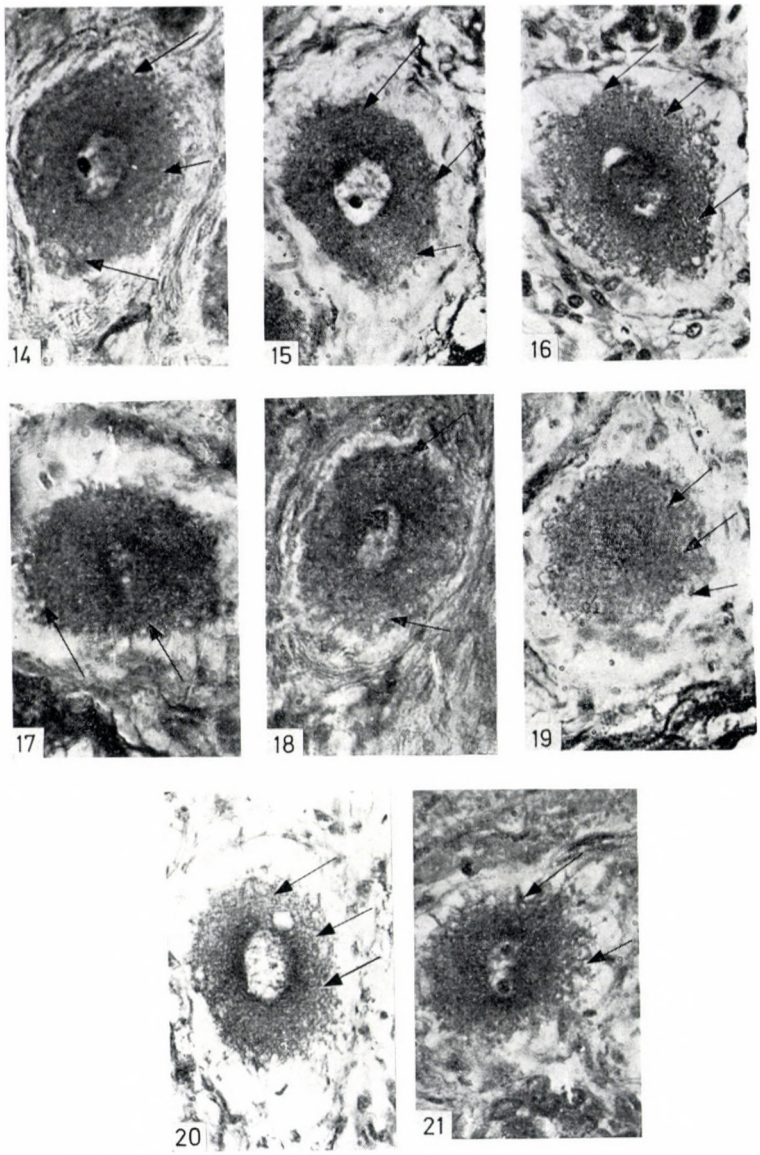


Figs 8—11. Horizontal sections through the thoracic ganglion of adult crabs, *Potamon magnum magnum*, showing giant neurosecretory cells without axons. Arrows indicate acrolein-Schiff positive neurosecretory material. Specimens collected in summer *(Fig. 8)*, autumn *(Fig. 9)*, winter *(Fig. 10)* and spring *(Fig. 11)*. Acrolein-Schiff ×400

Figs 12—13. Horizontal sections through the thoracic ganglion of an adult crab *Potamon magnum magnum* collected in winter. A giant neurosecretory cell with axon *(Fig. 12)* and another without axon *(Fig. 13)*. Arrows indicate gallocyanin–chromalum-positive neurosecretory material. Gallocyanin-chromalum × 400

types stain pale blue, indicating the presence of little amounts of cystine. In adult crabs, the concentration of cystine in the neurosecretory material did not show seasonal changes.

(7) Methyl green–pyronine technique for the demonstration of RNA. In the juvenile crab, both cell types stain dark red, indicating the presence of significant amounts of RNA-positive material. The RNA-positive material is distributed all over the cytoplasm. In adult crab, too, the cells stained dark red, indicating the presence of significant amounts of RNA-positive material in all seasons. The chromatin material stains red, while the nucleoli dark blackish-red. The RNA-positive material was distributed all over the cyto-



Figs 14—17. Horizontal sections through the thoracic ganglion of adult crabs, *Potamon magnum magnum*, showing giant neurosecretory cells with axons. Specimens collected in summer *(Fig. 14)*, autumn *(Fig. 15)*, winter *(Fig. 16)* and spring *(Fig. 17)*. Arrows indicate RNA-positive neurosecretory material. Methyl-green–pyronine. ×400

Figs 18—21. Horizontal sections through the thoracic ganglion of adult crabs, *Potamon magnum magnum*, showing giant neurosecretory cell without axons. Specimens collected in summer *(Fig. 18)*, autumn *(Fig. 19)*, winter *(Fig. 20)* and spring *(Fig. 21)*. Arrows indicate RNA-positive neurosecretory material. Methylgreen–pyronine. ×400

plasm (Figs 14—21). In autumn, giant cells were more abundant in RNA than in other seasons.

(8) Periodic acid-Schiff (PAS) technique. This test demonstrates the presence of carbohydrates and a number of related substances, such as, neutral polysaccharides, glycolipids and glycoproteins. The neurosecretory granules in both cell types stain faint purplish-red, indicating the carbohydrate moiety. After diastase treatment, the secretory granules stained purplish-red, it gives an indication of the absence of glycogen in both cell types.

(9) Alcian blue (AB) technique for acid mucosubstances. Both cell types showed an intense reaction. The neurosecretory granules are clear blue-green, indicating the presence of large amounts of acid mucosubstances (Fig. 2).

(10) Acriflavine test for sulphate esters (sulphomucosubstances and sulpholipids). The neurosecretory granules stain reddish-brown, indicating the presence of significant amounts of sulphate esters.

(11) Sudan black B (SBB) technique for lipids and lipoproteins. The secretory granules in both cell types are stained dark black. This indicates the presence of significant amounts of lipids and lipoproteins (Fig. 3).

The results of the three additional tests applied in adult crabs only

(1) DDD technique. This technique is considered to be specific for the demonstration of sulfhydryl groups by blocking the free sulfhydryl groups, followed by reduction of disulphide to sulfhydryl. The reaction was positive in both cell types. The secretory granules stained purplish to red. After blocking the sulfhydryl groups, the neurosecretory granules stained faint pink, indicating a weak positive reaction.

(2) Modified gallocyanin–chromalum technique. Both cell types stained faint blue, indicating the presence of small to moderate amounts of nucleic acids in spring, autumn and summer. The nucleoli are stained deep blue to black, but the nuclei appeared very faint. The perinuclear region in both cell types showed concentration of nucleic acids. In winter, the neurosecretory material of both cell types stained more or less deep blue, indicating the presence of significant amounts of nucleic acids (Figs 12, 13).

(3) Millon's reaction. The secretory granules in both cells stain greyish to black, indicating the absence of tyrosine-containing proteins in all the seasons.

Discussion

The results of the present histochemical tests carried out on the giant neurosecretory cells of juvenile crabs clearly indicate that the neurosecretory

material in these cells is proteinaceous in nature. It contains significant amounts of proteins having NH, NH_2, imidazole reactive groups and little amounts of disulphide groups. There are considerable amounts of lipids and lipoproteins, and little amounts of acid mucosubstances and sulphate esters have been reported but no glycogen could be detected. The neurosecretory material is associated with significant amounts of RNA. The histochemical tests carried out in the giant neurosecretory cells of adult crabs have shown that the neurosecretory material is proteinaceous in nature, with considerable amounts of proteins having NH, NH_2, imidazole groups, moderate amount of sulfhydryl groups and a small amount of disulphide bonds. However, proteins containing tyrosine proved to be absent. The neurosecretory granules were associated with significant amounts of nucleic acids.

Barnes and Gonor [6] and Rehm [40] reported little or no staining of neurosecretory material in crustaceans with Sudan black B. However, McGregor [24] found that the neurosecretory material reacted in a number of species of barnacles only with Sudan black B and Luxol fast blue. Lake [22] found neither phospholipid nor lipoprotein in the neurosecretory material of *Chirocephalus diaphanus*. Significant amounts of lipids and phospholipids [37] have been reported in the giant neurosecretory cells of the adult crab, *Potamon magnum magnum*.

On the contrary, the neurosecretory material in the giant neurosecretory cells of the juvenile crab, *Potamon magnum magnum* showed strong positivity to Sudan black B, indicating the presence of considerable amounts of lipids and lipoproteins. This difference may be due to species of seasonal variations.

It appears that the Gomori-positive material in the giant cells of the juvenile crab, *Potamon magnum magnum*, may contain a PAS-positive fraction. The presence of PAS-positive components in the neurosecretory material of crustaceans has already been reported [13, 14, 22, 28, 29, 30, 38, 40].

The presence of diastase-resistant PAS-positive and amylase-digestible PAS-positive [6] neurosecretory material has also been reported [22, 25, 26, 38] in *Pollicipes polymerus*.

In the present study, weakly to moderately PAS-positive neurosecretory material was detected, in addition to the diastase resistant PAS-positive neurosecretory substance. This may indicate the absence of glycogen.

Alcianophilic and acriflavine – positive neurosecretory material was observed in the giant cells of the juvenile crabs, *Potamon magnum magnum*, indicating the presence of significant amounts of acid mucosubstances and sulphomucosubstances. Rehm [39] reported the presence of alcianophilic neurosecretory material in *Carcinus meanas*.

In arthropods, several workers have reported the presence of neurosecretory material rich in disulphide bonds. Rehm [40] reported the presence of axon ending containing neurosecretory material rich in disulphide bonds

in the sinus gland of the crab, *Carcinus meanas*. Using electrophoresis and paper chromatography, Otsu [31] and Otsu and Sonobe [32] found chromactivating substances extracted from the thoracic ganglion of the crab *Eriocheir japonicus* to contain cystine and suggested that these substances are polypeptides with S–S bonds.

According to the theory of neurosecretion, the stainable neurosecretory material which occurs throughout the neurosecretory system is believed to be either the histological representation of the hormones or of some carrier substance to which hormones are attached. In insects, there is some correlation between the nature of the neurosecretory material as deduced by histological techniques and the known composition of the hormones [19, 20, 41]. On the other hand, some results suggest that the stainable components of the neurosecretory material are carrier substances from which the active hormones, in some way or other, are released [1, 2, 44]. Hormones from the thoracic ganglionic mass of crabs (probably giant cells) have been reported to control the migration of water from the haemolymph into the gut [7, 23]. Sharrer [42], on the basis of electron microscopieal studies, has shown that the brain hormones of insects and the content of the intrinsic secretory cells of the corpora cardiaca are proteinaceous in nature.

High content of cytoplasmic nucleic acids in the neurosecretory cells has been noted [30, 15, 34]. It is well known that the basiophilia of the extranuclear basophilic material and of the Nissl bodies is due to RNA [10, 16,43]. The sites of active protein synthesis are also abundant in RNA [9, 12, 35] and the RNA seems to be involved in the production of neurosecretory material [43]. This appeared to be true for the giant neurosecretory cells of *Potamon magnum magnum* as revealed by the present study, where large amount of RNA-positive material has been found in the sites of neurosecretory material.

In general, the various histochemical reactions were stronger in summer and winter than in spring. Clark [11] thus postulated that "the presence of stainable material in annelid neurosecretory cells indicates only that the rate of synthesis has outpaced its rate of release and that the accumulation of it in the cell may be caused by a change in either rate". In spring and autumn, the physiological demand of the animal seems to be more than in other seasons and, therefore, it may be reasonable to assume that during these seasons, there occurs a depletion of neurosecretory material in the cells.

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EFFECT OF MUTATION ON THE PERIPHERAL RETICULUM IN *TRADESCANTIA ALBIFLORA* CHLOROPLASTS

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Abstract

In the defective plastids of *Tradescantia albiflora* cv. aureo-vittata, a C_3 plant, the mutation (localized with high probability in the plastom) increases the quantity of the peripheral reticulum (PR). The enhancement of PR may be connected with the mutation of a repressive gene or with some metabolic change caused by the mutation. In the early phase of the plastid destruction, the PR can be distinguished from the thylakoid derivatives on the basis of its DAB-negativity. The positive DAB-reaction in the thylakoid membranes is explained by their PS I activity.

Introduction

The peripheral reticulum (PR), in its most conspicuous form, characterizes chloroplasts of the C_4 plants [9], but less prominently it occurs in several C_3 plants, too [4]. Concerning its function it is supposed to increase the inner surface of the plastid envelope and, consequently, to be in correlation with the intensity of certain transport processes. How it is genetically controlled, this is an open question. Our investigations carried out on *Tradescantia* chloroplasts demonstrated the effect of a plastid mutation on the PR; this is reported in the present paper.

Material and methods

Normal (green) and mutant (pale green or colourless) segments from variegated leaves of *Tradescantia albiflora* cv. aureo-vittata Kunth em. Brückn. [3] were used.

For morphology, leaf pieces were fixed in 1% glutaraldehyde (cacodylate buffer, pH 7.2) for 1/2—1 h on ice. The fixed samples were washed in the same buffer for 2 h, postfixed in 1% OsO_4 for 2 h then rinsed in distilled water for 10 min. Dehydration was carried out in an ethanol series, embedding in Araldite. Sections were made by Reichert ultramicrotome, stained with uranyl acetate and lead citrate, examined in Siemens electron microscope.

For cytochemistry, the preparation described by NIR and PEASE [11] was applied. Material was fixed in 4% freshly depolymerized paraformaldehyde (0.05 *M* K—Na phosphate buffer, pH 7.5) for 30 min in darkness. The fixed samples were washed in buffer containing 5% sucrose for 30 min in the dark. Incubation was carried out in buffer containing 1 mg/ml diaminobenzidine (DAB) for 60 min under strong illumination, then for 30 min in darkness in buffer containing also sucrose. (Part of each group was kept as a control without illumination.) Incubation was followed by osmification in 1% OsO_4 (0.05 *M* K—Na phosphate buffer, pH 7.0) for 60 min. Dehydration and further steps were the same as in the preparation for morphology, except that sections were not stained.

Results

The light and electron microscopic structure of normal and mutant plastids of *Tradescantia albiflora* cv. aureo-vittata and the change of this structure during ageing of leaves has been described [6, 8]. However, the $KMnO_4$ fixation used in electron microscopy prevented the examination of PR.

In thin sections from normal *Tradescantia* chloroplasts only minute quantities of PR could be observed even after glutaraldehyde + OsO_4 fixation, not more than that seen in Fig. 1.

Plastids with macrograna from the young mutant leaf segments (Figs 2 and 3) contain much more PR than the normal chloroplasts. The profiles of invagination do not differ from the usual shape; round vesicles or tubules of different length can be observed in sections.

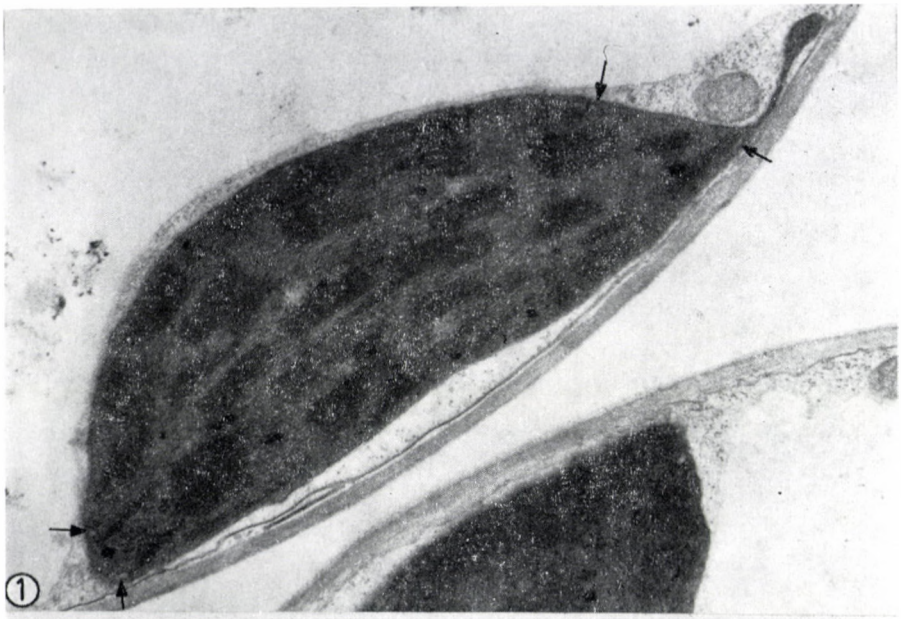
In those plastids, however, which contain dilated or fragmented thylakoids, the PR is hard to recognize as the whole organelle is filled with vesicles of different size (Fig. 4).

It is known from earlier investigations that the plastids of the pale green leaf segments — although incapable of producing O_2 — do perform PS I activity (a reversible photo-oxidation of P_{700}) [7]. There is no evidence, on the other hand, suggesting any photosynthetic activity in the membranes of PR. Therefore we made an attempt to distinguish between vesicles originating from destroyed thylakoids and the PR by DAB-reaction. As Figs 5 and 6 show this is possible in the early phase of plastid destruction; derivatives of thylakoids oxidize DAB when illuminated while PR is unable to do so. In the stage of progressed plastid destruction, however, this difference disappears; all the plastid vesicles are DAB-negative (Fig. 6).

Discussion

Vigil et al. [13] found both photosystems to participate in DAB-reaction in *Chlamydomonas*. Seeing positive DAB-reaction in *Tradescantia* plastid membranes known to be PS II-deficient [7], PS I must be considered sufficient to photo-oxidation of DAB. On the basis of the present results we cannot exclude the possibility that PS II also acts in the photo-oxidation of DAB in normal chloroplasts, but its role does not seem to be essential besides an active PS I.

In those instances when PR could be observed in C_3 plants, attempts were made to relate it to the transport or accumulation of some metabolites. So Hilliard and West [5] found low rates of photorespiration in PR-containing varieties of *Dactylis glomerata* and high rates in varieties without PR. Laetsch [10], however, does not regard this difference in the quantity of PR as a constant and significant one.



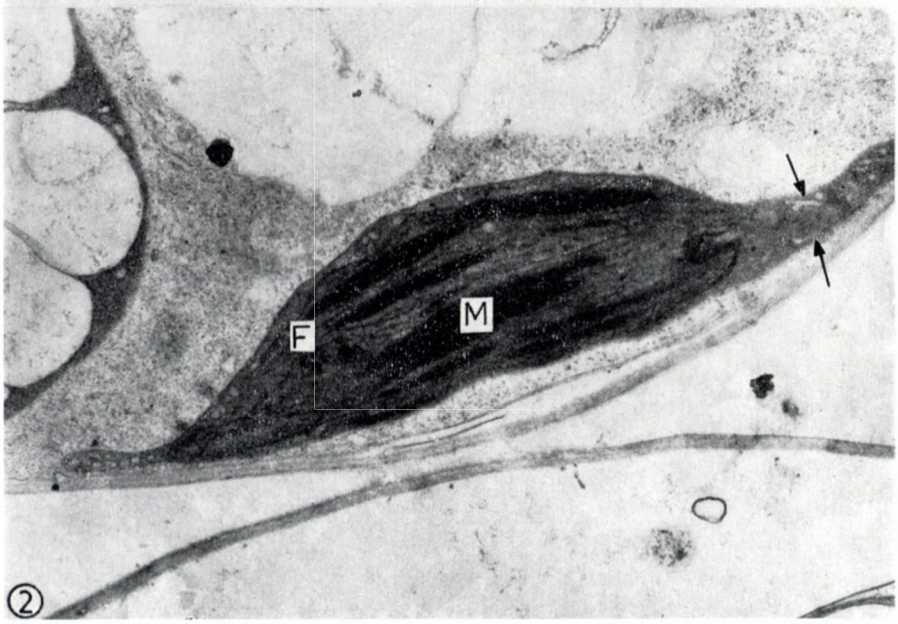


Fig. 1. Normal *Tradescantia* chloroplast. Arrows point to the peripheral reticulum (PR), ×16,000

Fig. 2. Mutant plastid with macrograna (M) and fret membranes (F). Arrows mark PR tubules cut longitudinally. ×14,000

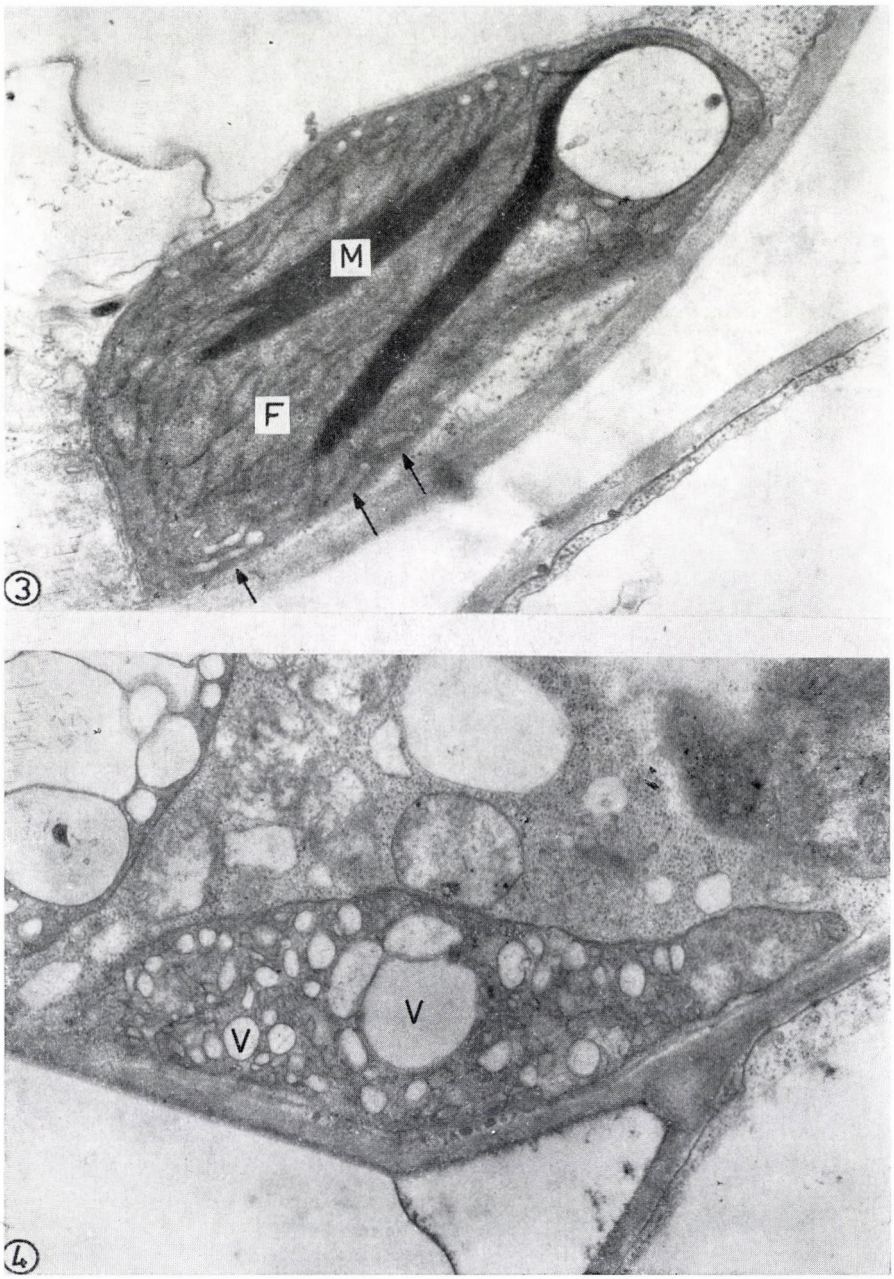
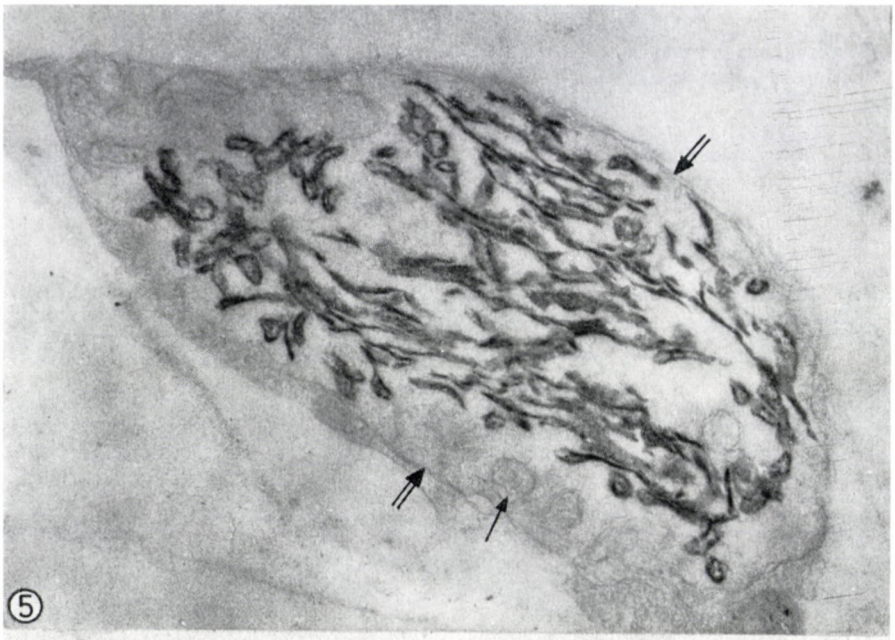


Fig. 3. Mutant plastid with macrograna and fret membranes. (For labelling see Fig. 2.) ×24,000

Fig. 4. Destructive mutant plastids with vesicles (V). ×20,500



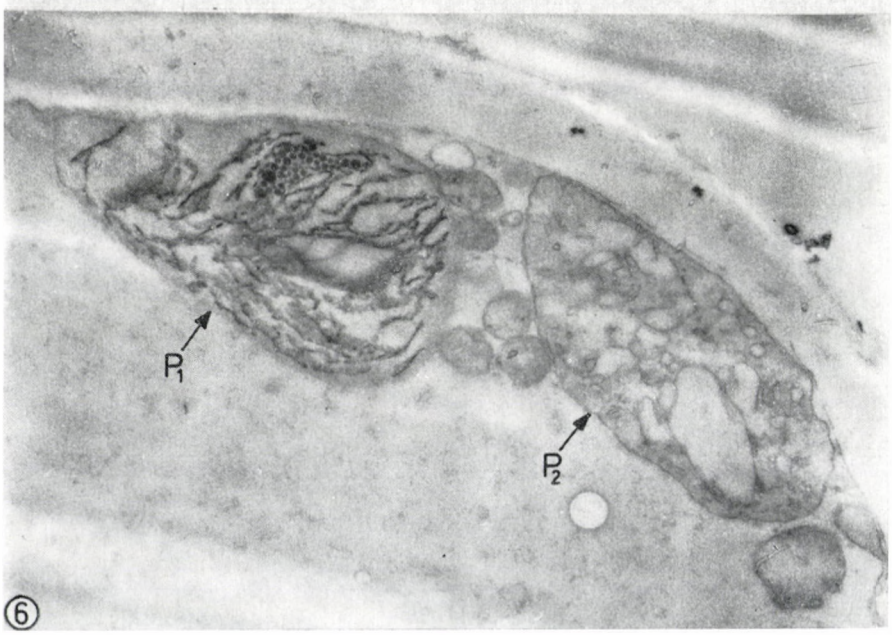


Fig. 5. DAB-reaction in a mutant plastid. The strong osmiophilic reaction product marks the thylakoid membranes, while the PR (arrow) and the plastid envelope (double arrow) are of much lower contrast. ×26,000

Fig. 6. DAB-reaction in mutant plastids. P_1=DAB-positive plastid with thylakoid membranes; P_2=DAB-negative plastid with vesicular inner structure. ×13,000

Reports on the presence of PR in guard cell chloroplasts (but not in mesophyll chloroplasts) of *Vicia faba* and *Allium porrum* [2, 12] seem to be more equivocal [10]. An interesting observation in this respect is that malate accumulates in guard cells of *Vicia faba* during stomatal opening [1].

The question arises, with what the enhancement of PR in the mutant plastids of our C_3 plant can be brought into connection. The simplest supposition is that the mutation switched off a repressive gene. As in this *Tradescantia* species, a plastom mutation was rendered probable on the basis of the occurring mixed cells [6], so the gene in question should be localized in the plastom. Alternatively, it is possible that the PR was not directly affected by the mutated gene but rather by a metabolic consequence of the mutation.

Acknowledgements

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THE EFFECT OF SOME PESTICIDES ON THE RHYTHMIC ACTIVITY OF ADDUCTOR MUSCLE OF FRESH-WATER MUSSEL LARVAE

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Abstract

The effect of some pesticides on the rhythmic and tonic activity of adductor muscle of fresh-water mussel *(Anodonta cygnea)* larvae was investigated in short-term experiments. In the examined concentration range, Dimecron-50 does not influence the rhythmic activity of adductor muscles, while high concentrations of Gramoxone (> 1 ml/l), Thimet-10 G ($> 5 \times 10^{-1}$ g/l) and Hungaria L-7 ($> 10^{-1}$ g/l) cause a slight increase in it. These substances also induce a prolonged tonic contraction of a small proportion (10%) of larvae. Hungaria L-7 makes an exception, causing considerable contracture (30%). The concentrations inhibiting the TA activation by 50% are: 6×10^{-1} ml/l Dimecron-50; 2.5×10^{-1} ml/l Gramoxone; 10^{-1} g/l Hungaria L-7 and 8×10^{-2} g/l Thimet-10 G, while the 50% inhibition of the nonspecific ACh activation is resulted by 2×10^{-1} ml/l D-50. It is concluded that contamination of environmental waters with Dimecron-50, Thimet-10 G or Hungaria L-7 may be injurious also for *Pelecypoda* larvae, that may contribute to the unbalancing of the water ecosystem.

Introduction

Owing to their wide-spread occurrence, bivalve molluscs participate almost in every water ecosystem. They have been used for a long time to investigate the pollution of the environmental water by different chemicals (heavy metal salts, pesticides, surface-active agents) as they have filtering and accumulative function [1]. The resistance of different species are not the same, nevertheless, they seem to be suitable for these investigations because of their high sensitivity to some agents, and an extraordinary resistance against others.

The majority of the earlier examinations have been performed on marine species, mainly on adult specimens, but a few observations have been made on their eggs, larvae and juvenile forms. As an indicator of the effect the mortality [2—9], the development of eggs [2, 5, 10, 11], the growth of the larvae and juvenile forms [5, 11] or the change in body weight [12] have been used. Furthermore, under the influence of the toxic substances other physiological alterations may also occur, *e.g.* the byssus formation is decreased [13], the movement of shells is changed [14, 15], tissue swelling of the adductor occurs [16] or the rate of heart beat declines [17].

In our earlier publications, the change in the duration of active periods of the adult fresh-water mussels were considered as indicator of heavy metals and pesticides [18, 19]. The present experiments were carried out on larvae of the fresh-water mussel, *Anodonta cygnea*, and the changes in rhythmic activity of adductor muscles were used as an indicator.

Glochidiae manifest a period of the larval development of *Anodonta cygnea* L., they are bounded by two shells coupled by larval adductor muscles. The rhythmic motor activity of glochidiae is due to an alteration of phasic contraction and relaxation, while the steady closure is the consequence of a tonic contraction. The nervous elements are in an early state of differentiation [20, 21].

KCl and tryptamine (TA) significantly increase the rhythmic activity of glochidiae [22, 23], and the sensitivity to TA shows a decreasing tendency with the age. In addition, a number of salts of alkali metals and alkali earth metals, bioactive substances and pharmacons have been tested on the rhythmic activity of adductor muscles of glochidiae [24—27]. Some of them proved to be effective.

Considering that the mussel larvae live in the environmental water, they are exposed to different injurious factors, like other organisms. The present short-term investigations were intended at clearing up the sensitivity of *Anodonta cygnea* L. larvae to some pesticides, which may contaminate water by human activity.

Material and methods

The experiments were performed on the glochidiae of fresh-water mussel, *Anodonta cygnea* L., in the months March to May. The lamellae of the gill containing glochidiae were dissected from the animals, then the larvae were washed out from the gills. The fragments of gill and the mucous material were removed by repeated washing. The larvae originating from different parents were separated and were also stored separately. The glochidiae were kept in Balaton water at +3— +5 °C until used. The water was refreshed every second day.

Before the experiments, the larvae were selectively grouped into 25 individuals and each group was placed in a perspex cell of 8 mm diameter containing 0.15 ml filtered Balaton water. Thereafter, the larvae were allowed to stay under binocular microscope for 30 min at room temperature (20—23 °C) illuminated with a 15-W wolfram lamp through BC-17 infra-filter. This was necessary the glochidiae to adapt to light and temperature [24].

Following this, in the first cell the frequency of spontaneous contraction and the number of tonic contractions were noted in every minute for 5 min, then the water was changed for a solution containing the given concentration of substances to be tested, and counting was continued for 25 min with the same method. Afterwards, another concentration was tested on the larvae of the next cell as above. So, the effect of the same substances was examined in increasing and decreasing concentrations, respectively. Each concentration was tested on 200—300 specimens originated at least from 4—5 different parents.

The control experiments showed that the spontaneous rhythmical activity of glochidiae was inhibited by low concentrations of most of the substances. As the frequency of the spontaneous contractions of the temperature- and light-adapted larvae was in the 0—0.02 cpm range, the inhibitory effect of substances was also investigated on larvae activated by 100 mg/l tryptamine. The control group was only treated by TA, while the other groups received the given concentration of the test substances in addition to TA.

In some cases, the age-dependent TA-sensitivity [23] influenced also the effect of the test substances, therefore, in such cases the effect of chemicals was examined separately in two groups (TA-sensitive and insensitive).

On one occasion, the inhibition of the nonspecific ACh-activation [23] was examined besides that of TA-activation, when ACh was applied in 200 mg/l concentration.

For evaluation of the results in one respect the number of contractions of 100 larvae per min (a/min) was calculated, and the percentage of those being in tonic contractions was given (C%). The results obtained are graphically presented.

According to another method, the number of contractions of 100 larvae per 25 min was summarized and the inhibition caused by the different substances was expressed in the percentage of TA- or ACh-induced activity. This value was given as a function of the concentration, and the concentration required to an inhibition of 50% was calculated from this curve.

The chemicals used were as follows: tryptamine hydrochloride (Fluka); acetylcholine chloride (Sigma); Hungaria L-7 containing 7.8% lindane made by Hungarian Chemical Industry, Budapest; Dimecron-60 (fluid pest-destroying spray containing 50% phosphamidon made by NIKE from CIBA-Geigy effective agent); Thimet-10 G (granulatum containing10% phorate, American Cyanamid Co. USA and Werfft Chemie, Austria) and Gramoxone (fluid pest-destroying spray containing 25% paraquat-dichloride made by Alkaloida, Budapest, from effective agent made in England, ICI Plant Prot. Div.).

The solutions were prepared in filtered Balaton water. The pH of the solutions: Balaton water 8.4; Hungaria L-7 in 10^{-1}—10^{-3} concentration 8.3; Dimecron-50 in 1 ml/l 8.0; 10^{-1}—10^{-3} ml/l 8.3; Thimet —10 G 10^{+1}—10° g/l, 8.1; 10^{-1} g/l, 8.2; 10^{-2} g/l, 8.3; 10^{-3} g/l 8.4; Gramoxone in 10 ml/l 8.3; 10^{-1}—10^{-3} ml/l, 8.4.

Results

Hungaria L-7

In concentrations higher than 10^{-1} g/l, this preparation has an excitatory effect on the rhythmic activity of adductor muscles (Fig. 1). The effect is concentration-dependent, and shows a maximum in the early phase. In

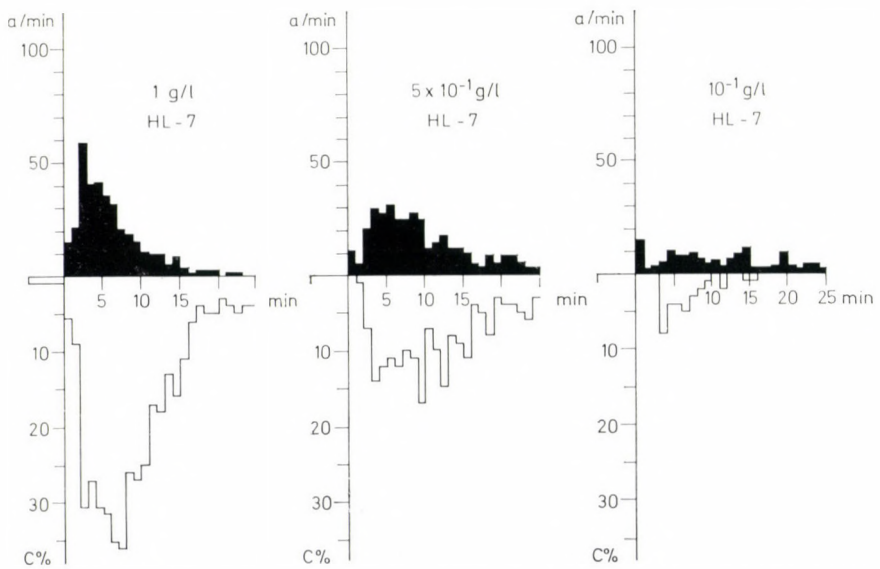

Fig. 1. Effect of different concentrations of Hungaria L-7 on the rhythmic and tonic activity of adductor muscle of glochidiae; a/min = number of contractions of 100 larvae/min; C % = percentage of larvae being in tonic contraction

addition, tonic contraction of several minutes duration was also observed on some of the glochidiae. This latter effect is also depending on concentration, and its maximum is somewhat delayed as compared to that of the excitation of contractile activity.

10^{-2} g/l or less concentrations of HL-7 caused practically no effect on the spontaneous activity. Thus, the next step was to examine the character and degree of its influence on the excitation induced by 100 mg/l TA. Figure 2 shows the effect of 100 mg/l TA on a TA-sensitive and a TA-insensitive group of glochidiae, further, the influence of this excitation by 5×10^{-1} g/l HL-7. As to the number of contractions, the excitatory effect of TA was highly different in the two different groups. This difference is equalized in the presence of HL-7; only a small difference remained in the time-distribution of contractions. In the TA-sensitive group, the maximum of contraction was reached in the 9th min, while in insensitive group it was reached within the first 5 min. In Fig. 3, the inhibition of TA activation by different concentrations of HL-7

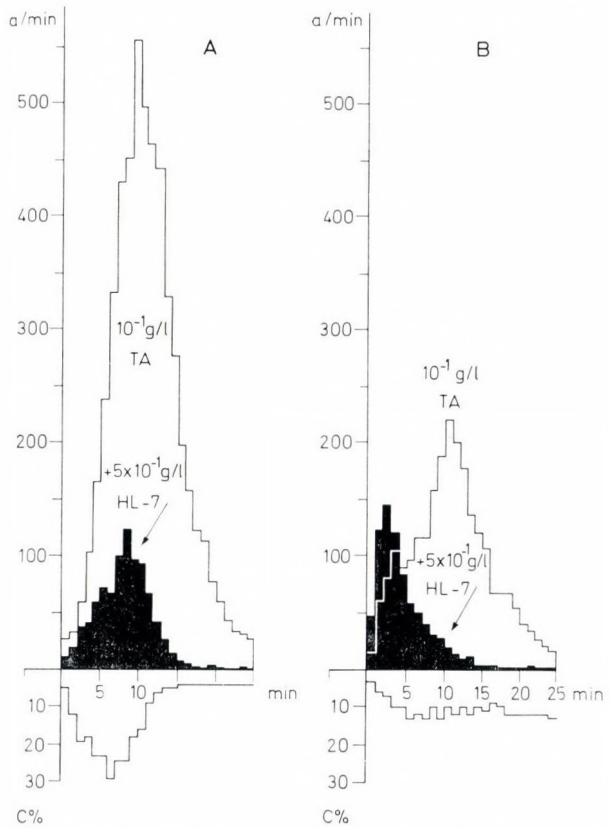

Fig. 2. Excitation induced by 100 mg/l tryptamine and inhibition on it caused by 5×10^{-1} g/l Hungaria L-7. TA-sensitive (A) and TA-insensitive (B) glochidia population. The values of C % refer to the simultaneous application of TA and HL-7

is presented in the case of a group of variable sensitivity. It is shown that an increase in the concentration of HL-7 is followed not only by a decrease in the maximum TA activation, besides, the maximum activation occurs earlier.

Figure 4 shows the inhibition of TA activation as a function of the HL-7 concentration, considering the contractions of 100 glochidiae per 25 min treatment. As HL-7 contains 7.8% lindane as effective agent, the concentration of the latter is also demonstrated. It should be noted that the effect of 7.8×10^{-2} g/l and 3.9×10^{-2} g/l lindane somewhat differs, though its solubility is about 1.2×10^{-2} g/l, consequently, in the above concentrations part of the

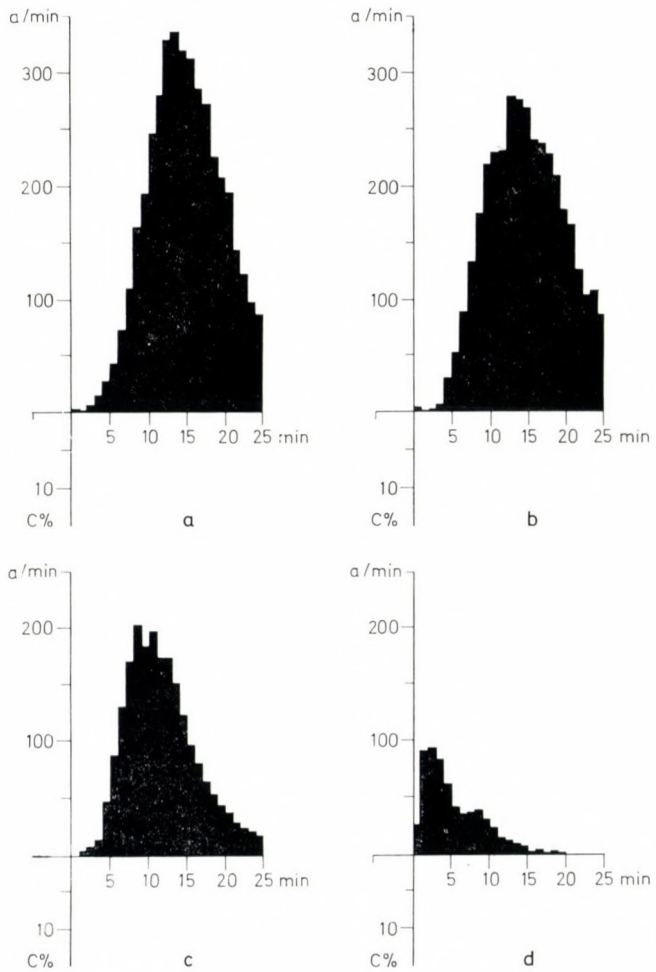

Fig. 3. Inhibition of TA activation by different concentrations of Hungaria L-7 in a population of variable TA-sensitivity; *a* : TA; *b*, *c*, *d*: effect of TA + 10^{-2}, 10^{-1} and 1 g/l HL-7, respectively

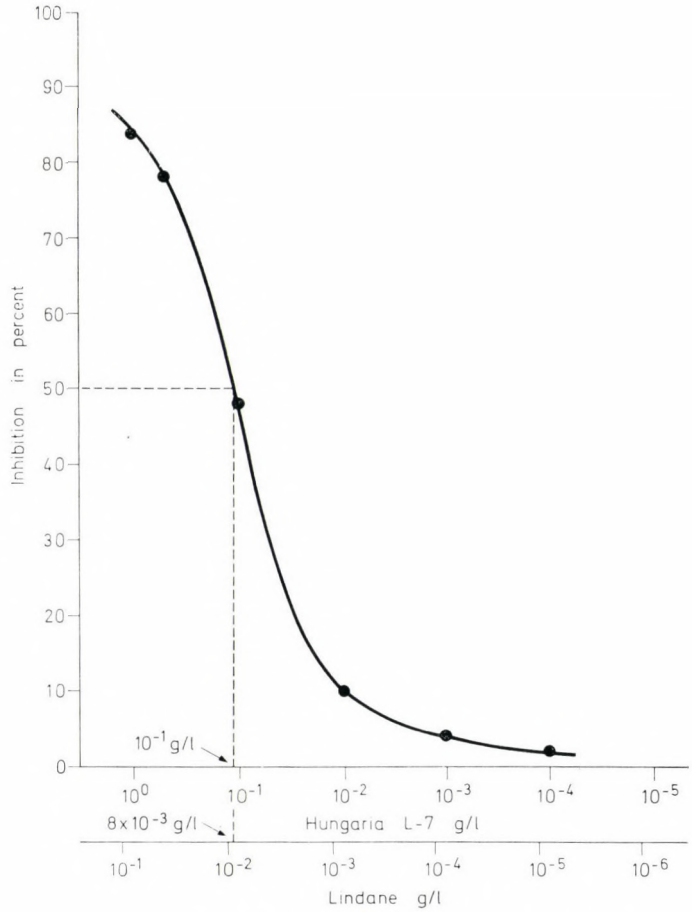

Fig. 4. Inhibition of TA activation in per cent as a function of concentration of Hungaria L-7 and the effective ingredient lindane considering the total contraction number of 100 larvae per 25 min

lindane is present in suspension. A 50% inhibition of the TA activation required 8×10^{-3} g/l lindane in the case of the population of variable sensitivity. Considering the slope of the middle section of the curve the effect can be realized in a narrow interval.

As HL-7 contains a large amount — 92.2% — of fine granulated inert water-insoluble material, its influence on the TA activation was also examined. Following sedimentation of the insoluble inert material there was no significant difference in the degree of inhibition of TA activation (the difference was less than 10% for each concentration).

Dimecron-50 was tested in the $1—10^{-4}$ ml/l concentration range. Within this range it practically does not affected the spontaneous activity of glochidiae,

but decreases the TA activation, the time course of which is demonstrated in Fig. 5 for a population of variable sensitivity. In more than 10^{-2} ml/l concentration, this substance decreases the time course of TA activation and the degree of that is considerably decreased, too, because in the case of some glochidiae a tonic contraction occurs.

Figure 6 shows the inhibition of TA activation as a function of concentration of D-50 and its effective agent (phosphamidon). The relatively gently sloping curve includes a wide concentration range. A 50% inhibition requires 6×10^{-1} ml/l concentration of D-50, *i.e.* 3×10^{-1} ml/l phosphamidon. No difference in the effect can be found between the TA-sensitive and insensitive groups.

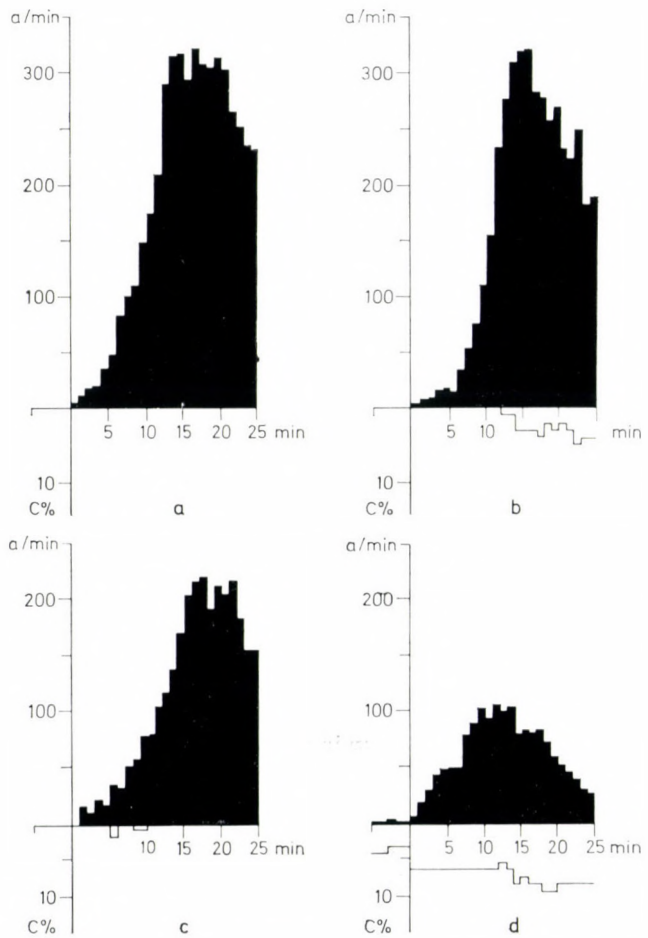

Fig. 5. Inhibition of TA activation by different concentrations of Dimecron-50 in a glochidia population of variable sensitivity; *a* : TA; *b*, *c*, *d*: effect of TA + 10^{-2}, 10^{-1} and 1 ml/l D-50, respectively

Considering that the influence of D-50 is mainly realized through a cholinesterase blockage, it seemed to be necessary to examine its effect on the ACh-induced adductor activation, too. This effect is shown in Fig. 6 as a function of the concentration of the effective agent. The inhibition has a very

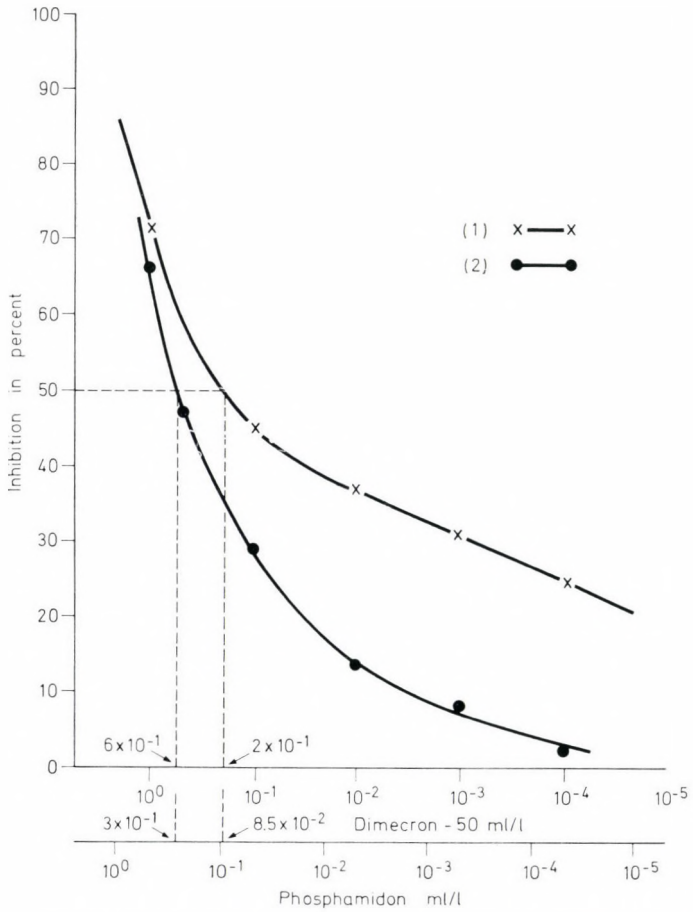

Fig. 6. Inhibition of TA and ACh activation as a function of concentration of Dimecron-50 and the effective ingredient phosphamidon in a population of variable TA-sensitivity. (1) Inhibition of ACh activation; (2) inhibition of TA-activation

wide spectrum: in a concentration of 5×10^{-5} ml/l inhibition of 25%, in a higher concentration of 5×10^{-1} ml/l 72% is obtained. A 50% inhibition of the ACh-excitation is found at 2×10^{-1} ml/l D-50, *i.e.* 8.5×10^{-2} ml/l phosphamidon.

Thimet-10 G was examined in the 9×10^{-1}—10^{-4} g/l concentration range. It has a slight own effect only at a concentration of 5×10^{-1} g/l or more, which

is about 3—4% of that of TA. An inhibition of TA activation requires a concentration exceeding 10^{-2} g/l (Fig. 7.).

The effect seems to be the same both for the TA-sensitive and the insensitive group; the difference in the degree of inhibition did not exceed 10%.

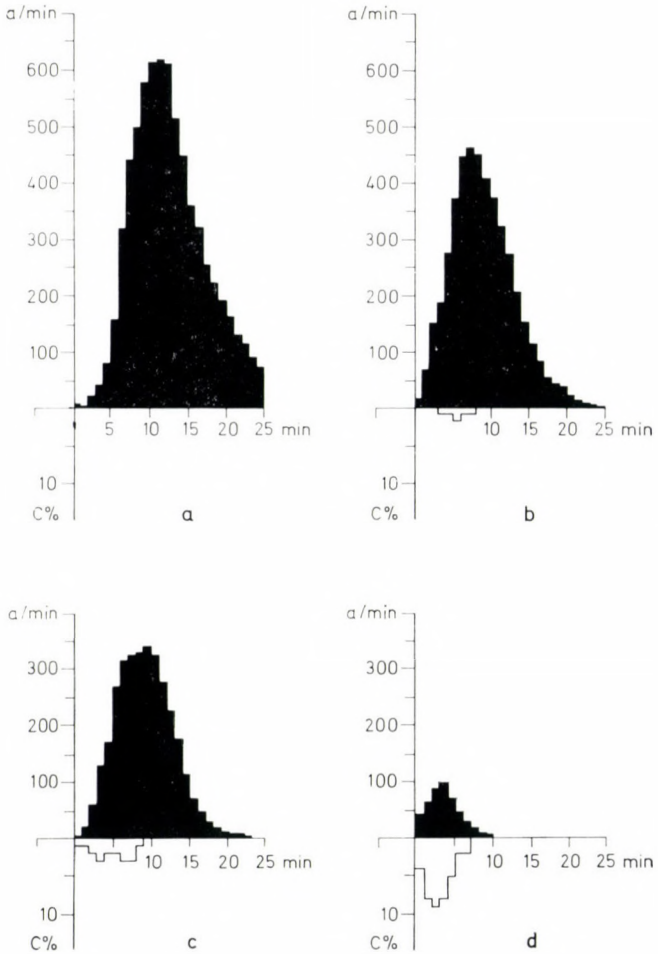

Fig. 7. Inhibition of TA activation by different concentrations of Thimet-10 G in a TA-sensitive population; *a:* TA; *b, c, d:* effect of TA + 5×10^{-2}, 10^{-1} and 5×10^{-1} g/l Thimet, respectively

In Fig. 8, the inhibition of TA activation is presented as a function of the concentration of Thimet and the effective agent phorate. The curve is quite steep, *viz.*, a 50% inhibition is caused by 8×10^{-2} g/l Thimet, *i.e.*, 8×10^{-3} g/l phorate.

Thimet-10 G applied in a 5×10^{-1} g/l or higher concentration considerably prolongs both the contraction and the relaxation of the adductor muscle, sometimes by more than 1 min.

Gramoxone was tested in the 10^{-1}—10^{-4} ml/l concentration range. In concentrations of 1—10 ml/l it has an own excitatory effect on the activity of

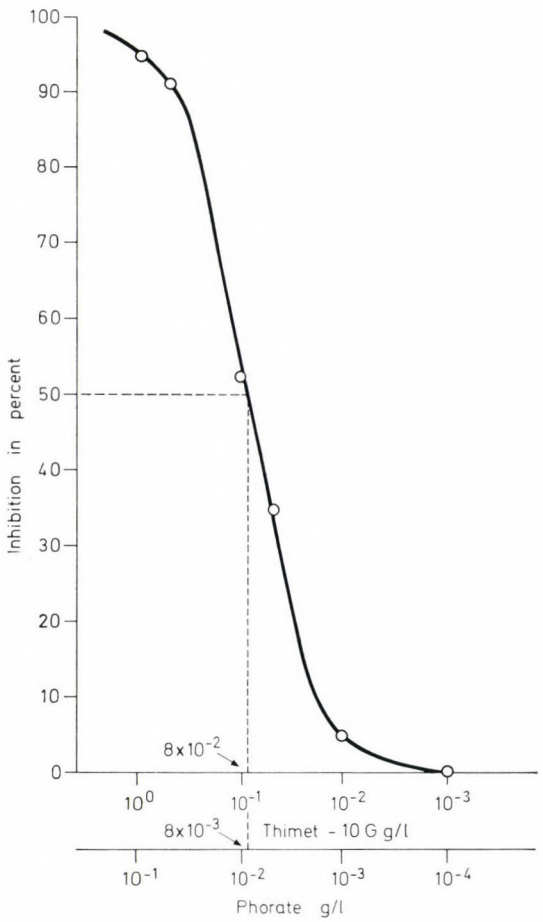

Fig. 8. Inhibition of TA activation as a function of the concentration of Thimet-10 G and the effective ingredient phorate in a TA-sensitive population

glochidiae, though, this does not exceed a few per cent of TA-induced activity. On the other hand, in the first 10 min a tonic closure of 20—35% of the larvae was evoked. In a concentration of less than 10^{-2} ml/l it is ineffective on the TA-induced activity, but in higher concentrations it shows an inhibition. In Fig. 9, the inhibition of TA activation is presented as a function of the concentration of Gramoxone and its effective agent (paraquate-dichloride)

contained in 25%. A 50% inhibition required 2.5×10^{-1} ml/l Gramoxone, *i.e.*, 6.5×10^{-2} paraquate-dichloride.

As to the time course of the effect, in presence of high-concentration of Gramoxone the TA activation is only induced in the first few minutes; at the same time, an immediate tonic closure of some glochidiae ensues (Fig. 10), which does not significantly differ from the own effect of Gramoxone.

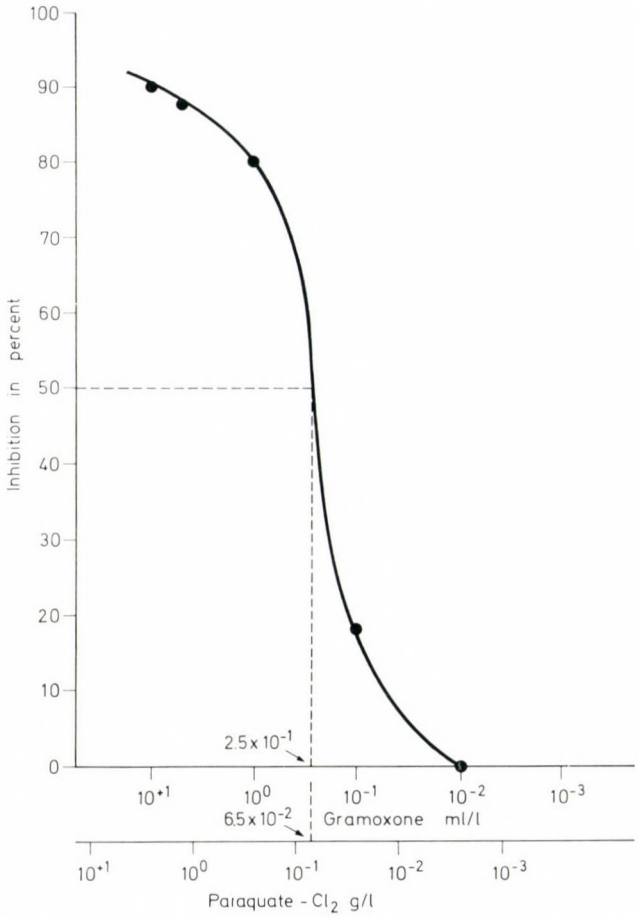

Fig. 9. Inhibition of TA activation as a function of the concentration of Gramoxone and the effective ingredient paraquate-dichloride in a TA-sensitive population

In the case of Gramoxone, no difference can be found between the TA-sensitive and insensitive populations.

In addition it is notable that in the case of 1 ml/l or higher concentration of Gramoxone, applied in itself or together with TA, after 10—15 min treatment a withish colour appeared surrounding the ganglion anlage of glochidiae.

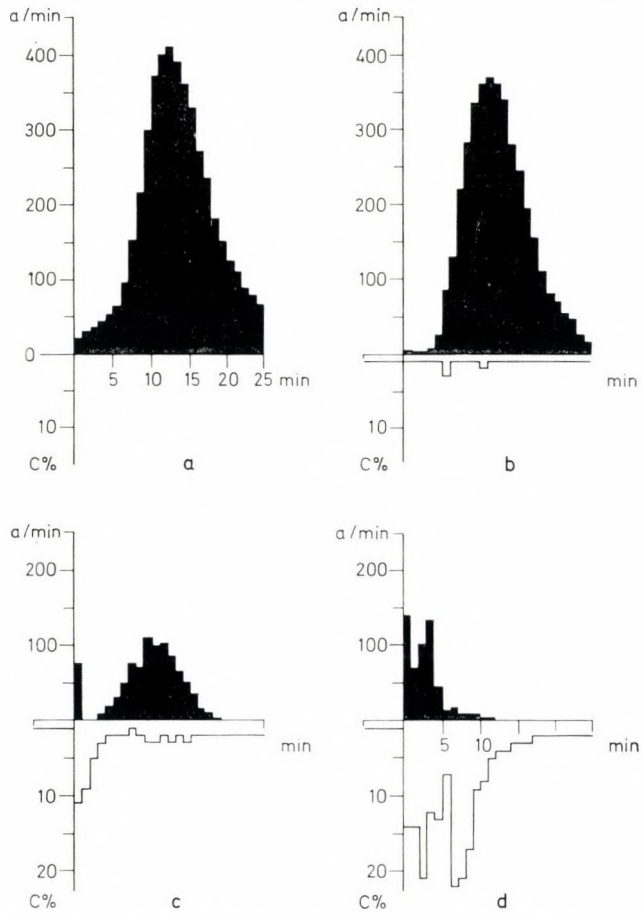

Fig. 10. Effect of different concentrations of Gramoxone on TA activation in a TA-sensitive population; *a:* TA activation; *b, c, d:* TA + 10^{-1}, 1 and 10 ml/l Gramoxone

Discussion

In the short-term experiments, among the examined substances, the high concentrations of HL-7 ($>10^{-1}$ g/l), Gramoxone (>10 ml) and Thimet-10 G ($>5\times10^{-1}$ g/l) exhibited an own excitatory effect on the rhythmic activity of the adductor muscle. Concerning effectivity, the Gr<Th<HL-7 sequence can be given, which does not show any change when the concentrations of the effective agents are considered.

In addition to these own effects, the influence on the TA activation was examined. In order to evaluate and compare the latter influences we had to determine the concentrations which caused a 50% decrease in the number

of contractions of 100 larvae per 25 min in the presence of 100 mg/l TA. These concentrations are as follows: HL-7: 10^{-1} g/l; D-50: 6×10^{-1} ml/l; Th-10 G: 8×10^{-2} g/l; Gr: 2.5×10^{-1} ml/l. On the basis of the effectiveness on TA activation, the D-50<Gr<HL-7<Th-10 G sequence was obtained, which remains the same when the concentrations of the effective agents are considered.

Further, the efficacy of the examined substances can be characterized by their threshold concentrations which cause a 10% inhibition of TA activation. These concentrations are: Gr: 6×10^{-2} ml/l (paraquate: 1.5×10^{-3} ml/l); D-50: 3×10^{-3} ml/l (phosphamidon: 1.5×10^{-3} ml/l); Th-10 G: 2×10^{-2} g/l (phorate: 2×10^{-3} g/l) and HL-7: 10^{-2} g/l (lindane: 8×10^{-4} g/l).

The effect of TA on glochidiae appears to be specific and also the presence of TA has been suggested [28]. The functioning of an adrenergic excitatory system [27], as well as occurrence of adrenergic neuromuscular synapses [21] have been made probable. At the same time, the slight excitatory effect of ACh on the rhythmic activity of glochidiae has been considered to be non-specific [23, 25]. In recent investigations D-50, containing phosphamidon, which considered to be a cholinesterase blocking agent, proved to be somewhat more effective inhibitor of ACh-induced excitation as compared to the TA-induced one.

When the substances were applied together with TA they sometimes caused a tonic contraction of larvae. This effect was insignificant in the case of Th-10 G and D-50, not more than 10% of the larvae were involved even at the highest concentrations (Figs 5 and 7), but in the case of Gr used in a high concentration (10 ml/l) it sometimes exceeded 20% (Fig. 10). Even more pronounced increase in the tonic contraction was caused by HL-7 of 5×10^{-1} g/l or higher concentrations applied either by itself or together with TA (Figs 1 and 2). This effect was especially significant on the TA-sensitive populations, that is, it may be more dangerous in an earlier stage of ontogenesis.

Lindane is a halogenated hydrocarbon, whose γ-isomer is the most active as insecticide. According to BUTLER [30], bivalve molluscs are able to accumulate this chemical following its uptake from diluted solution. DAVIS [2] demonstrated a normal development of 60% of *Venus mercenaria* eggs and of 43% of *Crassostrea virginica* eggs in lindane solution of 10 mg/l concentration. For the eggs of clams, the 48-h tolerance maximum (TL_m) exceeds 10 ppm, for the eggs of oyster it is 9.1 ppm, and the 12 days TL_m for clam larvae also exceeds 10 ppm [5]. The TL_m is defined as the concentration which does not cause any damage on 50% of the eggs or larvae. For 50% inhibition of TA-activation of *Anodonta* larvae we measured 8 ppm, a value close to the above values. Lindane has an insecticide effect as neurotoxic substance affecting mainly the central neurones [30]. In the case of glochidiae

it may exert an influence on the neurones of the ganglion anlage, which is in synaptic contact with the adductor muscles, or the neurones of the diffuse neuronal network. Even a direct influence on the adductor muscle cannot be excluded.

Paraquate is a dimethyl-bipyridyl herbicide, which causes a membrane damage and, as a consequence, increases membrane permeability [31]. Both its own effect and the inhibition of TA activation might be realized through this mechanism.

Phosphamidon and phorate are noncyclic organic phosphate derivatives which have an insecticide effect as cholinesterase inhibitors [32]. The activity of cholinesterase in glochidiae appears to be low, further, the role of the cholinergic system in the regulation of adductor contraction is not probable [21, 23, 26]. Nevertheless, on the basis of recent findings, this mechanism cannot be entirely excluded, though, the values of effective concentrations rather suggest a nonspecific influence.

It should be taken into consideration that the treatment of larvae lasted altogether 25 min. It is evident that in the case of a long-term exposure the injourious effects similar to those obtained here would be induced by lower concentrations.

The effects of pesticides may be lethal partly owing to their direct toxicity, partly as a result of inhibition of the functional opportunities required for the further development. For instance, both the inhibition of rhythmic activity and the initiation of a tonic contraction hinder the ontogenesis of the glochidiae, as one of the conditions of development is the sticking to the skin of fishes. For this purpose, the rhythmic movement of the shells and, following the contact with the skin of fish, the manifestation of the tonic contraction is needed. In the absence of sticking, the ontogenesis is broken off and the larvae perish. The thinning of *Pelecypoda* fauna in the environmental water occasionally may well be back to this mechanism. The diminution of the number of individuals has a harmful influence on the natural clearance of water.

On the basis of the present results the glochidiae can be considered to be suitable test objects for investigating the pesticide pollution of the fresh water, so they can complete the series of the species used at present routinely for this purpose.

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EFFECT OF THE ISOLATED REMOVAL OF THE MEDIAN EMINENCE (ME) AND PITUITARY STALK (PS) ON THE IMMUNOHISTOLOGY AND HORMONE RELEASE OF THE ANTERIOR PITUITARY GLAND GRAFTED INTO THE HYPOPHYSIOTROPHIC AREA (HTA) AND/OR OF THE *IN SITU* PITUITARY GLAND+

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Abstract

Isolated removal of the median eminence (ME) and pituitary stalk (PS) of female rats was performed under visual control, using a new instrument to open up the 3rd ventricle. Atrophy of the uterus, the follicles and the interstitial tissue in the ovaries was accompanied by persistent corpora lutea and persistent diestrous vaginal smears in rats which had undergone a successful removal of ME and PS. No change was, however, detected in the weight of the thyroid and adrenal glands at the end of the six weeks experimental period. An adenohypophysis implanted in the place of the ME at the time of the surgery, could not prevent these changes. In animals, in which the removal of the ME was not complete, the changes of the gonadal system were less pronounced.

Immunocytology of the pituitary LH-, FSH-, TSH- and prolactin-cells in animals with completely removed ME and PS showed inactive LH- and FSH-cells both in the grafted and *in situ* pituitaries, while the TSH- and prolactin-cells appeared to be in a stimulated state. In animals with ME remnant, LH—RH axon terminals were localized only on the blood vessels of the remnant. The part of the pituitary graft in contact with these blood vessels, as well as some areas of the *in situ* pituitary gland, contained active LH cells as judged from their size and immunohistological appearance. Since in the absence of the ME, the hypophysiotrophic area is not able to exert its regulatory effect on the gonadotrophs of the pituitary implant in this area, the authors suggest that this effect is mediated by the blood circulation of the ME which is rich in releasing hormones and is drained toward both the anterior pituitary and the medial basal hypothalamus.

Introduction

In immunocytological studies on anterior pituitary homografts [38, 39] we substantiated the earlier findings of Halász et al. [18, 20], Knigge [26] and Flament-Durand [11, 12] on the hypophysiotrophic effect of the medial

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basal hypothalamus (MBH). Although we found hormone-containing cells in all pituitary transplants irrespective of their location, cytological characteristics of the grafts were closely correlated with their location. Hypertrophied gonadotrophic cells were found exclusively in grafts located in the HTA. The relatively low hormone content in these hypertrophied gonadotrophs suggested an increased release of the hormones synthesized by the cells, while the small gonadotrophs in grafts located outside the HTA or under the renal capsule appeared to be in an inactive storing stage. These immunohistological observations are in accordance with the findings of HALÁSZ et al. [18, 20] that only pituitary grafts located in the HTA were able to maintain the functions of the target glands.

In our studies mentioned above, we questioned the so-called "diffusion theory" [18, 20] as a possible explanation of the hypophysiotrophic effect of the HTA. According to this theory, the HTA can regulate the anterior pituitary because it contains the neurons synthesizing the hypophysiotrophic hormones. Consequently, a pituitary graft located in the HTA would have access to the hypophysiotrophic hormones by simple diffusion. Our objection was based on the following observations: (1) luteinizing hormone–releasing hormone (LH—RH) synthesizing and/or–containing neuronal elements are located also outside the HTA, however, without exerting hypophysiotrophic effect [38, 39, 40]. (2) In serial sections of hypothalami bearing pituitary grafts and stained alternately for gonadotrophic cells and LH—RH, no correlation was found between the presence of LH—RH-positive neuronal elements and active, hypertrophied gonadotroph cells of the grafts [38, 39]. This finding appears to be indicative that the presence of, and direct contact with the LH—RH-containing structures of the HTA could not be the essential factors for the maintenance of the secretory activity of the gonadotroph cells in the grafts. (3) We have also demonstrated that in the rat (in contrast with earlier findings of other investigators) the MBH cannot maintain the tonic secretion of the gonadotrophic hormones if it is completely isolated from the rest of the hypothalamus. Female rats with successfully deafferented MBH show persistent diestrous vaginal smears, and atrophic uteri and ovaries. Disappearance of LH—RH from the hypothalamic island has been demonstrated both with immunohistochemistry [39, 40] and by radioimmunoassay methods [6].

The possible participation of the cerebrospinal fluid (SCF) and ependymal cells in conveying hypophysiotrophic hormones to the grafts has already been ruled out by the observation of HALÁSZ et al. [18] that pituitary grafts in the HTA but surrounded by ependymal cells, or grafts outside the HTA but in contact with the CSF, had not maintained normal histological structure and function. Our immunocytological studies [38, 39] substantiated these observations.

As an alternative explanation for the hypophysiotrophic effect of the HTA, we suggested [38, 39] that this effect is mediated by a vascular link existing between the capillary loops of the ME and the vascular bed nourishing the MBH. TÖRÖK [43, 44] and AKMAYEV [1] have already described this vascular connection, and TÖRÖK has demonstrated *in vivo* in dogs that the blood flow in this vascular system was directed upwards, *i.e.*, from the ME toward the MBH. Recently, AMBACH et al. [2], using an elegant ink-infusion technique, have demonstrated that the MBH is supplied by blood vessels emerging nearly exclusively from the pituitary circulation. Since the nerve terminals of the parvicellular neurosecretory system release their neurohormonal substances into the capillary loops of the ME, both the pars distalis and MBH is irrigated by blood rich in hypophysiotrophic hormones. Although the functional significance of this circulation of the MBH is unknown, we suggested that it might be the anatomical basis of an ultra-short feedback mechanism [9, 22, 38, 39]. Nevertheless, the special vascularization of the MBH offers an explanation for the hypophysiotrophic effect of the HTA, based on blood circulation.

The aim of the present experiments was to study the immunocytology of anterior pituitary implants grafted into the HTA of female rats having no ME and PS. Well-maintained, hypertrophied gonadotrophs in such grafts would be in favour of the "diffusion theory", while inactive, small gonadotrophs would indicate that — in the absence of the ME and PS — the grafts could not be in contact with the LH—RH present in neuronal elements of the surrounding brain areas.

Material and methods

Adult female rats (from our inbred strain, derived from a Wistar stock) with regular vaginal cycle were used. They had free access to standard laboratory diet and water during the whole experimental period.

Vaginal smears were taken and recorded every morning except Sunday. The animals were divided into three groups. The 3rd ventricle of the animals in each group was exposed by means of a device designed by SÉTÁLÓ and made in our departmental workshop. The device (Fig. 1) could be attached to a stereotaxic instrument. In the body of the instrument (a), two L-shaped supports (a_1 and a_2) could be synchronously moved to either direction from the midline in a groove by turning any of the two knobs (b_1 and b_2). Exchangeable blades (c_1 and c_2) could be attached to supports a_1 and a_2 with small screws. The blades were cut from a brass-plate, 0.5 mm in thickness, and individually shaped for animals with different body weights. For the animals used in this experiment (200 g body weight), blades with 3 mm width and 15 mm length were used. A portion of the skull of the anaesthetized animals, approximately 6×6 mm in size, was removed together with the sagittal sinus. The blades, while closed, were then lowered into the brain in the midline, just behind the anterior commissure. Special care was taken not to press the sharpened edges of the blades against the ME, but to get close to it as much as possible. Using knob b_1 or b_2, the blades were then separated from each other until the walls of the 3rd ventricle were about 1 mm off from each other (Fig. 2). At this phase of the experiment, animals belonging to the different experimental groups were treated differently. In group A, a whole adenohypophysis from a 3 weeks old donor was placed onto the floor of the 3rd ventricle. This group served as

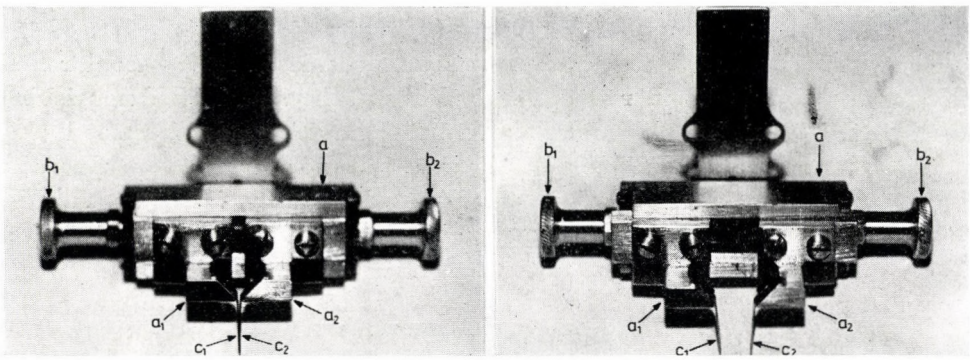

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Fig. 1. Device for the exploration of the 3rd ventricle. *a)* Body with the blade-supports (a_1 and a_2); (b_1 and b_2) turning knobs to move the blade-supports. The blades (c_1 and c_2) are closed
Fig. 2. The same as Fig. 1, but with open blades

control in the experiment. In group B, the ME and the PS were removed under the control of a dissection microscope, using a 22 gauge needle, conically shaped at the tip, and attached to a vacuum aspirator. In group C (and also D), a whole adenohypophysis was replaced in the site of the removed ME. (Group D was treated like group C, and was separated from Group C at evaluating the completeness of ME-ectomy in serial sections.)

41—44 days after the operation, the animals were sacrificed. Under sodium pentobarbital anaesthesia, a cannula was inserted into the ascending aorta and the animal was perfused with 100 ml phosphate buffered saline (PBS) followed by 100 ml picric acid–formaldehyde solution [45]. Before the perfusion was started, a ligature was placed at the beginning of the descending aorta, so that only the pituitary and the thyroid among the endocrine glands were perfused. All endocrine glands were dissected and weighed. Brains and the pituitaries were dehydrated in graded alcohols, embedded into Polywachs 1000 (Chemische Werke, Hüls) and processed for immunohistology. The rest of the endocrine glands were fixed in 10% formaldehyde solution. Frozen sections were cut from the ovaries and stained with Scharlach-R-hematoxylin. The uteri, thyroids and adrenals were embedded into celloidine–paraffin. A series of coronal brain sections was stained with hematoxylin–eosin.

Anti human β LH (rabbit; Calbiochem), anti rat FSH (rabbit; NIAMDD-A-Rat FSHS-6), anti human βTSH (rabbit; Calbiochem) and anti rat prolactin (rabbit; No. 221; a gift from P. K. NAKANE) antibodies were used at different dilutions to detect the respective cells both in the grafted and *in situ* pituitaries. LH–RH-containing neuronal elements were located in serial sections of the hypothalami using anti-LH–RH No. 442 antibodies raised against synthetic LH–RH in rabbits by A. ARIMURA. Anti rabbit gamma globulin (sheep; Serono) antibodies were used in the staining procedure as bridging antibodies, and horseradish peroxidase–antihorseradish peroxidase (rabbit; PAP Dako Immunoglobulins) complex served as the label in the system. Incubated sections were developed for peroxidase, using H_2O_2 as a substrate and diaminobenzidine (free base, Sigma) as the capturing agent.

Student's *t*-test was used for the statistical evaluation of the results.

Results

Changes in the vaginal smears and in the weight of the target glands are summarized in Table 1.

In group A, irregularity of the estrous cycle followed the surgery, however, weight data of the endocrine glands remained within the limits characteristic of intact females of the same age at the end of the six weeks experimental period.

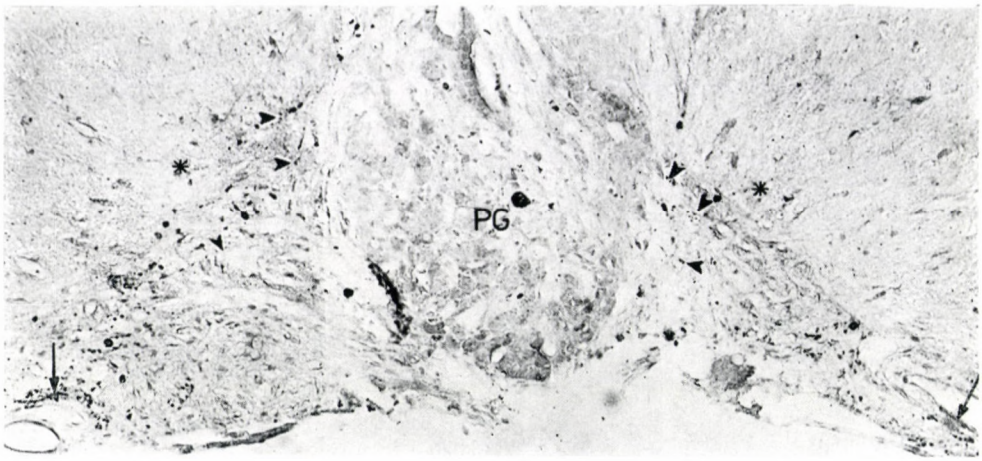

Fig. 3. Coronal section of the hypothalamus of a rat from group C with a pituitary implant and ME and PS removed. The pituitary graft (PG) occupies the lowest part of the 3rd ventricle. LH–RH staining. LH–RH fibres can be seen close to the surface of the hypothalamus (arrows) and in the vicinity of the graft (arrow heads). Asterisks indicate the arcuate nuclei

In group B, removal of the ME and the PS was followed by persistent diestrous smears and by changes in the weight and histology of some endocrine glands. The uterus weight was 70% less in group B than in group A ($P < 0.001$). The anterior pituitary glands showed about 35% reduction in weight ($P<0.05$). Decrease of the weight of the ovaries (20%) and thyroids (15%) was found to be not significant. No change was observed regarding the weight of the adrenals. In the ovaries, severe atrophy of the follicles and of the interstitial tissue was accompanied by the presence of multiple persistent corpora lutea.

In group C, where the removed ME and PS were replaced by a whole adenohypophysis, persistent diestrous smears were recorded during the whole experimental period. Weight changes of the endocrine glands and histology of the ovaries were the same as in group B.

The majority of animals belonging to group D showed persistent diestrous smears. Persistent diestrous smears were interrupted by occasional estrous smears only in two cases. The uterine weight in this group showed about 50% reduction compared with data of the group A. This weight reduction was highly significant ($P<0.001$). The ovaries of these animals showed some sign of a retarded follicle development, and moderate atrophy of the interstitial tissue. With only one exception, anterior pituitary weights were greater in this group than either in group B or C. Regarding the weights of the thyroids, ovaries and adrenals, no changes could be detected if compared with the respective data of group A.

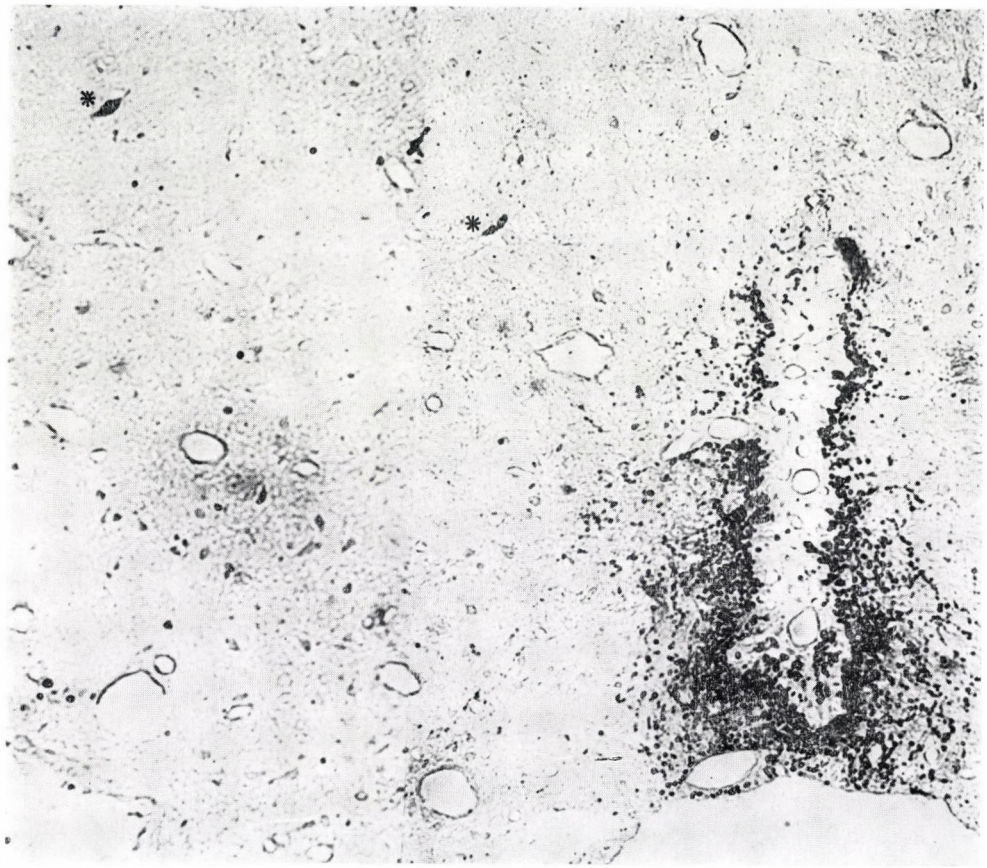

Fig. 4. Well preserved LH–RH axons and axon terminals in the OVLT of an animal from group C. Asterisks indicate cells containing LH–RH. ×150

Investigating the serial sections of the brains stained with hematoxylin–eosin, we could conclude that removal of ME and PS was complete in the animals of groups B and C. In the brains from group D, smaller or larger remnants of the ME were found. In all animals having surgery, the arcuate nuclei were found to be anatomically intact and in most cases the ependymal cell layers did not adhere to each other. Anterior pituitary grafts in group A were found to be in firm contact with the floor of the 3rd ventricle. With only one exception, in which the grafted pituitary was found in the thalamus, pituitary grafts in groups C and D had vascular connection with the rest of the MBH.

In the serial sections of hypothalami representing groups B and C stained for LH—RH, no axon terminals could be identified. Fibres containing LH—RH

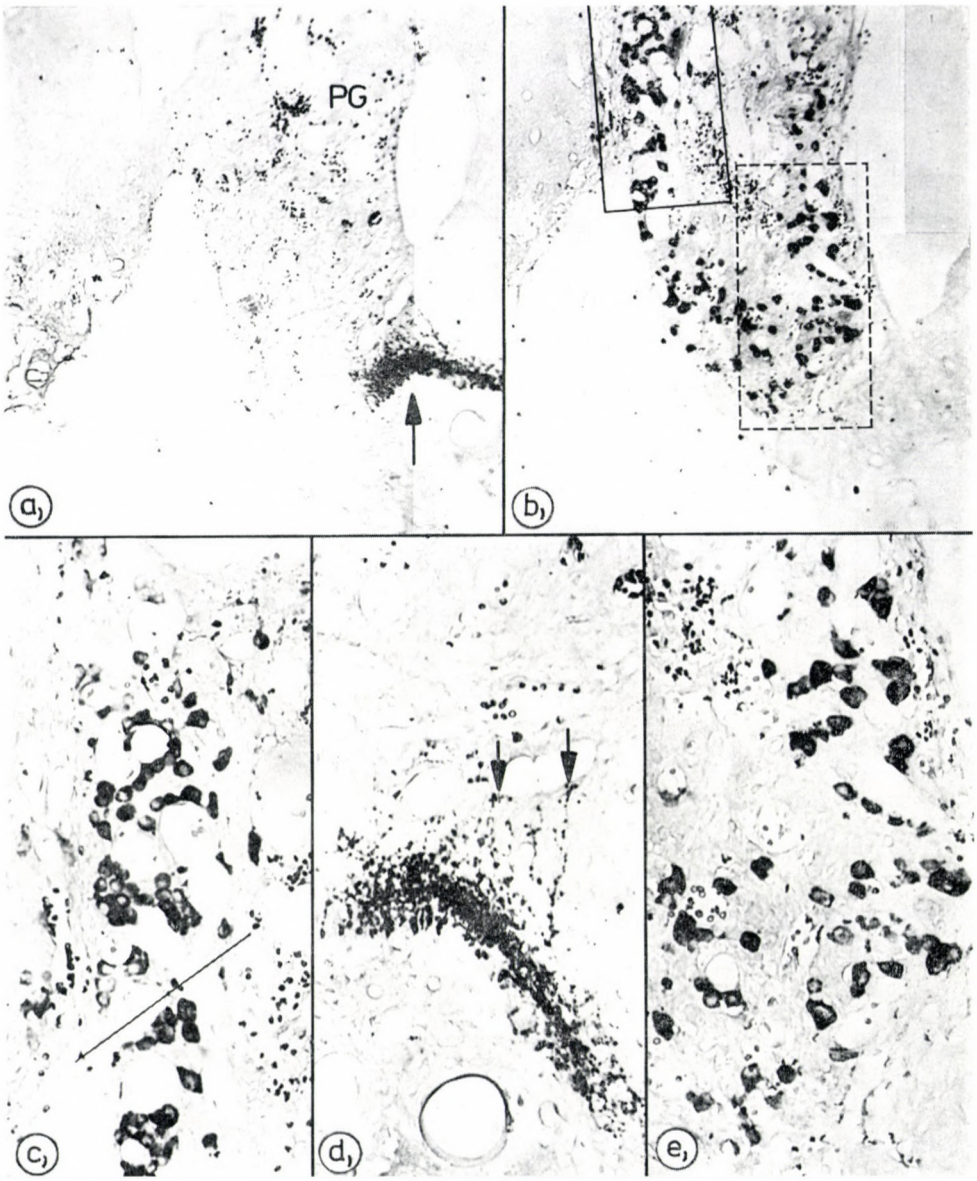

Fig. 5. Coronal section of the hypothalamus of a rat from group D with removed ME and PS and with pituitary implant. *a)* LH–RH staining. The ME was completely removed from the left side. On the right side, the lateral third of the ME remained intact. Bundle of LH–RH fibres is indicated by the arrow. Dark spots in the pituitary graft (PG) are red blood cells. ×95. *b)* Adjoining section to the one illustrated in *(a)*, stained for LH cells. Squares surrounded by solid or broken lines are also shown in *(c)* and *(e)* with higher power. ×95. *c)* LH cells of the area surrounded by solid lines in *(b)*. The cells above the solid line are smaller than those shown in *(e)*. ×240. *d)* Enlarged view of the LH–RH fibre bundle shown in *(a)*. Note LH–RH axon terminals approaching blood vessels (arrows). ×240. *e)* LH cells of the area surrounded by broken lines in *(b)*. The cells are larger than those shown in *(c)*. ×240. For details see also text

Table 1

Daily vaginal smears, body and organ weights of rats with anterior pituitary graft in the HTA[1] *graft in the HTA*

Label of animal	Daily vaginal smears
Group A	
528	○○○ ●●○○○● ●○○●○○ ●●●●●● ●●○●○● ●●●●
529	○○○ ●○○○●● ○○●●○○ ●●○○○○ ○○○○●● ●●○●
530	○○○ ○○●●●● ○○○●●● ●●●●○● ●●○○●● ●●●
531	○○○ ●○○○○● ○○○○○○ ○○○○●○ ○○○○●○ ○○●○
Group B	
519	○○○ ○○○○○○ ○○○○○○ ○○○○○○ ○○
520	○○○ ○○○○○○ ○○○○○○ ○○○○○○ ○○
521	○○○ ○○○○○○ ○○○○○○ ○○○○○ ○○○○
523	○○○ ○○○○○○ ○○○○○○ ○○○○○○ ○○○○○
524	○○○ ○○○○○ ○○○○○○ ○○○○○○ ○○○○○
Group C	
526	○○○ ○○○○○○ ○○○○○○ ○○○○○○ ○○○○○○ ○○
527	○○ ○○○○○○ ○○○○○○ ○○○○○○ ○○○○○○ ○○
532	○○○ ○○○○○○ ○○○○○○ ○○○○○○ ○○○○○○ ○○○○
553	○ ○○○○○○ ○○○○○○ ○○○○○○ ○○
570	○○○○ ○○○○○○○ ○ ○○○○ ○○○○○○
572	○○○○ ○○○○○○○ ○○○○○○ ○○○○○○ ○○○
573	○○○○ ○○○○○○ ○○○○○○ ○○○○○○ ○○○
574	○○○○ ○○○○ ○ ○○○○○ ○○○○○○ ○
Group D	
554	○ ○○○○○○ ○○○○○○ ○○○○○○ ○○○○○○
555	○ ○○○○○○ ○○○○○○ ○○○○○○ ○○○○○○ ○○○
556	○ ○○○○○○ ○○○○○○ ○○○○○○ ○○○○○○ ○○○
571	○○○○ ○○●○○○ ○○○○○○ ●○○○○○ ○○○
575	○○○○ ●○○○○○ ●○○○○○ ○○○●○● ○●●
533	○○○ ○○○○○○○ ○○○○○○○ ○○○○○○○ ○○○○○○ ○○○○

○: diestrous smear
●: estrous smear

were present all along the base of the hypothalamus and in the arcuate region. LH—RH fibres were also present around the pituitary grafts, but there were no signs to indicate that they had a tendency to form new axon terminals in the vicinity of the grafts. No LH—RH cell bodies could be detected in the MBH (Fig. 3). Well preserved LH—RH fibre system was detectable in the vascular organ of the lamina terminalis (OVLT) (Fig. 4) of each experimental group. In a few cases, cell bodies containing LH—RH could be detected in the medial preoptic area of the ME-ectomized groups. In the animals in group D, smaller or larger portions of the ME were found to be intact (Fig. 5a). True axon terminals approaching blood vessels could be recognized in these areas (Fig. 5d).

(group A). with ME[2] and PS[3] removed (group B), with ME and PS removed and with pituitary (groups C and D)

Duration of survival after the operation (days)	Body weight at autopsy (g)	Organ weights (mg)				
		uterus	ovary	adenohypophysis	adrenal	thyroid
43	301	617	50	14	45	25
43	382	658	63	12	52	21
43	431	592	40	9	52	17
43	268	528	53	7	34	16
41	293	152	40	7	64	16
41	245	242	37	7	43	17
42	296	150	40	8	45	14
42	308	96	39	7	25	21
42	316	199	49	7	38	16
44	363	197	42	8	31	18
44	386	163	34	9	43	19
44	309	148	46	5	62	15
42	389	94	19	7	29	23
42	375	173	52	9	50	10
43	288	174	60	7	30	15
43	240	133	33	7	38	15
43	285	107	27	7	33	16
44	302	455	47	12	87	13
44	293	282	52	11	48	18
44	329	201	35	12	51	19
42	258	192	32	10	35	13
43	248	221	52	10	45	22
44	389	345	39	7	63	13

[1] hypophysiotrophic area of the hypothalamus
[2] median eminence
[3] pituitary stalk

The grafted pituitaries of the animals in group C contained only small (8.4 μm) gonadotroph cells (Figs 6a and 7a). Such small diameter is characteristic of the LH cells of all pituitary grafts implanted outside the HTA (nonpublished observation). Both the LH and FSH cells were small also in the *in situ* pituitary glands of this group (Figs 6c and 7c). (LH and FSH cells remained small in the pituitary grafts of the ME- and PS-ablated rats even if the *in situ* pituitary was removed. We had three such cases, however, the data of these animals are not included in this paper). On the other hand, TSH cells in group C had normal size in a few cases both in the grafted (Fig. 6b) and the *in situ* pituitary glands (Fig. 6d).Prolactin cells were well preserved both in the grafted and/or the *in situ* pituitaries of all animals with removed ME and PS (Figs 7b and d).

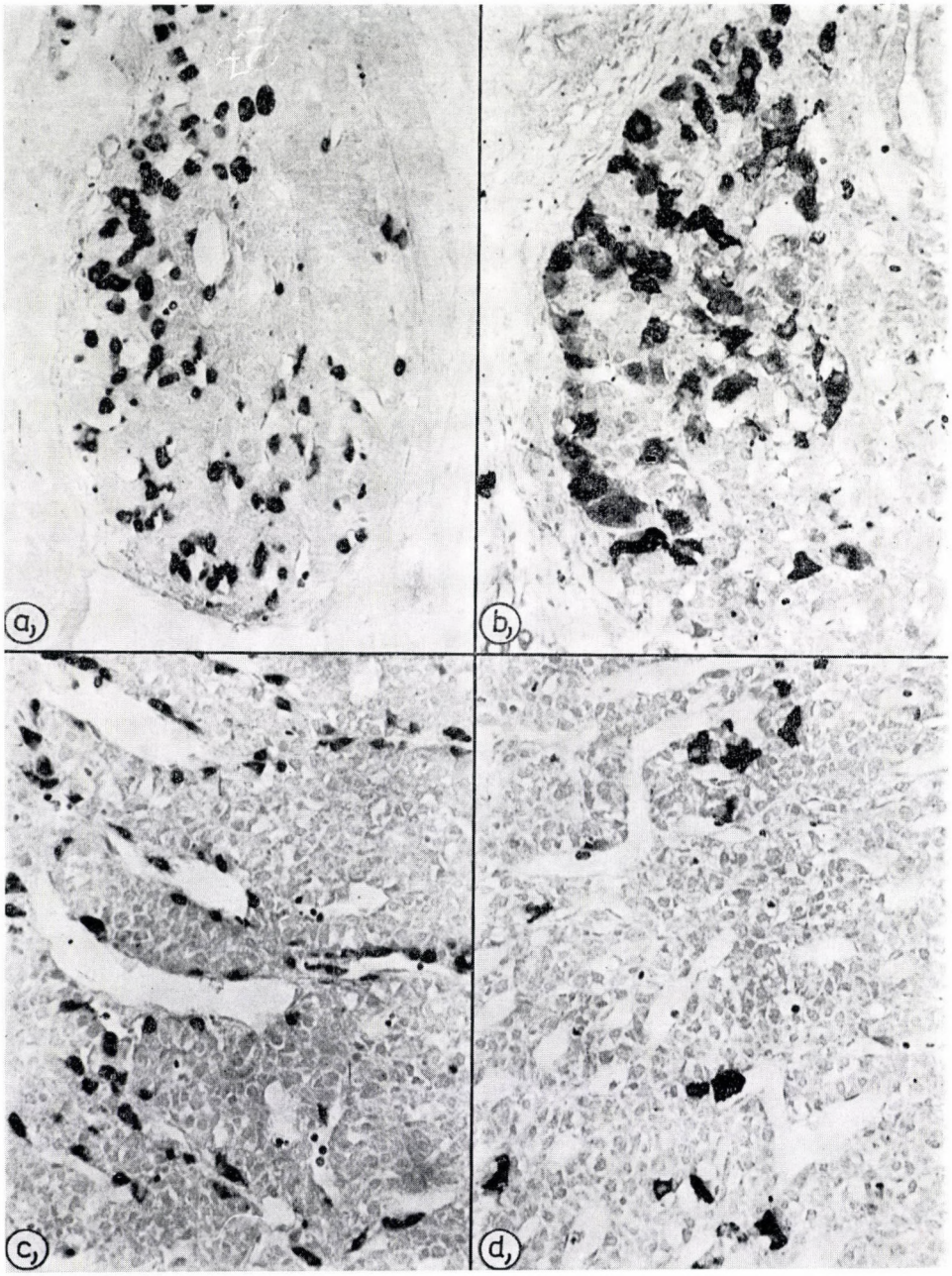

Fig. 6. LH cells of the graft *(a)* and the *in situ* pituitary *(c)* from the animal shown in Fig. 3. Note that the cells are small and loaded with LH. ×240. TSH cells of the graft *(b)* and the *in situ* pituitary *(d)* of the same animal. Note that TSH cells are larger than LH cells. × 240

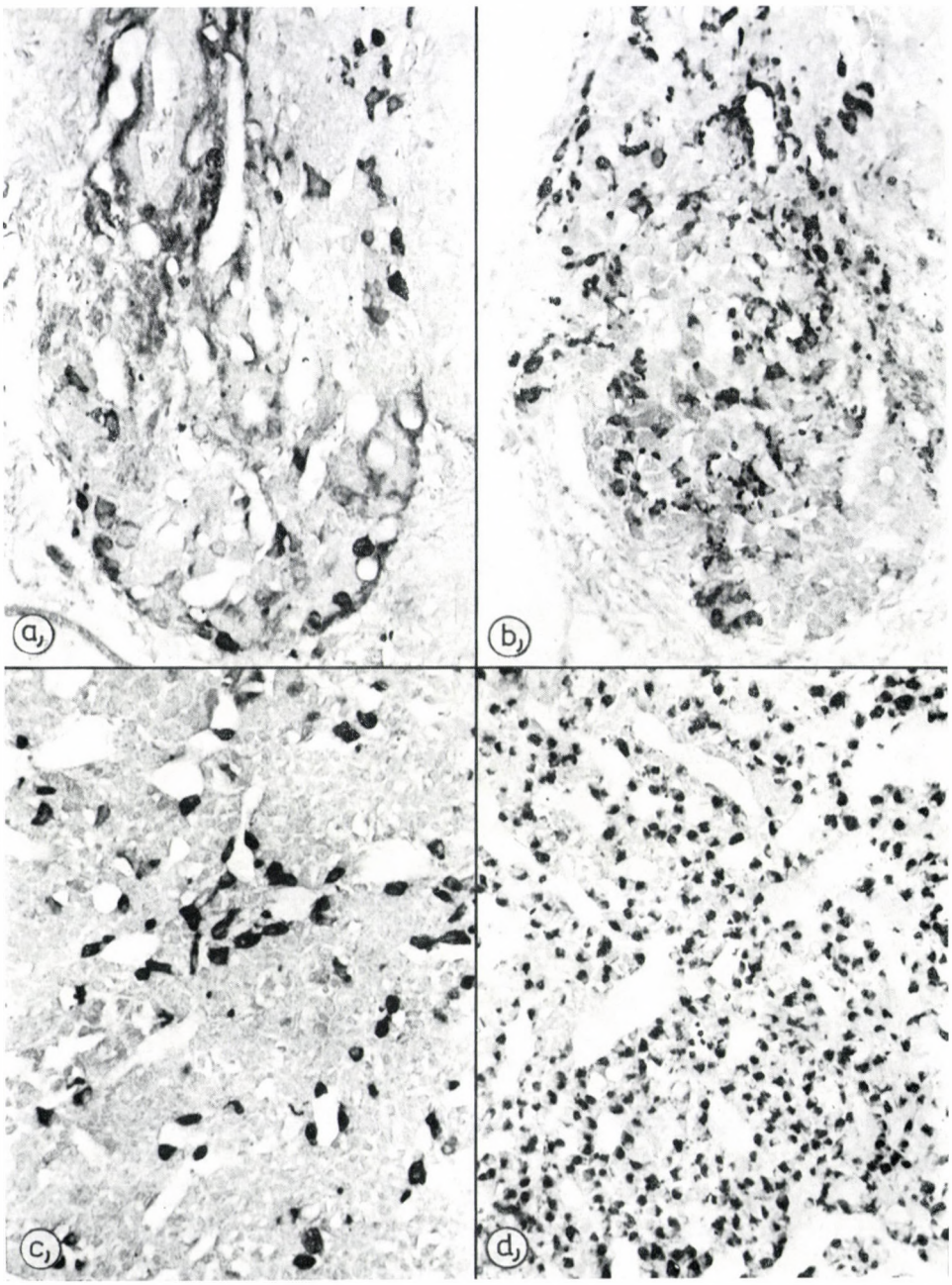

Fig. 7. FSH cells of the graft *(a)* and the *in situ* pituitary *(c)* from the animal shown in Fig. 3. Note that the cells are small and loaded with FSH. ×240. Prolactin cells of the graft *(b)* and the *in situ* pituitary *(d)* of the same animal. Note the large amounts of prolactin cells. ×240

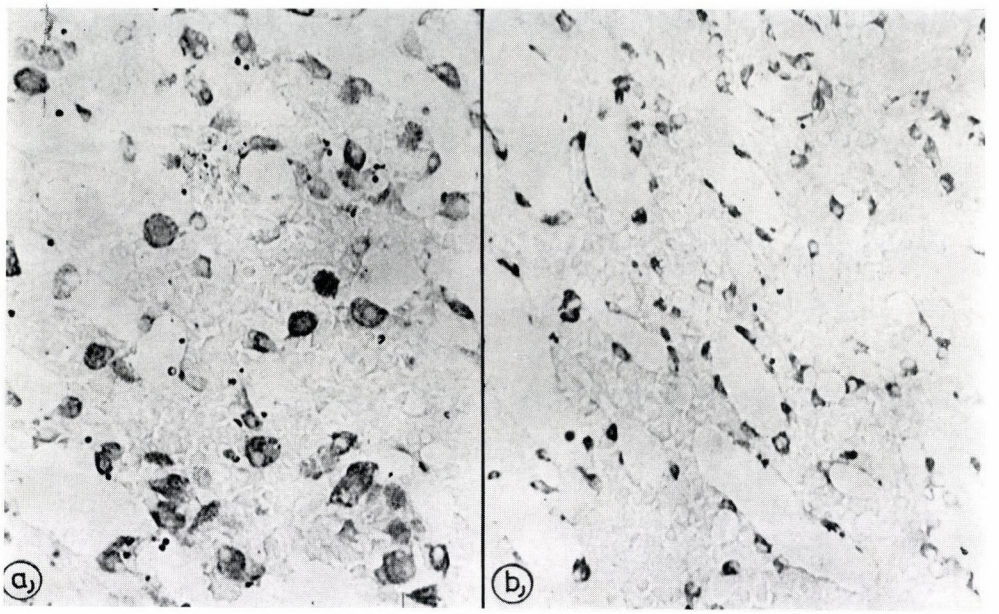

Fig. 8. LH cells from the *in situ* pituitary of the animal illustrated in Fig. 5. *a)* LH cells with normal size from an area of the gland. *b)* Small LH cells representing the general trophic state of the LH cells in the gland. For details see also text.

In the grafts in animals belonging to group D, the LH cells had a larger diameter (10.8 μm) at those regions and on that side where the ME remained partially intact (Fig. 5b, c and e). However, the portion of the graft in contact with the half of the hypothalamus with completely removed ME contained only small LH cells (8.4 μm) also in these cases (Fig. 5b, c and e). In the *in situ* pituitary glands of such animals, islands containing larger LH cells (12.7 μm) could be seen among the generally small (8.4 μm) LH cells (Fig. 8a and b). Small necrotic area was detected only in one *in situ* pituitary gland.

Discussion

The most spectacular observations of the present experiment were the following. (1) Removal of ME and PS resulted in the isolated atrophy of the gonad system of female rats, while the weight of the thyroid and adrenal glands remained unchanged at the end of the six weeks experimental period. Atrophic change of ovaries were detected only by histological examination, because persistent *corpora lutea* maintained the weight of the ovaries. (2) An anterior pituitary grafted into the HTA of the animals with removed ME and PS was not able to enhance the weight of the uterus relative to the uterine weight of rats which have undergone only a removal of ME and PS. Neither

was it able to initiate follicle development in the ovaries and to induce cyclic change of vaginal smears. Immunocytology of the *in situ* pituitaries showed that the gonadotroph cells became inactive and were characterized by small cell size and relatively dense staining. While the LH cells in control pituitaries have 13 μm mean diameter, in the ME- and PS-ablated animals this value was only 8.4 μm. LH cells of pituitaries grafted in the HTA of the ME- and PS-ablated animals also had a mean diameter of 8.4 μm, and were not larger even if the *in situ* pituitary was removed. LH cells of pituitaries grafted in the HTA of hypophysectomized animals had 16.4 μm mean diameter (unpublished data). (3) The presence of LH—RH-containing neuronal elements in the vicinity of the pituitary grafts was not enough to supply the gonadotroph cells of the grafts with that hormone. In other words, LH—RH did not diffuse out from the nerve cell bodies synthesizing it, or from the nerve fibres transporting it. We have already published similar observations [38, 39].

There are some contradictions between our present findings and the results published earlier upon pituitary changes following PS section or electrolytic lesion of the ME and PS. It has been shown that the transection of the PS resulted in pituitary and target gland atrophy if regeneration of the portal vessels was prevented by inserting a barrier between the hypothalamus and the pituitary [14]. Similarly, electrolytic lesion of the ME and PS in the rat resulted in the atrophy of the ovaries [8, 10, 13], the thyroid glands [41] and the adrenals [4, 17, 27, 29, 30, 31, 41]. In the experiment of HALÁSZ et al. [19], significant atrophy of the pituitary gland and the target glands was detected within three weeks, if the PS was completely severed by the lesion.

In our experiment, removal of the ME and PS by vacuum aspiration resulted in the isolated atrophy of the gonadal system. Persistent corpora lutea found in the ME- and PS-ectomized animals were most probably due to elevated prolactin secretion. KANEMATSU and SAWYER [23] have described an immediate and prolonged elevation in plasma prolactin levels of rats with PS section. Increase of the prolactin secretion was also detected in rats treated with drugs blocking dopaminergic fibres [36]. Since dopaminergic fibres of tuberal origin terminate in the ME [21], removal of the ME may cause similar effect. Although the reduction of the weight of the anterior pituitary gland was only moderate, it was, however, highly significant. Decrease of the weight of the adenohypophysis might have been caused by the inactivation of the gonadotroph cells accompanied by the decrease of their size. No significant change could be detected regarding the weight of the thyroid and adrenal glands. Since maintained target gland weights cannot be explained without the effect of the respective hypophysiotroph hormones, we have to assume that in some way CRH and TRH can reach the *in situ* pituitary in a high concentration even in the absence of the ME and the PS.

The degree of atrophy of the endocrine glands of animals with ME-lesions or section of PS never reaches the degree one can observe in hypophysectomized animals [32, 42]. Such observations already indicated that the *in situ* pituitary gland was able to release limited amounts of trophic hormones even if its normal connection with the MBH was disrupted. To some extent, the maintained anterior pituitary functions could be explained by the possible revascularization of the anterior lobe with the portal vessels. Other experiments, however, indicated that the hypophysiotroph hormones are available for pituitaries from the general circulation, even if they are grafted to remote sites of the body [16, 25, 29]. Detectable CRH [5], gonadotrophin releasing hormone [7, 15, 34, 45, 37] and GH—RH activity [28, 33] were demonstrated in the plasma of hypophysectomized rats. Furthermore, experiments, in which several pituitary glands were grafted into the abdominal wall of hypophysectomized rats, showed that although the weight data of the endocrine glands of these animals were far from the normal, they were high above the hypophysectomized values [3]. Following the electrolytic lesion of the ME of hypophysectomized rats bearing 10 ectopic pituitary grafts, the weight gain of the testicles was diminished, however, there was no change in the weight of the thyroids, and the adrenal glands became even larger [3]. KENDALL and ALLEN [24] have reported that electrolytic lesion of the ME of hypophysectomized rats bearing pituitary implants did not decrease the ACTH secretion maintained by the grafts.

All the observations listed above indicate that TRH and CRH are relaesed into the general circulation even if the ME is lacking, although, at a reduced rate. This is reflected by the thyroid and adrenal weights maintained above hypophysectomized level in animals having either intact *in situ* pituitary gland but electrolytically destroyed ME, or ectopic pituitary homograft(s) and destroyed ME. Our animals were different from those just mentioned in two respects. (1) Their ME and PS were not electrolytically destroyed, but removed. (2) They had an adenohypophysis grafted into the HTA and/or an *in situ* pituitary gland.

That functional revascularization of the *in situ* pituitary gland is impossible, or, at least, is restricted following the electrolytic lesion of the ME is indicated by the observations that the functional capacity of such pituitary glands remains permanently impaired, and that large areas of such pituitary glands become necrotic. This is probably caused by the scar tissue formed after the lesion, which seems to be impenetrable for the regenerating blood vessels. In our experiments, the complete lack of pituitary necrosis indicates that, either the few supratuberal vessels surviving the surgery, and the posterior pituitary arteries could provide the pituitary with sufficient amounts of blood, or, in the absence of scar tissue, regeneration of the pituitary circulation could be completed very rapidly. Since the pituitary gland remains in

the sella, and is the animal's own pituitary, hazardous events, like physical and chemical injuries and immunologic reactions will not interfere with the chances of the *in situ* pituitary to recover to a possible utmost extent. What these pituitaries will lack, is the ME as a specialized structure to convey hypothalamic neurohormones to the pituitary gland. From our results it is obvious that gonadotrophin releasing hormone(s) are not available for such pituitaries in the amounts necessary to maintain follicle development. The observation that in the present experiment the anterior pituitary grafted into the HTA was not able to prevent the atrophy of the uterus and gonads, even in presence of the *in situ* pituitary, indicates that in the absence of the ME, the HTA cannot exert its hypophysiotrophic effect on the gonadotroph cells. Since LH—RH-containing nerve fibers are present in the MBH of the animals with ME and PS removed, the absence of the hypophysiotrophic effect on the gonadotroph cells could not be caused by the general lack of gonadotroph releasing hormones. In the knowledge of the peculiar blood circulation of the MBH described in the introduction, the most obvious explanation of this finding is that without this circulation LH—RH is not available either to the graft or to the *in situ* pituitary. This is so, because LH—RH is probably not released from the axons which had lost their terminals when the ME was removed. We can also assume that the LH—RH axons cannot form new, functional terminals, not at least within the six weeks experimental period. This would again support our earlier supposition that the hypophysiotrophic effect of the HTA on gonadotroph cells is provided by the vascular system of the MBH.

On the other hand, this experiment showed that CRH and TRH do reach the *in situ* pituitary in a concentration enough to maintain normal adrenal and thyroid weights even in the absence of the ME and PS. This was indicated also by the TSH cells having normal size and cytological characteristics both in the grafted and *in situ* pituitaries of rats with ME and PS removed. Functional capacity of these adrenal and thyroid glands was not tested in the present experiment, it has to be investigated in the future. Nevertheless, we have to suppose that CRH and TRH can be released not only into the capillary loops of the ME, but also into other capillaries having connection with the portal circulation. Alternatively, the possible functional regeneration of the nerve fibres transporting these hormones onto the regenerating capillaries has to be taken into consideration. Immunohistology would certainly help us to explain these observations, however, in the lack of specific antibodies against these hypothalamic hormones, these investigations have to be postponed.

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DIFFERENTIATION OF ATYPICAL MITOCHONDRIA INDUCED BY BORON APPLICATION

SHORT COMMUNICATION

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(Received 1977—02—08)

The mechanism of action of the physiological activity of boron in plants has not been elucidated so far. Recently it has been proposed that the physiological role of boron in plants may be associated with the process of phosphorylation. Plants made deficient in boron were observed to have a decreased ATP content [1, 2]. This fact led to the supposition that boron might affect the energy metabolism of the cell. To ensure the normal course of biosynthetical reactions during cell growth and division, the cell has to be sufficiently supplied with energy. In cells, ATP serves as a universal carrier of energy and mitochondria have proved to be the main place of ATP synthesis. The relationship between boron and ATP together with the fact that ATP synthesis occurs in mitochondria, prompted us to study the relation of boron to mitochondrial differentiation.

Plants of *Vicia faba* L. cv. Považský were grown in a half-concentrated Knop's solution with addition of boric acid in doses of 100 and 500 mg/l. Root tips fixed in permanganate and osmium were used for electronmicroscopic studies.

The mitochondria in meristematic cells of *Vicia faba* root tips, as in other plant cells, are oval-shaped (Fig. 1), and the cristae in them appear to be well-developed. After application of boron, changes in mitochondrial differentiation were observed. In the cytoplasm, apart from typical oval-shaped forms we observed atypical mitochondria (Figs 2—3), namely, elongated, differently branched and, in some cases, T-shaped ones. In the elongated mitochondria we could observe constrictions, but no mitochondrial division by constriction after application of boron. The division seems to be inhibited by boron treatment, which results in differentiation of various atypical mitochondria. We have never observed any atypical mitochondrial cristae to differentiate following boron treatment. The changes as to shape and size of mitochondria observed after application of boron suggest that boron affects mitochondrial morphogenesis and, most likely, mitochondrial division as well. Attention is drawn to the fact that after boron application both normal

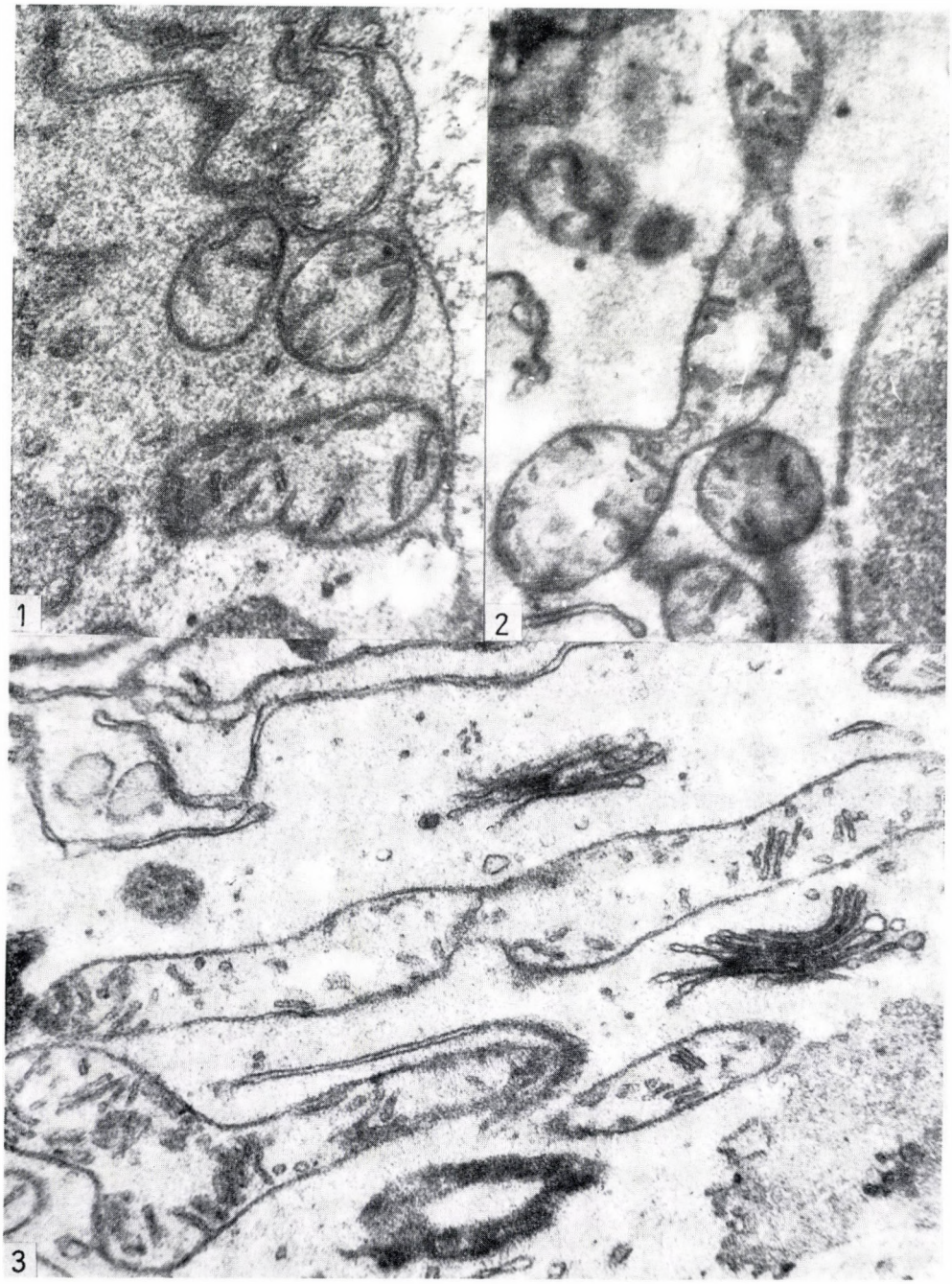

Fig. 1. Mitochondria in control. ×25,000
Figs 2—3. Atypical shape of the mitochondria after the 48-h treatment with boron (100 and 500 mg/l). ×24,000 ×28,000

and atypically-shaped mitochondria were observed. Thus, boron did not affect all of the mitochondria available. We assume this phenomenon to be conditioned by the ontogenetical cycle of mitochondria. Ontogenetically older mitochondria, which differentiated prior to boron treatment, preserved their typical shape. The younger mitochondria, which had developed in the presence of the added boron, were atypical in shape.

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PHYSIOLOGICAL AND ULTRASTRUCTURAL INVESTIGATIONS OF AN IDENTIFIED NEUROSECRETORY CELL OF *LYMNAEA STAGNALIS*

SHORT COMMUNICATION

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(Received 1977—05—09)

In the CNS of Gastropoda, neurosecretory cells widely occur (cit. in [1]). Certain types of them are involved in the regulation of the sexual cycle [2], while others take part in the osmoregulation. The examination of their electrophysiological properties started only in the last decade [3, 4, 5, 6], and up to now just a few neurosecretory cells have been identified individually [5, 6, 7].

In the past years a number of neurones producing secretory material have been discovered in the CNS of *Lymnaea stagnalis* [1, 3, 6], however, they have not been identified except the so-called Canopy cells [6].

Neurone Al0 located in the visceral ganglion [8] probably corresponds to the VD1 neurone of Benjamin and Ings [9], which can be well distinguished from the surrounding yellow-orange, pigmented neurones owing to its whiteness. Usually, this property is characteristic of the neurosecretory cells. Our investigations were aimed at clearing up the neurosecretory character of this neurone in a morphological way and, at the same time, to analyze its electrophysiological properties. We wished to answer the question if there is any speciality in the potential generation of this neurosecretory cell.

The experimental animals were collected in different seasons and were kept in aquarium in continuously running Balaton water until used. For localization of the cell and demonstration the main axonal branches intracellular $CoCl_2$ injection was performed without any intensification method [10]. The electron microscopic examinations were carried out in spring and autumn, altogether on 12 animals. Cell A10 was isolated with a fine forceps, then was fixed in a solution containing 2.5% glutaraldehyde and subsequently in 2% OsO_4. The resting and action potential of the cell was recorded with microelectrodes [8]. The micropipettes used for injection of the mediator substances were filled with 1 *M* ACh or 0.1 *M* 5-HT and dopamine, respectively. The electrophysiological mesurements were performed within a five-year period on 100 animals.

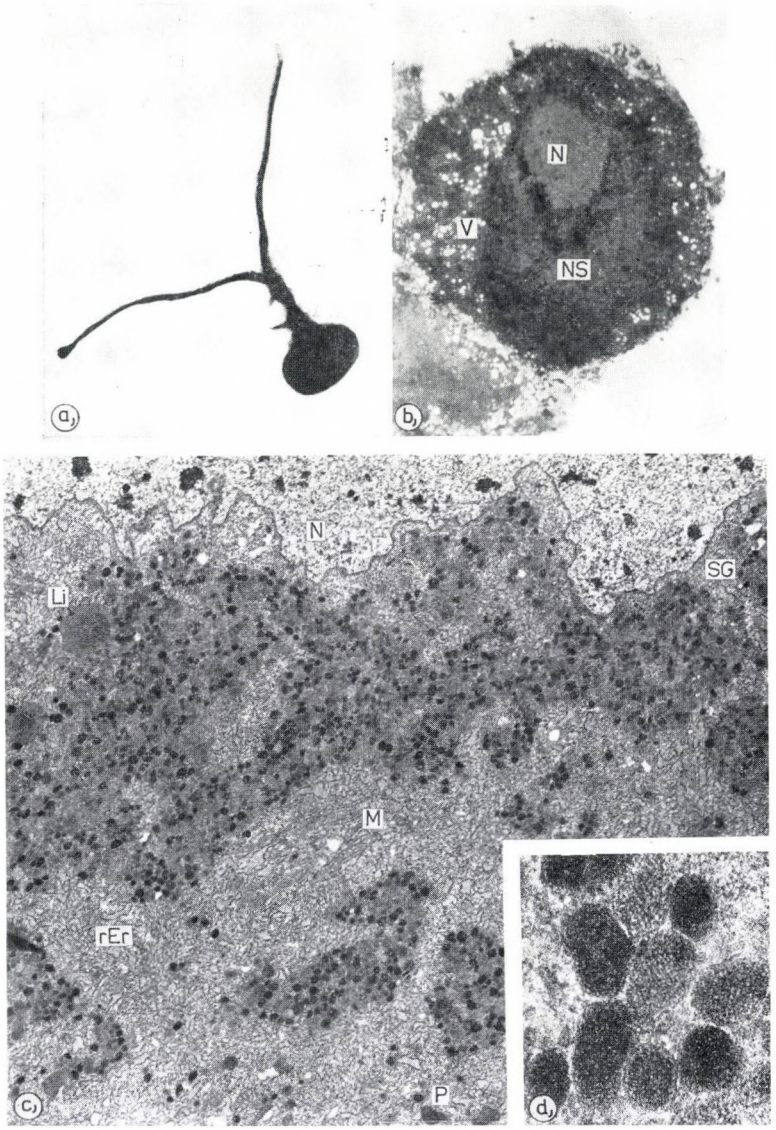

Fig. 1. a) Neurone A10 marked with intracellular $CoCl_2$ staining. *b)* A light microscopic section of neurone A10. The nucleus (N) is poor in chromatin. Note the piles of dark-stained neurosecretory material (NS) in the cytoplasm. The cell is highly vacuolated. Toluidine blue staining. ×330 *c)* Electron micrograph taken from cell A10. In the surface of nucleus (N), there are a number of invaginations. In the immediate vicinity, a large amount of secretory granules (SG) of variable appearance can be seen. The cytoplasm is rich in rough-surfaced endoplasmic reticulum (rEr). Besides, a couple of mitochondria (M), lipid droplets (Li) and pigment granules (P) occur in the cell. ×8000 *d)* High-power electron micrograph shows, that the granules are variable in shape, their fine-granulated inner content is bounded by a unit membrane ×72,000

The neurone A10 as filled up with cobalt proved to be unipolar, with branching axon (Fig. 1a). The proximal region of the axon is thickened to a high degree and several dendritic spines are originated from it. The longer axonal branch runs into the right parietal ganglion, while the shorter one is terminated in the visceral ganglion showing a swelling like that described as characteristic of some neurosecretory cells.

In semithick sections, neurone A10 has a spheroid appearance (Fig. 1b.) The cytoplasm is vacuolated to a high degree, on the surface membrane glial processes can be seen. After staining with toluidine blue there are coherent, compact areas in the soma showing the presence of the neurosecretory material. The largest diameter of the cell is 150 μm. In the electron micrographs (Fig. 1c), a great number of secretory granules and rough-surfaced endoplasmic reticulum can be found in the immediate vicinity of the nucleus. The secretory granules are embedded in a fine-granulated matrix, they have spheroid or ovoid form, with an inner content of varying electron density.

In addition, tubules and cisternae of endoplasmic reticulum, a number of mitochondria, lipid droplets and pigment granules can also be seen in the soma. High-power magnification (Fig. 1d) showed that the number of secretory granules of regular spherical form is relatively small, granules of ovoid, drop-like and irregular appearance occur more frequently. The membrane surrounding the granules proved to be unit membrane in every case. Usually the granules of high electron density occur most frequently in the neuroplasm, besides, there are many medium dense or electron transparent granules too. Here and there in the soma, fusion of neurosecretory granules of irregular form can also be found. Crinophagy was not observed in the cytoplasm.

On measuring 500 granules, their average diameter appeared to be 1600Å.

Cell A10 is a spontaneously active neurone, its activity is generated in an endogenous manner. Concerning the firing pattern, three main activity types can be distinguished (Fig. 2). *a)* Frequency of continuous regular distribution, *b)* burst activity, *c)* frequency of continuous irregular distribution. From early spring until autumn the proportion of type *c* was continuously decreasing, at the same time that of type *a* and *b* was increasing. So, toward the autumn months, the significance of the exogenous synaptic influence was declining, while that of the endogenous potential generation was increasing.

Several excitatory inputs and one inhibitory input resulted in an inhibition of long duration (ILD) converge onto the neurone A10 [12]. Serotonin and dopamine applied either on perfusion or with microiontophoresis can mimic excitation and inhibition, respectively (Fig. 2d—h). Acetylcholine seems to be less important, nevertheless, it may play a role as a modulator depending on the functional state. This is because of the equilibrium potential

of the ACh effect estimated is equal to the resting potential (see in Table 1). Qualitatively it is demonstrated in the Fig. 2f—h.

The results suggest that neurone A10 may be a peptidergic neurosecretory cell of type "C" [13]. According to the works of ROUBOS et al. [3] and BENJAMIN and SWINDALE [14] as well as the ultrastructural and electrophysiological

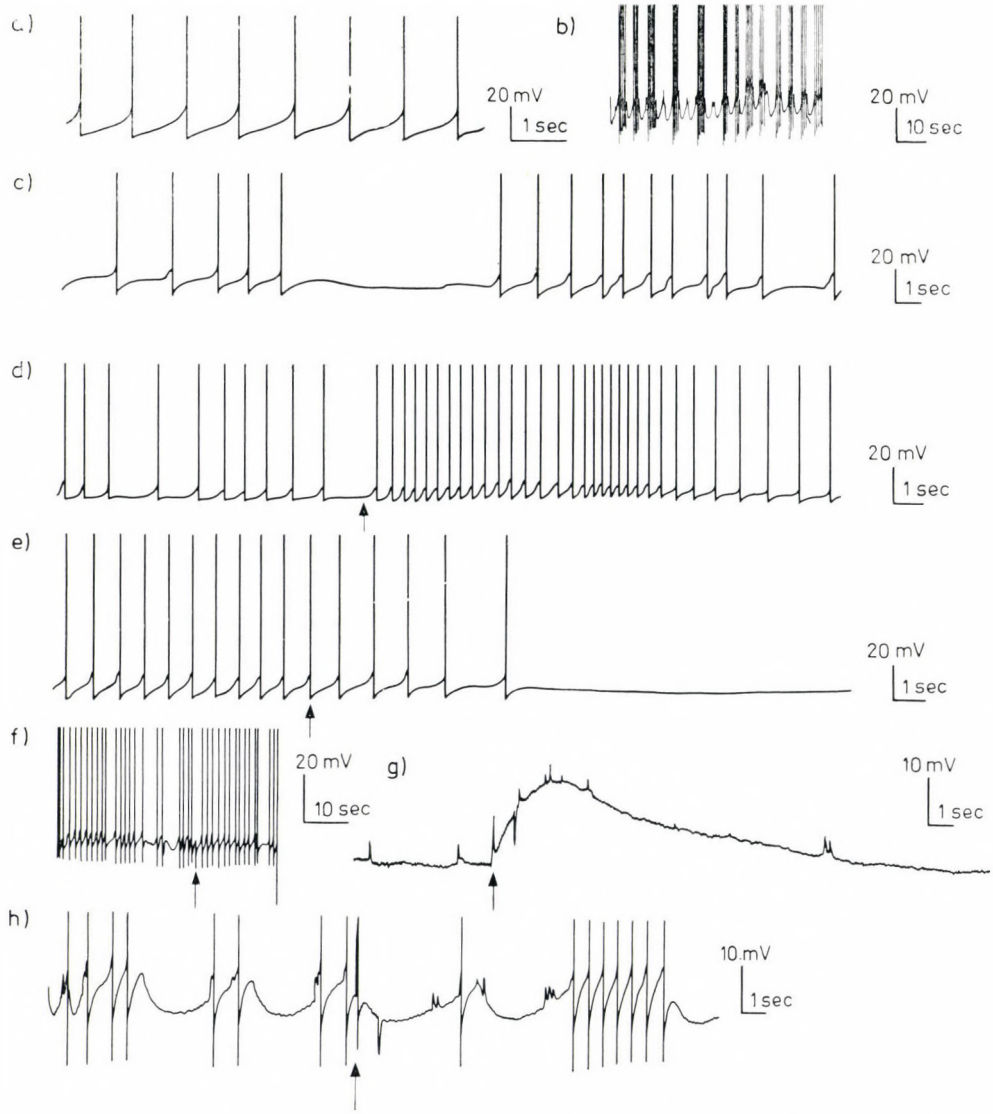

Fig. 2a, b, c) Spontaneous activity patterns of cell A10. *d)* Effect of 5-HT. *e)* Effect of dopamine. *f, g, h)* Effect of ACh at different membrane potential levels. The values of membrane potential: *f)* 40 mV = resting potential, *g)* 50 mV (—10 mV artificial hyperpolarization), *h)* 30 mV (+10 mV artificial depolarization). Arrows mark the moment of the application of substances

Table 1

Resting membrane potential (mV)	Frequency of spontaneous activity (cps)	Amplitude of action potential (mV)	Overshoot (mV)	Spike duration (msec)
37.9±6.9*	1.15±06.	72.9±9.7	33.9±12.5	12.5±3.3

* SD

properties found in the recent work neurone A10 is quite similar to the "dark green" cells which are involved in the osmoregulation [3]. Concerning the electrophysiological properties, there is a crucial point where neurone A10 differs from the "dark green" cells: it receives a rather characteristic ILD input, and can be defined as CILDA cell. From morphological point of view, the most pronounced difference is in the diameter of the soma. While "dark green" cells are not more a 100 μm in diameter, cell A10 is a giant neurone whose diameter exceeds 100 μm.

Comparing our data to those described for the giant neurosecretory cells identified in other gastropods [5, 6, 7] a number of similarities can also be found concerning the average diameter of granules and their variable form, the irregular shape of nucleus, the high degree of vacuolization of the cytoplasm, and the soma surface, which is rich in glial processes. According to the pharmacological classification, some of the giant neurosecretory neurones proved to be of CILDA type cell like neurone A10. Most of them exhibit burst activity of endogenous nature. In certain periods of year such a burst activity can be recorded from neurone A10, however, usually this does not show a high regularity like that of typical "Br" cells.

No axonal process of neurone A10 was demonstrated running into any nerve originating from the visceroparietal ganglion complex [8]. When recording its intracellular activity in pair with a number of other neurones it could not be observed up to now, that A10 would drive the firing of any examined cell with monosynaptic transmission. The only cell connected with A10 by electrical coupling is a sensory cell marked as P1 in the parietal ganglion. Comparing the above findings with the morphological ones obtained in the present work we suggest that the efferent function of this cell may be realized in a humoral way.

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RECENSIONES

The Evolution of Reproduction

Eds: C. R. AUSTIN, R. V. SHORT, Cambridge University Press, Cambridge (1976), pp. 189, £ 6.50 (H/C) £ 2.50(P/B)

The Evolution of Reproduction is the sixth volume in the series of Reproduction in Mammals. Like the previous volume it is a clear, readable and informative small book aiming the needs of undergraduates reading Biology, Medicine or Agriculture and Veterinary Science. The contents is the following:

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Each of the five chapters is a respectable venture into an important area of biological thinking. A succinct treatment of topics in the field of evolution of mammalian reproduction.

V. CSÁNYI (Alsógöd)

Calcium in Biological Systems

Ed. C. J. DUNCAN, Cambridge University Press (1976), pp. 485, £ 180.

The volume contains the papers presented at the 30th Symposium of the Society for Experimental Biology (9—12 Sep. 1975). In 20 chapters, the authors give a comprehensive review of Ca chemistry, discussing also the methodical problems of Ca demonstration, its first and second messenger properties and, to a smaller extent, its role played in mineralization. The chapters are written by the distinguished scientists of this field of research.

In his introductory paper, WILLIAMS discusses the protein binding of Ca and Mg which is important for membrane stabilization. The chapters concerned with the chemical structure of natural and synthetic Ca ionophores (TRUTER), with the possible application of aequorin Ca luminescence (SHIMOMURA and JOHNSON), as well as with laser measurements (PIDDINGTON) are of particular interest. These techniques render the visualization and recording of intracellular movements of Ca possible. The most exciting, though most skeptical, chapter is the one entitled Ca ions and mitochondria (CARAFOLI and CROMPTON). At first the reader thinks that mitochondrial Ca transport may play a regulating role, but the author's opinion is not quite explicit in this respect which is wholly justified by the varying results obtained from mitochondria originating from different tissues. As regards the evaluation of transcellular Ca transport valuable data can be obtained from various model experiments, such as those using chorioallantoic membrane (TEREPKA et al.) and computer models (BERLE and ANDERSON).

One of the much debated problems is the correlation between intracellular Ca and control of membrane permeability (MEECH). According to a certain regularity, increase of the quantity of intracellular Ca enhances K conductance and depresses the permeability of the membrane to Na and Ca. This may be a first messenger role present, for example, in the muscle, neurons and sensory organs. The other control may be calcium-mediated potassium activation. The second messenger role of Ca is discussed in several chapters. FOREMAN et al. describe the role of Ca in secretory processes by means of model studies in mast cells. No antigen-induced histamine release occurs in the absence of calcium. A similar second messenger role is attributed to Ca by BERRIDGE when he suggests that there is an apparent involvement of the increased intracellular level of Ca and cyclic GMP in the control of cell division. According to ECKERT et al., Ca plays a role in regulating ciliary frequency in the *Paramecium*. Further chapters are concerned with the second messenger role of Ca in muscle activity, comprising studies on the structure of Ca-binding troponin C (COLLINS), on Ca regulation of muscles in mammals (SZENT-GYÖRGYI), on the regulation of contraction of smooth and striated muscle (EBASHI et al.), on the correlation between Ca messenger release and contraction, on the role of external (extracellular) and internal (terminal cistern) reservoir of Ca (TAYLOR and GODT; NIEDERGERKE et al.; ASHLEY et al.). Mineralization is discussed in two chapters. SIMKISS gives a detailed description of intracellular Ca granules present in a large number of invertebrate tissues. TEREPKA discusses the possible mechanisms of active transcellular Ca transport.

This excellent volume offers rich information on the role of Ca in the physiology of the cell. It may be of interest for investigators interested in Ca metabolism or membrane permeability, and for those dealing with problems of cytology, immunology and mineralization.

I. FÖLDES

Neural Principles in Vision

Ed. F. ZETTLER, R. WEILER. Springer–Verlag, Berlin–Heidelberg–New York, (1976) 430 pp, 293 figs, $ 39.40. ISBN 3—540—07839—8.

The book is a part of the series of Proceedings in Life Sciences and contains the papers presented at a symposium held in Munich in September, 1975. The symposium was organized with the intention to encourage dialogues between separate research groups engaged in the study of the vertebrate and the invertebrate retina. Accordingly, the book consists of three main chapters. The first chapter (9 contributions) deals with the vertebrate retina from fish to mammals inclusive. A general feature of the work presented is that most of them employ a combination of various morphological and physiological techniques in order to achieve an unambiguous identification of the retinal element under investigation. From this kind of complex approach, a number of fascinating results are described on the wiring diagram of the retina and on the problems of information processing in the retina, including synaptic transmission from receptors to neurons, and interactions between different types of receptors and neurons. The second chapter contains 9 papers on the retina of arthropods. The results of these works were obtained mainly from physiological experiments, but various labelling techniques were frequently used for the identification of the neuron studied. Discussing similar problems as with the vertebrate retina, the authors make several references to the similarity of the neuronal processing of visual information in the lens eye and compound eye. The last paper in this series offers an interesting comparison between the function of these two structurally different eyes. The third chapter gives an account of investigations performed on the

retina of molluscs (4 contributions). There are several interesting morphological and physiological data obtained from studies of this primitive lens eye.

The book truly reflects the success of a symposium which provided an opportunity to several outstanding scientists to explore the common principles in the neural mechanisms of structurally different eyes. It is unfortunate that discussions and comments to the individual papers are not published. The book is produced by photoprint processing of excellent quality. It is of special value to the students of neurobiology, sensory physiology and ophthalmology.

G. Székely (Debrecen)

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INFLUENCE OF CHRONIC DOSES OF MERCURIC ACETATE AND LEAD ACETATE ON THE NUMBER AND ACTIVITY OF GOMORI-POSITIVE GLIAL CELLS IN THE MOUSE BRAIN

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(Received 1977—02—25)

Abstract

Intraperitoneal injections of mercuric acetate or lead acetate in doses of 0.2 mg daily during 14 and 21 days caused statistically significant rise in the number of Gomori-positive glial cells around the third cerebral ventricle of mice. In addition, the nuclei of the Gomori-positive glial cells markedly increased in volume.

Introduction

In the brain of mammals and some other vertebrates, glial cells of a special type are present around the cerebral ventricles [6, 8, 13, 15, 16, 26, 27]. These cells differ from typical glia cells by the presence of large numbers of cytoplasmic granules with strong affinity to Gomori's chrome haematoxylin and aldehyde fuchsin after oxidation with potassium permanganate.

The Gomori-positive cytoplasmic granulation in the periventricular glia is unusually rich in cysteine and cystine detectable by histochemical methods [17, 18]. The glial Gomori-positive granules are large, peroxisome-like cytoplasmic organelles [18]. The Gomori-positive glial (Gpg) cells around the ventricles markedly increase in number after whole-body X-irradiations in rats [17]. Their pronounced increase in number was observed after irradiation of the rat's head with high doses of X rays, and in mice after UV-irradiation [19, 20, 21, 22].

It is well established that lead and mercury readily cumulate in the CNS [1, 3, 7, 25], causing a special type of degeneration in nerve and glial cells [4, 12]. Despite numerous studies on the influence of mercury and lead compounds on the CNS, there is no precise information about the effect of these elements on the quantitative and qualitative changes in Gomori-positive glial cells.

The present study was therefore concerned with the effect of chronic doses of lead acetate and mercury acetate on the number and activity of Gomori-positive glial cells in the mouse brain.

Materials and methods

Twenty-five male white mice aged 4 months, average weight 25 g, were divided into a control group and two experimental groups. The control group consisted of five mice, and each of the experimental groups of 10 mice.

Each experimental group was divided into two subgroups of five mice each. The mice of the first subgroup were injected i.p. with 0.2 mg of lead acetate daily for 14 days (group 1) and 21 days (group 2). Mice of the second subgroup were injected with the same daily doses of mercury acetate, for 14 and 21 days, respectively.

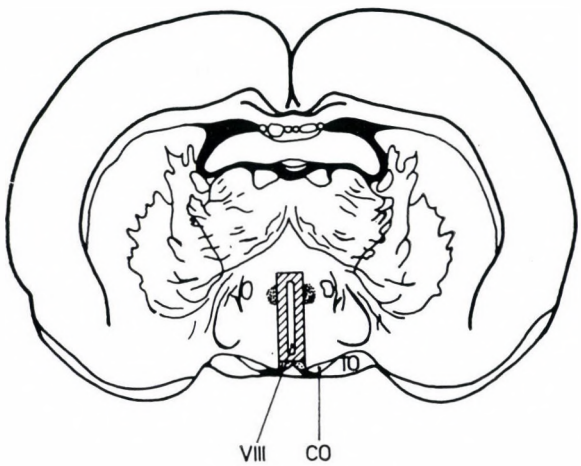


Fig. 1. Schematic drawing of a cross section of a mouse brain at the level of the anterior hypothalamus. The Gomori-positive glial cells were counted in the hatched area; VIII — third ventricle, CO — optic chiasm, TO — optic tract

Lead acetate and mercury acetate were dissolved in 0.5 ml saline, and i.p. injections were made always at the same time of day. After the last injection, the mice were killed by decapitation. The brains were fixed for 24 h in Bouin fluid. The paraffin embedded material was cut into complete series of 7 μm thick sections. GOMORI's chrome haematoxylin–phloxin method in BARGMANN's modification [14], and aldehyde fuchsin stain were used throughout [9]. The Gomori-positive glial cells were identified by the presence of strongly chrome haematoxylin and aldehyde fuchsin-positive cytoplasmic granules. The cells which possessed in their axons or cytoplasm concentrations 2 or more distinct granules 1—2 μm in diameter, stained with GOMORI's chrome haematoxylin method, were regarded as Gpg cells. This granulation is characterized by more intensive dark blue colour than that of the neurosecretory material and by a typical arrangement of granules. The Gpg cells were counted in a field of 0.125 mm^2 in each section in a series of adjacent sections, in the left and right half of the brain. The periventricular zone of the anterior hypothalamus was chosen for experiment. The volume of the nuclei of the Gomori-positive glial cells was calculated with the formula $V = \pi/6\ LS^2$, where L is the long and S is the short axis. Nuclei of 100 Gomori-positive glial cells were measured in each brain. Altogether 2500 cells were counted and their nuclei measured. The area which was taken under scrutiny is shown in Fig. 1. The arithmetical means for each group were calculated and compared with a two-sided "t" test.

Results

Figures 2 and 3—5 represent the numbers and volume of the nuclei of Gomori-positive glial cells from the neighbourhood of the third cerebral ventricle in control male mice and those injected with 0.2 mg of lead acetate. It is evident that chronic doses of lead acetate induce statistically significant increase in the numbers of Gomori-positive glial cells around the third ventricle, after 14 as well as 21 days of injections, compared with the control group.

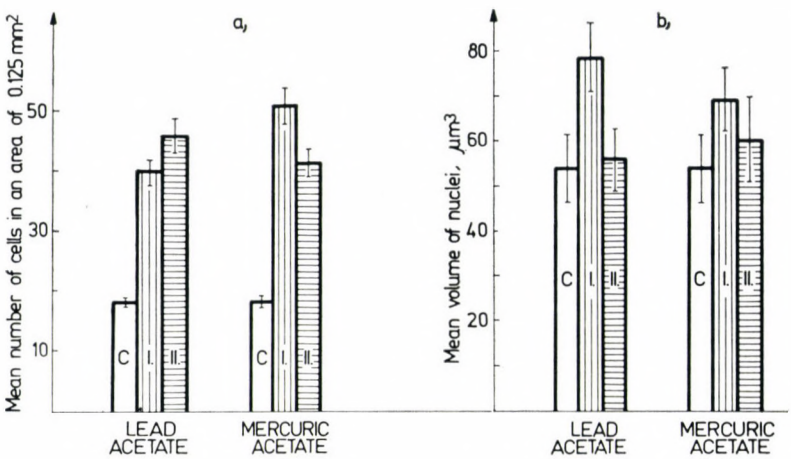


Fig. 2. Mean numbers of Gomori-positive glial cells (A), and mean volume of nuclei of the Gomori-positive glial cells (B) in the neighbourhood of the third ventricle in male mice injected daily with 0.2 mg of lead acetate or mercuric acetate for 14 and 21 days. C — control group, I — first experimental group (14 days), II — second experimental group (21 days)

Significant increase in the volume of the nuclei of Gomori-positive glial cells was noted after 14 daily injections of lead acetate. Prolongation of the injections to 21 days caused a drop in the nuclear volumes in these cells nearly to the control level (Fig. 2). I. p. injections of 0.2 mg daily doses of mercuric acetate for 14 and 21 days also caused statistically significant increase in the number of Gomori-positive glial cells around the third ventricle compared with the control values (Figs 2, 6—7). Moreover, mercuric acetate injected i.p. for 14 days caused significant increase in the volumes of Gomori-positive glial cells, but no significant increase was observed when the period of injections was prolonged to 21 days (Fig. 2).

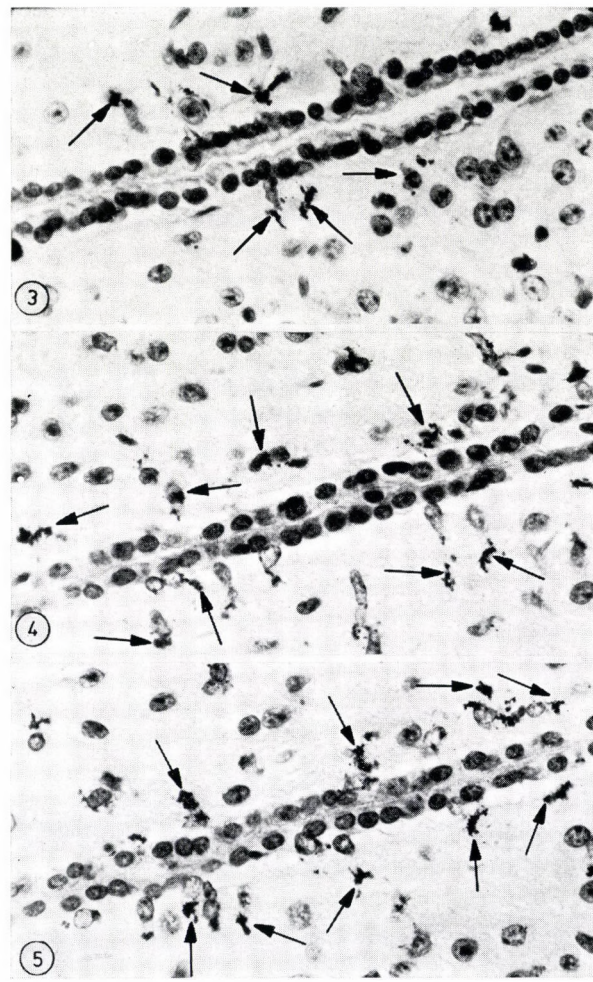
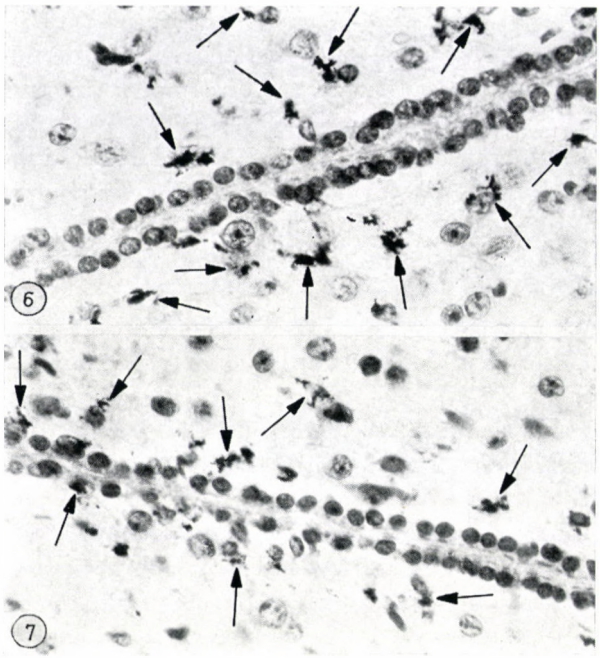


Fig. 3. Gomori-positive glial cells in the region of the third cerebral ventricle in control mice. Chrome hematoxylin — phloxine. ×535

Figs 4, 5. Increase in numbers of Gomori-positive glial cells in the region of the third ventricle in male mice injected with 0.2 mg of lead acetate daily for 14 days (Fig. 4) and 21 days with 0.2 mg/day (Fig. 5). Chrome hematoxylin — phloxine. ×535

Discussion

The findings indicate that i.p. injection of lead acetate or mercuric acetate in daily doses of 0.2 mg during a period of 14 and 21 days caused statistically significant increase in the number of Gomori-positive glial cells situated around the third ventricle of the brains of male mice.



Figs 6, 7. Increased numbers of Gomori-positive glial cells distributed around the third cerebral ventricle in the brains of male mice injected with 0.2 mg daily doses of mercuric acetate for 14 days (Fig. 6) or 21 days (Fig. 7). Chrome hematoxylin — phloxine ×535

Similar, statistically significant increase in the number of Gomori-positive glial cells had been observed in the brains of rats injected with 400 mg/kg body weight of salicyl-quinine-lithium (Togal) for 17 days [23]. However, the biological role of this cell type may be much more general, and not restricted to peroxide removal. For instance, it has been observed that these cells increase in number after prolonged administration of some drugs [23] and ether narcosis (unpublished observations). Thus, the Gomori-positive glia represents some kind of chemical protection system of the brain disposing of xenobiotics and toxins of endogenous or exogenous origin. It was reported by WISLOCKI and LEDUC [27] that chronic administration of silver nitrate to rats causes silver granule accumulation in the Gomori-positive glia. It is therefore probable that the Gomori-positive glia serves a protective function in the brain. A particularly dense coat of Gomori-positive glial cells is present around those sites in the nervous system where the blood–brain barrier is absent, namely, the subfornical organ and the area postrema [16].

It seems that the increase in number of Gomori-positive glial cells in the mouse brains under the present conditions was associated with the decomposition of organic peroxides formed in cerebral oxidative metabolism.

I.p. injections of lead acetate and mercuric acetate for 14 days also caused statistically significant increase in the volume of nuclei of Gomori-positive glial cells, but the same was not observed when the injections were prolonged to 21 days.

The increased volume of the cell nuclei after 14 days of injections indicates an increased activity of this form of glia and consequently, an enhanced protein synthesis in these cells.

STEINWALL and OLSSON [24] demonstrated that high doses of methyl-mercuric chloride can disrupt the brain–blood barrier. According to these authors, an increased incorporation of labelled amino acids in brain proteins *in vivo* under the influence of methyl mercuric chloride may be a result of dysfunction of the brain–blood barrier.

NIKLOVITZ and YEAGER [11] reported the interesting observation that prolonged administration of tetraethyl lead lowers the Cu, Fe and Zn levels in brain cells. The lowered level of copper first probably lowers the levels of ATP and cytochrome oxidase in the mitochondria [12]. Glial cells play an essential role under physiological conditions and are probably first to be intensely paralysed in lead poisoning as a result of disruption of the blood–brain barrier [5, 10], and they contain copper [12]. Presence of copper in glia cells suggest that copper containing enzymes, such as cytochrome oxidase, are depressed during prolonged administration of lead and, as a result, phospholipid synthesis is probably inhibited [2].

It seems likely that the lowered activity of Gomori-positive glial cells when lead acetate or mercuric acetate was injected for 21 days, compared with 14 days, may have been due to gradual reduction of activity of this form of glia.

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ORIENTATION OF *EUGLENA GRACILIS* BY ELECTROMAGNETIC FIELDS: THEORY AND EXPERIMENT

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(Received 1977—04—27)

Abstract

Computer data derived from theoretical treatments of the orientation of ellipsoidal particles in alternating fields is compared with experimental data from microscopic studies on *Euglena gracilis*, an elongated free-living flagellate. Computed data based upon a theoretical treatment by SAITO, SCHWAN and SCHWARZ showed good agreement with experimental results, predicting the relationship between cellular orientation, the frequency of the impressed field, and the conductivity of the suspending medium.

Introduction

The biophysical effects of alternating electrical fields include heat generation, exemplified by a fatal human exposure to radar [8], and nonthermal effects, such as particle translocation, aggregation, and orientation [3, 4, 5, 13, 14, 16]. While the experimental conditions used for study of nonthermal effects are adjusted to minimize increases in temperature, all *ac* fields produce heat. It is difficult to rule out possible local field foci (hot spots) in causing such "nonthermal" effects as the cytogenetic changes reported by HELLER and TEIXEIRA-PINTO [7]. Although some heat is doubtless produced, it does not appear that heat is directly involved in causing the particle translocation, aggregation, or orientation observed in *ac* fields.

Frequency-independent field effects on particles have been recognized since 1927 and include translocation and aggregation to form pearl chains. SHER [13, 14] summarized earlier work and presented extensive experimental and theoretical results describing translocation, dielectrophoresis, and conventional pearl chains (rows of particles aligned with the field). SHER [13] concluded that each particle had a dipole induced by the field and that a pearl chain distorted the impressed field less than did the component particles when separate. GRIFFIN and FERRIS [5] mixed living and nonliving particles in suspension and found that pearl chains formed across fields when adjacent particles were unlike. When like particles were adjacent, only conventional chains with the field formed. While they could not apply a rigorous theoretical

test, GRIFFIN and FERRIS [5] postulated that field distortion minimized when lossy dielectrics (living cells) were adjacent equatorially to good dielectrics (plastic spheres), by analogy to the detailed treatment of conventional pearl chains [13]. While pearl chains formed both with and across fields, the nature of the particles determined the orientation and this orientation was the same at all tested frequencies.

TEIXEIRA-PINTO et al. [16] first reported a frequency-related nonthermal response, that elongate living cells would, at different frequencies, orient both with and across *rf* fields, while nonliving particles oriented only with the field. GRIFFIN and STOWELL [3] and GRIFFIN [4] worked with suspensions of living cells in microscope chambers and found that, for a given population of cells, the variables controlling orientation were the frequency of the field and the conductivity of the suspending medium. Orientation–frequency relationships could be changed by osmotically swelling red cells [4].

In our preliminary considerations of the orientation of *Euglena*, a motile flagellate, it seemed possible that the direction of swimming was oriented in response to an environmental signal, as in the well known trophic responses to light, gravity, or chemical gradients. For the present analysis, tropisms seemed unlikely, since nonmotile bacteria (unpublished), red cells [4], and *Euglena* unable to swim [3], could all be oriented both with and across fields. *Euglena* fixed in glutaraldehyde and washed could also be oriented in both directions, although the turnover frequencies (frequencies at which the mechanical alignment of the cells changes) changed with time in suspension (unpublished). Thus, there was no requirement that the particles being oriented had to be motile or alive to orient across fields.

We assume that the interaction between the field and *Euglena* depended on the physical and electrical characteristics of the organisms their environment, and the induced field. The motility of living *Euglena* was of use as an indicator of viability and preservation of the electrical characteristics of the organism. Preliminary experiments in which specimens were deliberately heated indicated that no significant changes in the orientation of populations occurred until the living cells were damaged enough to stop movement.

In the present paper, we report the measured physical and electrical characteristics of *Euglena* and incorporate these values with experimentally determined values for orientation vs. frequency and conductivity into a computer program as a test of whether theoretical treatments predict the observed phenomena. FERRIS and GRIFFIN [1] noted a preliminary experimental verification of a theoretical treatment formulated by SAITO et al. [11]. A detailed analysis is presented herein.

Materials and methods

Euglena, a photosynthetic flagellate, was selected as an experimental subject because the cells are easily grown, resistant to handling, and are large and elongated so that their orientation can be seen readily.

Culture and handling

Euglena gracilis var. *bacillaris* were grown in bacteria-free nutrient media at pH 7.0, under moderate illumination. The medium used was the rather complex formulation developed by EBERSOLD and LAVINE. The cells were harvested 1 to 3 days after reaching maximal density. At this time, few dividing cells were present, but interphase cells maintained physiologic vigor by photosynthesis. The flagellates were washed by gently centrifuging three times, each time resuspending in an aqueous solution of the composition and conductivity needed for experiments (see GRIFFIN and STOWELL, [3]).

Light microscopy

The microscope chamber used to observe orientation of living cells was illustrated and fully described by GRIFFIN [4]. Figs 1—3 show *Euglena* in the chamber. A preparation in which about 90% of active cells were aligned together was classed as oriented*. The only stable orientations were with the field (Fig. 2) or across the field (Fig. 3). From photomicrographs similar to Figs 1—3, dimensions were determined indicating that the *Euglena* cells used could be assumed to be a population of prolate spheroids with a major axis of 65 microns and an axial ratio of 5.4 : 1. The effects upon the computations of assuming other physical dimensions are considered in a later section.

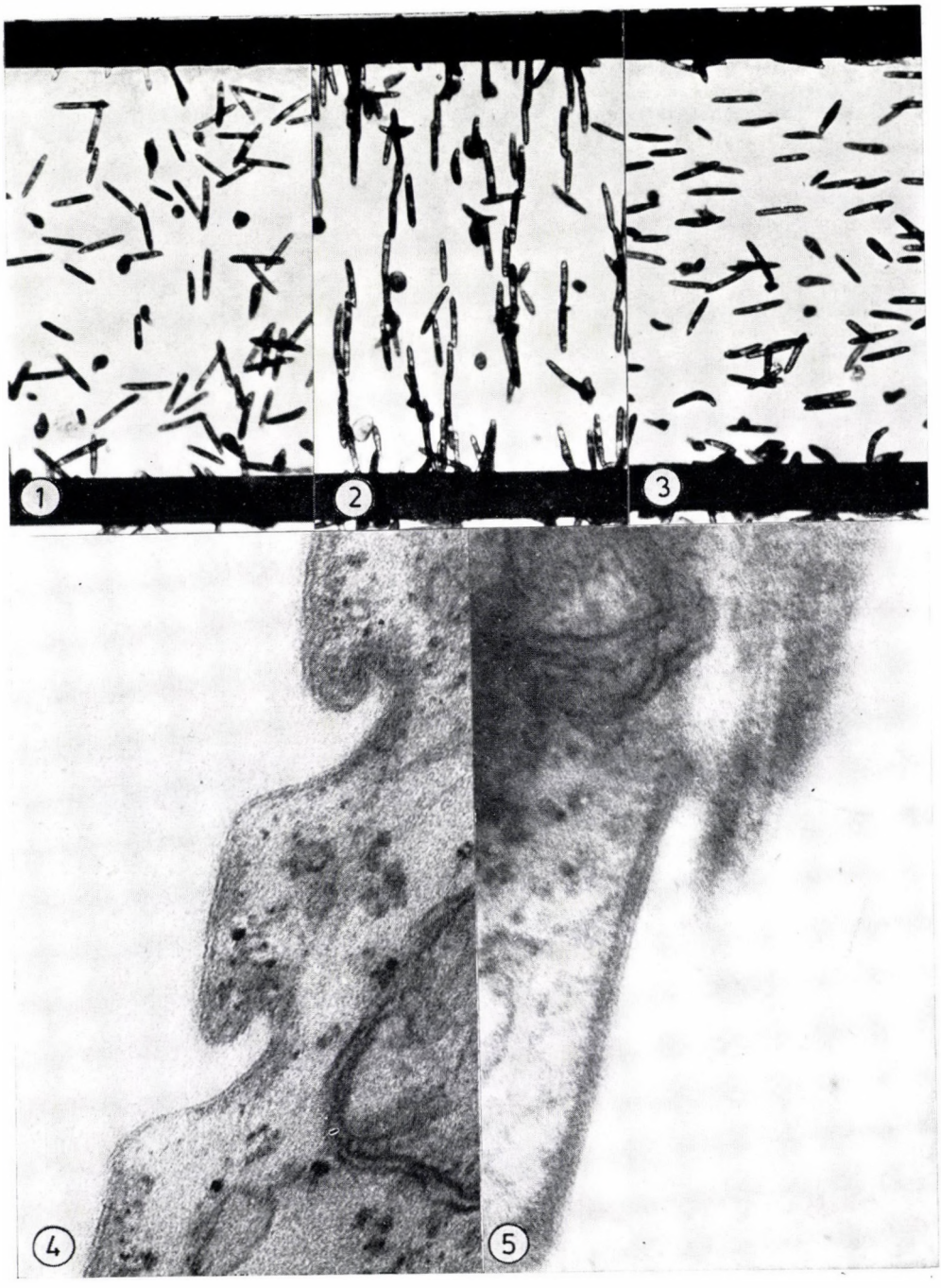
Electron microscopy

The ultrastructure of the *Euglena* membrane was studied in thin sections of a preparation fixed in 2% osmium tetroxide in distilled water and embedded in epon. Fixed cells were in a shortened or contracted state and exhibited surface folds (Fig. 4). In Figs 4 and 5, an external trilaminar membrane 8 to 12 nm in thickness can be seen. Beneath this external membrane and adjacent to it are bands of pellicle material, seen in cross section in Fig. 4 and in longitudinal section in Fig. 5. This material apparently contributes to the structural integrity of the *Euglena* pellicle, but is not assumed to influence significantly the electrical properties of the membrane, since it is not continuous. *Euglena* cells, elongated during locomotion, such as were used for light microscopy, presumably have less dramatic surface ridges than cells that contracted during fixation, but the actual microconfigurations of the surface are unknown. Since there was no simple way to compute the effect of surface irregularities, the basic computations assumed a smooth 10 nm lipoprotein membrane and also considered the effects of different membrane thickness.

Orientation

The cellular orientation was determined, relative to the electric field lines of an applied electromagnetic field, as a function of field frequency and the conductivity of the suspending medium. Criteria for evaluating orientation are described above and in GRIFFIN and STOWELL [3]. Figure 6 shows the relationship between orientation, conductivity, and frequency

* Orientation was determined by counting the numbers of aligned cells in photographs taken during experimentation. Approximately 300 feet of 16 mm motion picture film and 35 mm still film were used. Electronic flash was used to stop motion of the cells when the 35 mm photographs were taken. Alignment was determined from frame-by-frame counts. Since alignment is a dynamic situation, any numbers quoted must necessarily be estimates based upon visual observations. The experimental accuracy can be expressed as $87 \pm 5\%$.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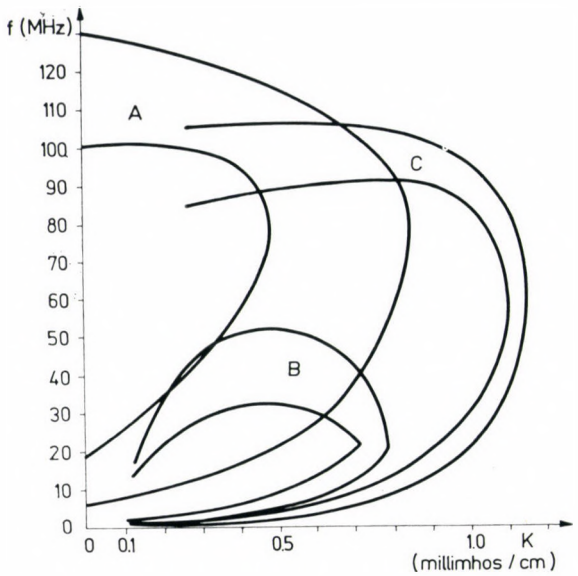

Fig. 6. Experimentally observed alignment of *Euglena* (A) as a function of the frequency of the applied electromagnetic field and the conductivity of the suspending medium, and calculated values (B and C). See text

for a specimen in which the external conductivity was varied by changing concentrations of sodium chloride. Other ions gave similar results [3]. Curve A of Fig. 6 applies.

At the transition frequencies, populations showed mixed orientations, an apparent reflection of biologic variability in the population. If a strong signal was suddenly applied at a transition frequency, almost all cells oriented either with or across the field lines. Thus, although a broad transition frequency range is seen for a population, a single cell probably exhibits a transition point on the frequency scale, rather than exhibiting a transition range. As has been noted previously, orientation is a dynamic process and one must depend upon visual estimates and photographic records. For a fixed value of conductivity $\varkappa$, the breadth of the transition frequency is estimated as ± 5 MHz about the central value.

The electrical properties (conductivity and dielectric constant) of the *Euglena* were determined. A Boonton Radio Corp. (Hewlett-Packard Corp.) type 250A R—X Meter was used with a special sample chamber to contain the *Euglena* preparation.

←

Figs 1—3. Photomicrographs of *Euglena* in the chamber used for light microscopic studies on orientation (conductivity of suspending saline, 0.2 millimhos). The platinum wire electrodes about 500 microns apart, cross the top and bottom of each picture. Fig. 1, no signal, random orientation. Fig. 2, field on at 15 MHz, orientation parallel to field lines. Fig. 3, field on at 35 MHz, orientation perpendicular to field lines. The field intensity was just enough to get a clear orientation. Since no cells in Fig. 2 were across the field and none in Fig. 3 were with the field, the samples as photographed were classed as oriented, even though some cells were not fully aligned. (Figs 1—5, AFIP Neg 72—5642.)

Figs 4—5. Electron micrographs of *Euglena* to show the external membrane, 80—120 Å in thickness. Beneath the membrane is additional material that apparently contributes to the integrity of the pellicle. Note, in Fig. 4, that this material seen in cross section, does not underlie the whole membrane, being absent below each cleft. In Fig. 5, the pellicular material is seen in longitudinal section, adjacent to the membrane near the bottom of a cleft. ×110,000

After experimenting with various designs the general configuration developed by PAULY and SCHWAN [10] was adopted for the sample chamber. As shown in Fig. 7, this is in effect a short transmission line. In practice, the chamber is filled to a level well above the ends of the electrodes to minimize field fringing and resulting errors in calibration.

The residual capacitance of the chamber is 1.2 pF and the conductance is too low to be measured on the R—X Meter. These values are thus well within the initial balance adjustments in the Schering bridge circuit of the R—X Meter and the meter can be adjusted to give direct readings of sample values.

The R—X Meter is designed to measure impedance as a parallel resistance–capacitance (R—C) combination. Corrections must be made, however, for the residual capacitance and conductance of the chamber and the lead inductance of the connectors from the R—X Meter to the platinum electrodes. These matters are discussed in the operation manual for the instrument. The R—X Meter measures all R—C impedances as if they are composed of two parallel components R_m and C_m as shown in Fig. 8 (a). These are the indicated dial readings. Figure 8 (b) shows what is actually measured and presented as the dial readings in terms of equivalent values for R_m and C_m. If initial balance offset is applied (by making the initial balance with the empty chamber connected to the bridge), the circuit of Fig. 8 (c) applies. Initial balance offset is most easily achieved by connecting the empty sample chamber to the R—X Meter and balancing the bridge with the initial balance adjustments. This does have the disadvantage that the chamber must be filled and emptied for each frequency setting of the bridge. When the chamber is filled with an unknown sample, the dial readings will give R_s and C_s, after the inductance correction, without any further correction being necessary for R_r and C_r, provided that initial balance offset has been applied.

The correction factors for the R—X Meter and sample chamber were determined experimentally as described in PAULY and SCHWAN [10]. Aqueous KCl solutions were used.

The corrected values for sample conductivity and dielectric constant are determined from the empirically derived expressions.

$$\varkappa = (1.81\ R_s)^{-1}$$

$$\varepsilon = 0.696 \times 10^{13}\ (C_s - 1.2 \times 10^{-12}),$$

where R_s and C_s are the measured values of sample resistance and capacitance, respectively.

For a suspension of *Euglena gracilis* cells in water, the values determined for $\varkappa$ and ε apply to an inhomogeneous sample. The variation of $\varkappa$ and ε with frequency is presented in Fig. 9. Our goal was to relate the observed turnover frequencies (frequencies at which orientation of the cells changed) for *Euglena* orientation to the electrical properties of the cells. It was next necessary to separate the electrical properties of the *Euglena* $\varkappa_e$ and ε_e from the values for the suspending aqueous medium $\varkappa_w$ and ε_w.

Because of the limited capacitance measuring range of the Boonton Radio Corp. R—X Meter (0—23 pFd), it was necessary to use a low volume concentration of cells in suspension. The volume concentration p, of the *Euglena* cells was determined by two methods to be $p = 0.0036$*. Because of this low value for volume concentration and the large axial ratio for the cells, the modified Clausius–Mossotti equation proposed by FRICKE [2] to find $\varkappa_e$ and ε_e was inapplicable. The electrical conductivity of the cells was thus determined by the approximation

$$\varkappa_e = (1/p)\ \ [\varkappa - (1/p)\ \varkappa_w].$$

The dielectric constant for the cells was determined grossly from a similar relation

$$\varepsilon_e = (1/p)\ [\varepsilon - (1/p)\ \varepsilon_w]$$

* The volume concentration was determined by two methods: 1. The dimensions of the cells were determined by microscopic examination using a calibrated eyepiece. From these experimentally determined dimensions, the average volume of the cells from a given population was calculated. The number of cells in suspension was determined using a hemacytometer. Total cell volume was computed by multiplying the experimentally determined number of cells by the average volume of a cell. Calibrated pipettes were used to determine the volume of the suspending fluid. 2. Volume concentration was determined optically using colorimetric and nephelometric methods. The value $p = 0.0036$ is considered accurate to $\pm 5\%$.

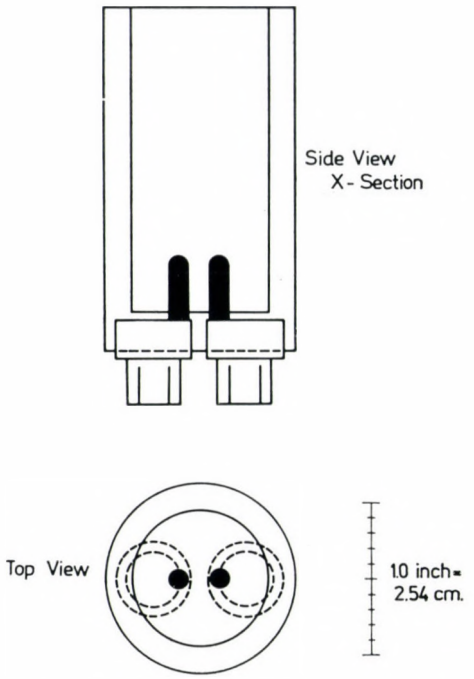

Fig. 7. Sample chamber for the R—X Meter

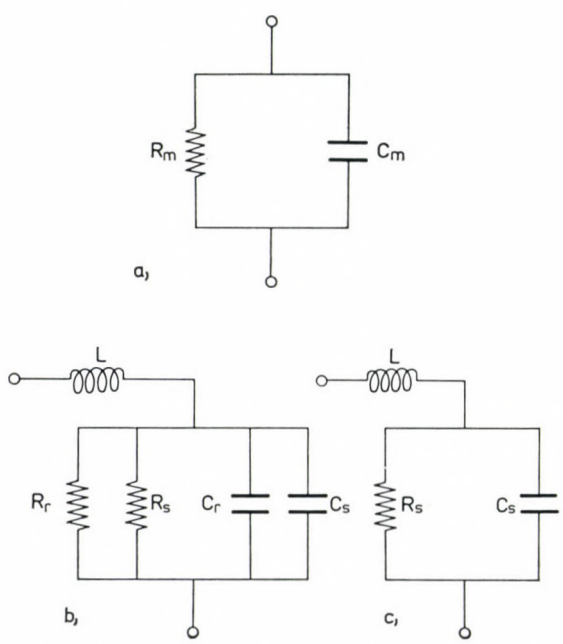

Fig. 8. Circuit equivalents at R—X Meter measuring terminals

and by the more exact method developed by TAYLOR [15] for prolate spheroids

$$\varepsilon_e = \{-(\varepsilon_w - \varepsilon - \beta\varepsilon_w + 5\beta\varepsilon) + [(\varepsilon_w - \varepsilon - \beta\varepsilon_w + 5\beta\varepsilon)^2 - 4\beta\varepsilon\ (\varepsilon_w - \varepsilon - 5\beta\varepsilon_w)]^{1/2}\}\ /2B,$$

where

ε = measured value for dielectric constant of suspension;
ε_e = dielectric constant for whole *Euglena*;
e_w = dielectric constant of water (78);
$\varkappa$ = measured value for conductivity of suspension (mhos/m);
$\varkappa_e$ = conductivity for whole *Euglena* (mhos/m);
$\varkappa_w$ = conductivity of suspending aqueous medium (variable) (mhos/m);
p = volume concentration of cells = 0.0036;
β = p/3 = 0.0012.

After the suspension of whole cells had been measured, the *Euglena* were sonicated to destroy the cells and the sample remeasured. The intent was to obtain an estimate of the electrical properties of the cytoplasm. Because of the low volume concentration of the cells and the concomitant high dilution of the cellular contents in the suspending fluid, only a rough estimate was possible.

It was not possible to increase the volume concentration of the cells, since high concentration produced a high capacitance for the chamber that was beyond the balance capabilities of the bridge in the R—X Meter.

SAITO et al. [11] have presented a theoretical treatment of orientation and turnover frequencies for ellipsoidal particles subjected to the influence of electromagnetic fields. When dielectric dispersion occurs, as indicated by the data presented in Fig. 9, a dimensionless energy-related quantity may be used to quantitate orientation. This is given by the relation

$$u_i = A_i + B_i\ (k_i^2 + e_i^2\omega^2)^{-1}, \qquad (i = a \text{ or } b)$$

where:

$A_i = (\varepsilon_0/e_i)\ (\varepsilon_m - \varepsilon_p)$
$B_i = (\varkappa_m\varepsilon_p - \varkappa_p\varepsilon_m)\varepsilon_0[2\varkappa_m\varepsilon_m(1 - L_i) + (\varkappa_m\varepsilon_p + \varkappa_p\varepsilon_m)L_i]/\varepsilon_m e_i$
ε_0 = permittivity of free space, 8.85×10^{-12} Fd/m;
ε_m = dielectric constant of medium in which ellipsoidal particles are suspended; ($\varepsilon_m = \varepsilon_w = 78$ for work reported herein);
ε_p = equivalent dielectric constant of ellipsoidal particle ($= \varepsilon_e$);
ω = radian frequency, rad/sec;
$k_i = \varkappa_m - (\varkappa_m - \varkappa_p)L_i$
$e_i = \varepsilon_0[\varepsilon_m - (\varepsilon_m - \varepsilon_p)L_i]$
$\varkappa_m$ = conductivity of suspending medium, mhos/m ($= \varkappa_w$);
$\varkappa_p$ = equivalent conductivity of particles, mhos/m ($= \varkappa_e$);
L_i is a geometry factor and is evaluated below.

Euglena are essentially prolate spheroids and for the population used in this work, the axial ratio is given by a : b : c = 5.4 : 1 : 1. Since b = c, L_i has only two values, one relating to each axis (a or b). For the condition a > b = c,

$$L_a = (b^2/2a^2e^3)\ (-2e + \ln[(1 + e)/(1 - e)])$$
$$L_b = (b^2/2a^2e^3)\ (e/(1 - e^2) - 1/2 \ln[(1 + e)/(1 - e)]),$$

where:

$e = [1 - (b/a)^2]^{1/2}$
a = half-length of major axis (32.5 microns in this case);
b = radius of cells (6.02 microns in this case).

Since *Euglena* exhibit a cell membrane (pellicle), the problem is complicated by the fact that we must consider an ellipsoid with a shell. SAITO et al. [11] have done this and have shown that a biological cell with a surrounding membrane can be considered in terms

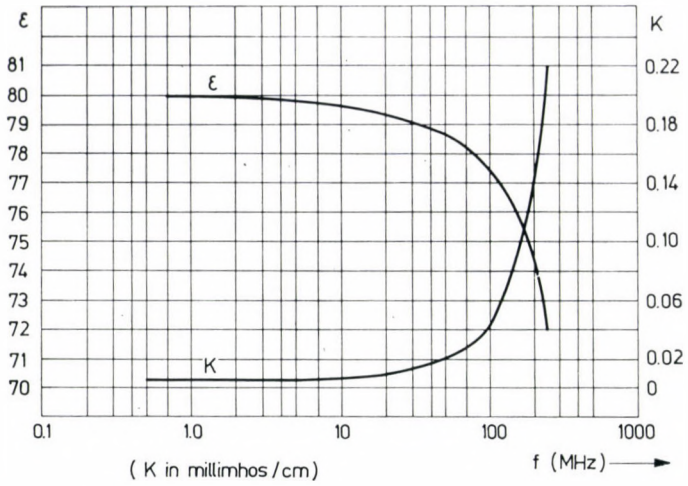

Fig. 9. Smoothed data for conductivity and dielectric constant of *Euglena* suspension

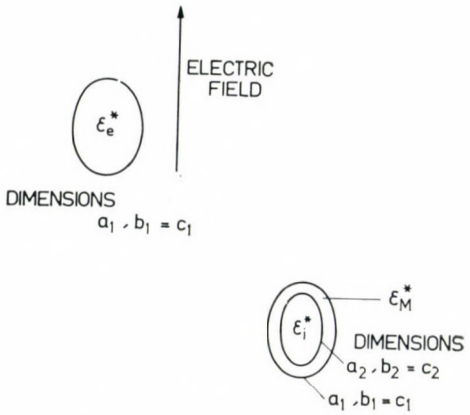

Fig. 10. Equivalent ellipsoids

of an equivalent ellipsoid without a shell. The geometry is indicated in Fig. 10. Generally in biological cells, the thickness of the membrane is much smaller than the radius or principal axis of the cell. With reference to Fig. 10 for dimensions, if the relation

$$\tau = a_1^2 - a_2^2 \ll b_2^2 = c_2^2$$

exists, then the desired equivalence between the two ellipsoids can be developed. For *Euglena*, $a_1 = 32.5\ \mu$, and the membrane thickness $(a_1 - a_2)$ is on the order of 10 nm, thus the condition is easily satisfied.

The equivalence between ellipsoids with and without a shell (membrane) is given by SAITO et al. [11]

$$\bar{\varepsilon}_e^* = \varepsilon_M^* \left[(2 - \tau M)\varepsilon_i^* + \tau M \varepsilon_M^*\right] / \left[\tau L_X \varepsilon_i^* + (2 - \tau N)\varepsilon_M^*\right],$$

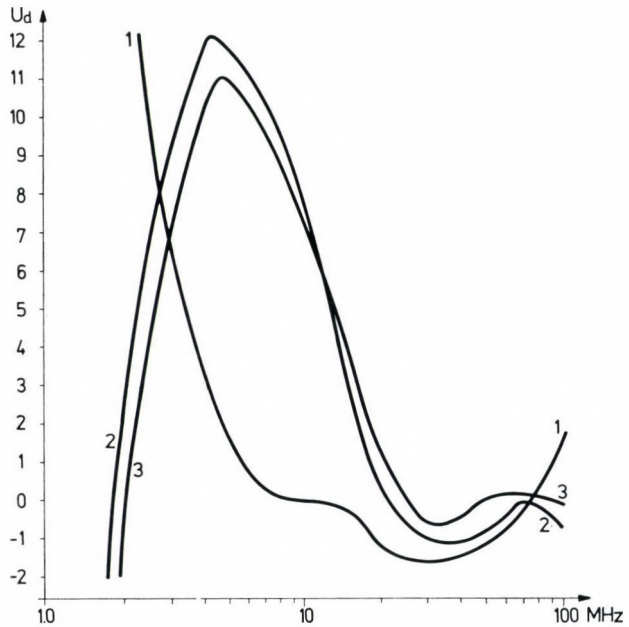

Fig. 11. Typical values of u_d as a function of frequency as determined experimentally for *Euglena*. $\varkappa = 0.8, 0.9, 1.0$ millimhos/cm respectively for curves 1, 2, 3. $5 < \varepsilon_M < 15$

where:

$\mathbf{M} = 1/a_2^2 + 2/b_2^2 - 1/x_2^2$
$\mathbf{N} = 1/x_2^2$ prolate spheroids, $a > b = c$;
$x_2 = a_2, b_2$ depending upon the direction of the impressed field;
τ = membrane thickness in direction of impressed field;
$L_x = L_a$ or L_b;
$\bar{\varepsilon}_e^*$ = complex dielectric constant of equivalent ellipsoid;
ε_i^* = complex dielectric constant of internal portion of ellipsoid;
ε_M^* = complex dielectric constant of membrane (shell).

$$\bar{\varepsilon}_e^* = \varepsilon_e - j\varkappa/(\omega\varepsilon_0)$$

with similar expression for ε_i^* and ε_M^*.

Experimentally, $\bar{e}^*$ is the quantity measured in terms of ε and $\varkappa$ as shown in Fig. 9. Thus

$$\bar{\varepsilon}_e^* = \varepsilon_e - j\varkappa_e/(\omega\varepsilon_0)$$

in terms of our previous notation.

Normally biological cells have a membrane which can be characterized as having a capacitance of 1 μfd/cm^2, [12], and a conductance of 10 millimhos/cm^2. The *Euglena* in this study had an average surface area given by

$S = 2\pi b^2 + 2\pi ab[(1/e') \sin^{-1} e'] \sim 1.9 \times 10^{-9} m^2$,
where $e' = (a^2 + b^2)^{1/2}/a$.

A rough calculation based upon $C_{sur} = 1$ μfd/cm^2 and $\tau = 10$ nm yields

$$10 < \varepsilon_M < 15.$$

Based upon this information, ε_i^* could be evaluated by solving Eq. (1) for ε_i^* in terms of $\bar{\varepsilon}_e^*$ and then taking the real and imaginary parts to obtain ε_e and $\varkappa_e$.

Data analysis

Fortran IV programs were developed to carry out the various data correction procedures and the calculations of ε_e and $\varkappa_e$ from the measured data. A final set of programs was written that calculated u_a, u_b, and $u_d = u_a - u_b$ (to find the energy crossover points = turnover frequencies), as a function of frequency. A sign change in u_d denotes a turnover frequency. Some typical plots of u_d *vs.* frequency are presented in Fig. 11.

Results

Various parameters in Eq. (1) were varied to determine the sensitivity of the computations for the energy functions and u_d. It was found that these functions and their difference, u_d, were quite insensitive to changes in the membrane properties of the *Euglena*. As a check, ε_M^* was varied by several orders of magnitude with only slight resultant changes in the energy functions. Variation of the axial ratio by $\pm$ 10% had insignificant effect upon the u's. Axial ratio is a quantity which can be measured quite accurately. Variation of the membrane thickness by 50% did not show any significant changes in the energy functions. When the *Euglena* parameters ε_e and $\varkappa_e$, and the medium parameters ε_m^* were varied, significant changes in the energy functions resulted. As to be expected, the turnover frequencies shifted radically. The parameters to which the computations were most sensitive were the conductivity $\varkappa_m$ of the suspending fluid, and ε_i^*, the complex permittivity of the cytoplasm. The dielectric constant of the suspending fluid was maintained at 78, the value for water.

Using Eq. (1) as developed in SAITO et al. [11], it was not possible to match exactly the experimentally observed turnover frequencies, as shown in Fig. 6 (A), to those predicted theoretically. Generally speaking, the agreement was good, considering the low sample concentration and the resultant data reduction processes which were necessary to convert the raw data to values for $\varkappa_e$ and ε_e. Our theoretically determined turnover frequencies, based upon the experimental data are shown in Fig. 6. Curve A is the experimental data. Curve B is the turnover frequency plot for *Euglena* modeled as homogeneous ellipsoids without shells. Curve C resulted when the shell model was used (Eq. 1). Curve C is quite similar in profile to curve A, but is shifted to the right by a factor of 1.4 in conductivity. Since Eq. (1) is quite sensitive to $\varkappa_e$, ε_e ,and $\varkappa_m$, small errors in calculating $\varkappa_e$ and ε_e, or in measuring $\varkappa_m$ could easily be responsible for the lack of exact agreement. In addition, the pellicle of the *Euglena* may not be a true shell electrically, which could also account for the discrepancy. Some typical energy function curves are shown in Fig. 11.

It should be noted that a general ellipsoid exhibits three turnover frequencies, one for each of the three axes, assuming $a > b > c$. Since for prolate spheroids, $a > b = c$, only two turnover frequencies are observed. Curve (3)

in Fig. 11, however, does exhibit three crossover points: 2.2 MHz, 25 MHz, 85 MHz. This may represent an artifact in the data, or it may suggest that the *Euglena* used in this study were not exactly prolate spheriods and the condition $a > b > c$ holds. We did not observe a second rotation relative to the b—c axes for *Euglena*, but such a rotation quite possibly could have occurred and have gone unnoticed. This rotation would have been very slight as b and c are nearly identical. If a second rotation did occur, this could also account for the lack of agreement between curves A and C in Fig. 6. We did observe three axis displacements for avian erythrocytes which are true ellipsoids ($a > b > c$) [4].

Several other theoretical hypotheses were tested with regard to orientation, [6, 9], but were not found satisfactory for this study. OGAWA's work was not directly applicable as his results were developed on the basis of a crossed field situation which we did not have experimentally. GRUZDEV's formulation did not yield the results which we observed experimentally with respect to *Euglena* orientation.

Discussion

The orientation of living elongate cells by *ac* fields is shown herein to be reasonably accounted for by a theoretical treatment devised by SAITO et al. [11]. This agreed fairly well with our experimental results when tested in a computer program. Orientation is a field-induced force effect, a direct result of the field acting on suspended particles with dielectric properties different from those of the suspending medium.

Early observations that only living cells oriented both with and across fields in a frequency-dependent manner, while nonliving particles oriented only with the field, are now explained by the difficulty in finding nonliving particles that were lossy dielectrics with intermediate conductivity. The inert particles tested were good insulators (plastic) or good conductors (metal) while only biologic material was of intermediate conductivity.

Both frequency-independent and frequency-dependent behavior of particles result from the physical interaction between the *ac* field and the suspended particle. How particles behave depends upon the physical and electrical nature of the particles and their environment, as well as upon the frequency and the strength of the impressed field. We have seen no evidence of tropic or indirect effects in addition to or competing with the electrical-physical effect. Our observations, however, were not designed to detect such effects and we can not rule out possible trophic effects at low field intensities or at the fringes of fields.

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STUDIES ON ADENOSINE TRIPHOSPHATASE ACTIVITY OF RAT CARDIAC MYOSIN IN ISOPROTERENOL-INDUCED CARDIAC HYPERTROPHY

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Abstract

Ca^{2+}- and K^+-activated ATPase activity of cardiac myosin from normal and hypertrophied rat hearts was investigated. Cardiac hypertrophy was induced by isoproterenol treatment. A nearly 40% increase in heart mass was seen after seven consecutive days of isoproterenol injection (5 mg/kg) as determined by either heart weight expressed as per cent of body weight or by dry heart weight and total protein content. The measurement of ATP hydrolysis revealed that cardiac myosin from isoproterenol-treated rats had a significant decrease ($P < 0.01$) in Ca^{2+}-activated ATPase activity at low ionic strength (0.05 *M* KCl) in the presence of 5 and 10 *mM* Ca^{2+}. In contrast, in a high ionic strength medium (0.50 *M* KCl) the K^+- and Ca^{2+}-activated ATPase activity of myosin prepared from hypertrophied myocardium remained unchanged. Comparative analysis of protein present in the light chains of myosin showed no alteration in the proportion of LC_1 to LC_2 in the myosin from hypertrophied hearts, however, a decrease in the absorption of myosin in the u.v. region was observed. On the basis of our results one can hypothesize that there may be some conformational change in the myosin molecule from hypertrophied myocardium, thereby modifying both Ca^{2+}-sensitivity and ATPase activity at a low KCl concentration.

Introduction

It is well-known that increased work load of the heart, — besides the activation of cardiac protein synthesis [11, 12], — leads to some changes in the specific ATPase activity of myocardial contractile proteins [1, 11, 13, 20]. Intermittent haemodynamic loading of rat myocardium due to swimming training for several weeks resulted in a significant increase in specific ATPase activity of myosin [10]. CONWAY et al. [4] found depressed ATPase activity for myosin from failing hearts, whereas elevated ATPase activity was obtained for myosin from non-failing hypertrophied myocardium of dogs.

Extensive studies on the properties of myosin from fast and slow skeletal and cardiac muscles of the same species revealed differences in ATPase activity [21]. The rate of ATP hydrolysis of myosin was found to be in a close correlation with the speed of muscle contraction [2, 5]. Differences in ATPase activity were reflected in the different pattern of light chains of myosin from heterologous muscles [21]. It has been suggested that these differences are involved in the control of ATPase activity of the molecule [6].

Injection of isoproterenol (IPR) is a potent stimulus for myocardial RNA synthesis [26]. The increase in RNA synthesis is the primary event leading to an increased protein synthesis and subsequent myocardial hypertrophy [3, 22]. The present work was undertaken to study that the IPR-induced increase in myocardial proteins resulted in alteration in both specific ATPase activity and subunit structural aspects of myosin.

Materials and methods

Male Wistar rats with initial body weights in the range of 235 to 275 g were used in these experiments. Care was taken to ensure similar initial body weights of rats in the control and treated groups. Cardiac hypertrophy was induced by daily i.p. injections of DL-Isoproterenol hydrochloride (Sigma) in a dose of 5 mg/kg, for seven days. The control animals were injected in each case with the diluent (isotonic saline).

The rats were killed by decapitation approximately 24 h after the last injection. The beating heart was immediately removed and placed in 50 ml of ice-cold water. Deionized water (resistivity above 2 MΩ) was used for all steps of experiments. The blood vessels and the atria were trimmed away from the hearts. Then the ventricles were opened and washed again in 50 ml of ice-cold water. Finally the ventricles were blotted dry, weighed and minced with scissors.

For the determination of ventricle dry weight and total protein, an aliquot of the minced ventricular tissue was homogenized in water to a 20% (w/v) solution. 0.5 ml aliquot of the homogenate was dried to a constant weight under infrared lamp and stored in an exsiccator. The protein was extracted from the heart according to the procedure of WANNEMACHER et al. [24] and determined with the biuret method [9].

Preparation of myosin

All manipulations for myosin isolation and purification were carried out at 0—4 °C. Since no difference was found in Ca^{2+}-activated ATPase activity and the low molecular weight fragment of myosin from right and left ventricles of rat hearts [8, 15], we isolated the myosin from the right and left ventricles together. The minced ventricular tissue of five hearts were pooled and homogenized in ten volumes of a Guba–Straub solution (0.30 *M* KCl — 50 *mM* K_2HPO — 100 *mM* KH_2PO_4 — 1 *mM* $MgCl_2$ — 10 *mM* $Na_4P_2O_7$, pH 6.9). The homogenization and extraction was performed for 15 min. The following steps of purification were carried out according to PERRY [16]. The myosin obtained was dialysed against 0.15 *M* phosphate buffer, pH 7.5 and purified on DEAE Sephadex A-50 [18]. A typical absorbance curve was obtained during chromatography of cardiac myosin from drug- or diluent-treated rats. Before chromatography the 280/260 ratio of myosin preparations was 0.98—1.05 for control hearts and 0.99—1.02 for IPR-treated hearts. After chromatography, this ratio had increased in the myosin peak and was 1.19—1.39 for control and 1.36—1.46 for IPR-treated hearts.

Measurement of ATPase activity

The standard conditions for ATPase assay was 0.50 *M* KCl or 0.05 *M* KCl and 50 *mM* Tris hydrochloride, pH 7.5, 5 *mM* ATP, 5 *mM* Na azid and 0.4 mg myosin. Ca^{2+}-activated ATPase of myosin was measured in the presence of 1, 5 and 10 *mM* Ca^{2+}. For K^+-activated ATPase the Ca^{2+} was replaced with 5 *mM* EDTA and 0.50 *M* KCl. Incubation was carried out for 15 min at 23 °C in a final volume of 2 ml. The reaction was stopped by 2 ml of 15% trichloroacetic acid. After filtration, the inorganic phosphate liberated was assayed according to the method of TAUSSKY and SHORR [23]. The protein concentration was determined by micro-Kjeldahl method.

To control the purity of the myosin preparation, the Mg^{2+}-ATPase was measured in the presence of 1 *mM* Mg^{2+} and 0.50 *M* KCl. Using these conditions we never obtained values superior to 50 nmol P_i/mg myosin/minute for our myosin preparations.

Polyacrylamide gel electrophoresis was carried out as described by WEBER and OSBORN [25]. The protein absorption curves were registered on a "Spectromom 202" apparatus in the u.v. region.

Statistical analysis of the results was made according to Student's t test.

Results

Changes in the parameters related to cardiomegaly are presented in Table 1. The results obtained show that the IPR-treated rats did not gain significantly less body weight than the control animals. The per cent increase in the heart mass, — calculated either on the basis of absolute heart weight or by determining the heart weight as percentage of initial or final body weight — was significantly different ($P < 0.001$) from controls. Cardiomegaly was also assessed by measuring dry ventricle weight and total ventricular protein. A similar increase (+ 39%) was found in these parameters for IPR-treated rats as seen in the case of absolute heart weight (Table 1).

The results of ATP hydrolysis measurements are summarized in Tables 2 and 3. Table 2 shows the Ca^{2+}-activated ATPase activity of cardiac myosin at high ionic strength (0.50 M KCl). We found that with an increase in the Ca^{2+} concentration of the incubation medium activation of ATPase activity of myosin from normal or hypertrophied hearts became more pronounced (Table 2). The Ca^{2+}-activated ATPase activity of myosin measured at low ionic strength (0.05 M KCl) demonstrated that in the presence of $1mM$ Ca^{2+} no difference was found in ATPase activity of the myosin isolated from control

Table 1

Assessment of cardiomegaly after seven days of isoproterenol treatment

	Control (n = 45)	Isoproterenol-treated (n = 40)	Difference per cent	P
Initial body weight (g)	239.42 ± 4.480	239.42 ± 4.620	0	—
Final body weight (g)	269.42 ± 8.100	265.00 ± 6.390	−1.64	N. S.
Heart weight (mg)	747.53 ± 14.750	1043.82 ± 25.800	+39.64	< .001
Heart weight as % of initial body weight	0.31 ± 0.002	0.43 ± 0.003	+38.71	< .001
Heart weight as % of final body weight	0.28 ± 0.002	0.39 ± 0.009	+41.07	< .001
Ventricle dry weight (mg)	141.64 ± 7.070	197.64 ± 8.090	+39.54	< .001
Total protein (mg)	91.31 ± 5.720	127.20 ± 6.520	+39.31	< .010
Ventricle dry weight as % of wet heart weight	19.74 ± 0.790	20.07 ± 0.770	+1.70	N. S.

Data are expressed as mean ± S.E.M.
N.S.: not significant
n = number of animals

Table 2

Ca^{2+}-activated ATPase activity of heart myosin in a high ionic strength medium (0.50 *M* KCl)

	Myosin ATPase in nmol P_i/mg myosin/minute		
	1 *mM* Ca^{2+}	5 *mM* Ca^{2+}	10 *mM* Ca^{2+}
Control	178.4 ± 17.9 (12)	383.8 ± 23.4 (11)	434.5 ± 22.2 (12)
Isoproterenol-treated	170.6 ± 27.3 (9)	398.8 ± 18.6 (8)	429.6 ± 14.1 (14)
P	N.S.	N.S.	N.S.
ATPase change (%)	−4.38	+3.90	−1.37

Conditions: pH 7.5, 23 °C, myosin 2 mg/ml
Each value represent the mean ± S.E.M. The number of individual determinations made on pooled hearts is in parentheses
N.S.: not significant

Table 3

Ca^{2+}-activated ATPase activity of heart myosin in a low ionic strength medium (0.05 *M* KCl)

	Myosin ATPase in nmol P_i/mg myosin/minute		
	1 *mM* Ca^{2+}	5 *mM* Ca^{2+}	10 *mM* Ca^{2+}
Control	190.1 ± 14.5 (12)	544.5 ± 20.8 (13)	628.6 ± 25.8 (13)
Isoproterenol-treated	167.5 ± 15.0 (14)	428.9 ± 35.1 (13)	516.1 ± 28.0 (13)
P	N.S.	$.001 < P < .01$	$.001 < P < .01$
ATPase change (%)	−11.92	−21.25	−17.90

Conditions: pH 7.5, 23 °C, myosin 2 mg/ml
Each value represent the mean ± S.E.M. The number of individual determinations made on pooled hearts is in parentheses
N.S.: not significant

or IPR-treated rat hearts. In contrast, at 5 and 10 *mM* Ca^{2+} a significant ($P < 0.01$) difference was observed between control and treated groups. Myosin from IPR-treated rat hearts showed a significantly lower ATPase activity (Table 3). The K^+-activated ATPase activity of myosin from hypertrophied hearts remained unchanged.

Comparative analysis of protein present in the light chains of myosin was made by electrophoresis on polyacrylamide gel. Figure 1 shows the pattern of the chains of myosin from control and IPR-treated rat hearts. There was no significant difference in the electrophoretic pattern nor in the proportion of LC_1 to LC_2 of myosin prepared from control or hypertrophied hearts (Fig. 1).

The optical characteristic of myosin from hypertrophied hearts was decreased throughout in the u.v. region (Fig. 2). The decrease in the extinction

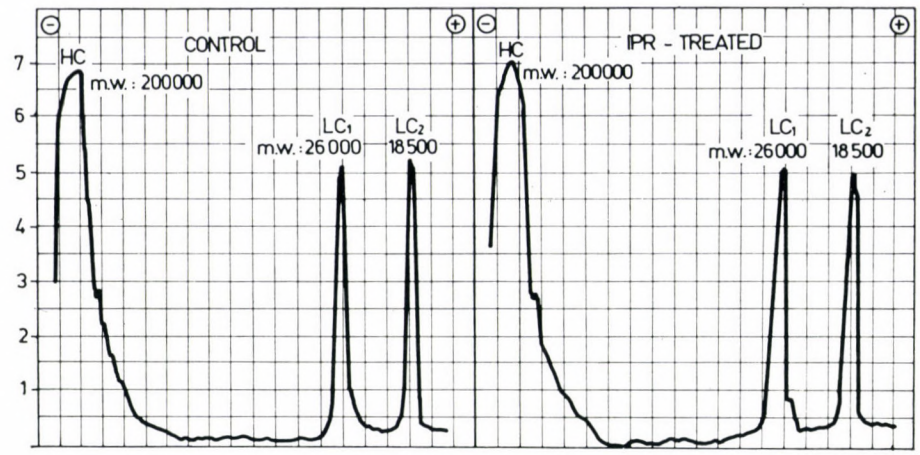

Fig. 1. Electrophoresis of rat cardiac myosin with SDS in 7.5% polyacrylamide gel

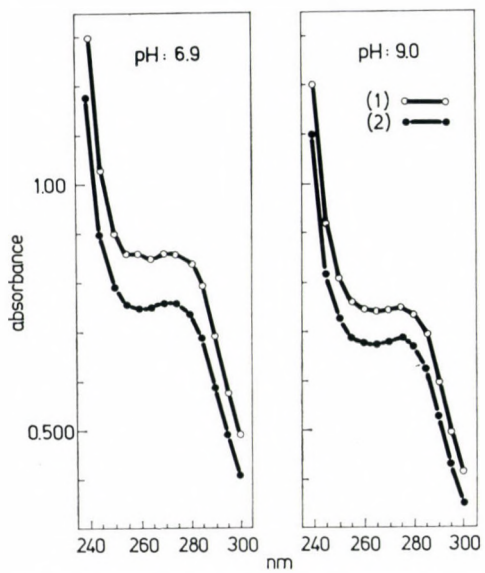

Fig. 2. The effect of pH on the u.v. absorption of myosin solution; 0.4 mg/ml protein concentration in 1 *M* KCl solution. (1) Indicates control and (2) isoproterenol-treated hearts

of the protein solution was not accompanied by a shift in the peak of maximum absorption (270 to 275 nm). An increase in the pH of the myosin solutions from 6.9 to 9.0 produced a moderate decrease in the extinction of both the normal and hypertrophied myosin without any displacement in the peak of maximum absorption (275 nm).

Discussion

Administration of IPR produces an increase in cardiac mass in response to increased work demand in animals [17]. Since after seven days of IPR treatment the cardiac mass was significantly increased [22], all analysis described here were carried out with animals treated for seven days.

Acute myocardial hypoxia and the resultant necrosis produced by high doses of IPR (80 mg/kg, once daily, for two days) induces formation of myosin aggregates which have a significantly lower ATPase activity compared with monomeric myosin [14]. The dose of IPR used in this study (5 mg/kg) did not cause macroscopically detectable myocardiopathies [3]. Chromatography of myosin from hypertrophied hearts showed that the IPR treatment did not produce any myosin aggregation since only the monomeric form was present. This suggests that neither the production of micronecroses nor the consequential formation of myosin aggregates was responsible for the observed decrease in ATPase activity of myosin following IPR treatment.

In spite of the identity of chromatographic pattern of myosin from IPR- or diluent-treated rats, the myosin prepared from hypertrophied hearts differed from the control, *viz.*, a significant decrease in the Ca^{2+}-activated ATPase activity could be observed. A similar degree of inhibition was found by GORDON et al. [7] in the ATPase activity of actomyosin in IPR-induced cardiomegaly.

The development of experimental cardiac hypertrophy is accompanied by activation of the synthetic processes in the heart (*e.g.* increased nucleic acid and protein synthesis) and gradual adaptation of the myocardial contractile system [11, 12, 13].The changes in theATPase activity ofmyocardial contractile proteins during the development of cardiac hypertrophy may be understood only if they are regarded as a function in severity of heart overload at a time when the tissue is studied. MEERSON [11] has formulated the concept of the fundamental stages and mechanism involved in the process of hyperfunction, hypertrophy and heart failure. On the basis of MEERSON's concept, OGANESSYAN et al. [13] in their study on cardiac hypertrophy in the rabbit following aortic stenosis have shown that at the stage of acute cardiac overloading (on the 7th to 10th days following the imposition of stenosis on the rabbits' aortas) there was a significant decrease in both the actomyosin ATPase activity and the contraction of glycerinated fibrils from the left ventricle. In the process of the development of relatively stable hyperfunction and hypertrophy of the myocardium — on the 28th to 30th days — actomyosin enzymic activity remained decreased, whereas contraction of glycerinated fibrils was almost completely restored. At the stage of stable cardiac hyperfunction (in the third month) the ATPase activity of actomyosin and the contraction of glycerinated fibrils was slightly increased.

We should like to stress that IPR-induced cardiac hypertrophy develops more rapidly than hypertrophy following aortic stenosis. In our opinion, cardiac hypertrophy induced by seven consecutive days of IPR treatment may be in the process of the development of relatively stable hyperfunction and hypertrophy of the myocardium, when ATPase activity is still decreased. At this stage the decrease in the Ca^{2+} activation of the myosin molecule might represent an adaptive mechanism to acute myocardial overloading to allow increased wall tension to be sustained at a lower energy cost. This adaptation does not require necessarily an alteration in the primary structure of the molecule [19]. However, the difference in the u.v. absorption of the normal and hypertrophied myosin may reflect some alteration in the secondary or tertiary structure of the molecule, which is manifested in the decreased Ca^{2+} sensitivity of myosin.

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EFFECT OF NEURECTOMY ON NUCLEASE ACTIVITY IN SKELETAL MUSCLES OF RATS

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Abstract

Author followed up the activity of the three enzymes involved in the catabolism of nucleic acids — acid deoxyribonuclease (DNase II), alkaline ribonuclease (RNase I), and acid ribonuclease (RNase II) — in the denervated gastrocnemius and soleus muscles of rats for 28 postoperative days. The activity of both acid nucleases increased in both types of denervated muscles, compared with the respective controls. Up to the 14th postoperative day, the activity excess of both acid nucleases was more significant in the m. gastrocnemius than in the m. soleus. The RNase I ran below the control activity during the whole period in the m. soleus and up to the 14th day in the m. gastrocnemius. The role of nucleases and nuclease inhibitors in the changes of nucleic acid catabolism in neurogenic muscular atrophies is discussed.

Introduction

The quantitative changes of total nucleic acid in the neurogenic atrophy of skeletal muscles has been studied by several authors. The results are contradictory. Mandel et al. [22], Hearn [12], McCaman and McCaman [23], further on, Hogan et al. [13] have reported an approximately constant absolute amount of DNA in limb muscles of mammals, whereas Hollósi et al. [14, 15, 16], Gutmann and Zák [11], and Shchesno [27] reported an increase of the total DNA level in skeletal muscles.

Data on the changes in total RNA amounts are also contradictory. Gutmann and Zák [11] reported a constant total RNA amount during the first months of neurogenic atrophy of mammalian limb muscles, while Hollósi et al. [14, 15, 16], Lissák et al. [21], Graff et al. [10], and Shchesno [27] found decreased RNA levels.

Few papers have dealt with the catabolic background of the nucleic acid changes in denervated muscles. Weinstock and Lukács [30] observed an increased DNase and RNase activity in denervated limb muscles of rabbits. An increased RNase activity has been reported by Pollack and Bird [25] in denervated rat muscles, by Epstein [3] in denervated pectoral muscles of pigeons, and by Goldspink et al. [8] in denervated red and white muscles of rats.

In our present work, we followed up the activity changes of acid DNase (DNase II; EC 3.1.4.6), of alkaline RNase (RNase I; EC. 3.1.4.22) and of acid RNase (RNase II; EC. 3.1.4.23) in the denervated gastrocnemius and soleus muscles of rats.

Materials and methods

Experimental animals. Male CFY albino rats (*Epimys rattus* var. albino) 250—350 g in body weight were used. The animals were kept on the standard rat food of the Laboratory Animal Centre, Gödöllő.

Operation techniques. A slight pentobarbital (nembutal) narcosis (8 mg/100 g body weight i.p.) was applied, the n. ischiadicus was isolated on the right back limb at the height of the trochanter, and a 8—10 mm section of the nerve was removed. On the contralateral side, a false operation was performed, *viz.*, the nerve was isolated, but not removed. The wounds were closed with surgical silk (2—0 silk). On each of the 3rd, 7th 14th and 28th postoperative days, three or four animals were killed by ether narcosis and their soleus (a red muscle) and gastrocnemius muscles (a white muscle) were removed (see FRANZINI and PELLEGRINO [5], YELLIN [32]). The muscles removed from the contralateral limbs served as controls.

Preparation of muscle homogenates. Cooled muscles were cut into small pieces and homogenized in 15 volumes of ice-cold distilled water in a Potter-Elvehjem type glass homogenizer (3 × 1 min cooled with ice-cold water).

The interstitial tissue was removed by filtering the homogenate through several layers of gauze. Then the homogenate was stored at 3 °C until used within 2 h.

Quantitative determination of non-collagen protein (NCP). One volume of muscle homogenate mixed with 9 volumes of 0.05 *N* NaOH was incubated at room temperature for 18 h. Hereafter, it was centrifuged (1050 *g*, 10 min) and the protein concentration of the supernatant was determined by the method of GORNALL et al. [9]. Bovine albumin (BDH Chemicals Ltd., Poole, England) was used as standard.

Assay of nuclease activity was based on the UV spectrophotometric assay of the acid-soluble oligonucleotides released from highly polymerized nucleic acids. Highly polimerized DNA Na salt ex calf thymus (Koch-Light Lab. Ltd. Colnbrook, England) and highly polymerized yeast-RNA Na salt (BDH Chemicals Ltd. Poole, England) were used as substrates. As commercial nucleic acid preparations may contain a significant amount of oligo- and mononucleotides (see: FIERS [4]) the substrates were purified by dialysis; the yeast-RNA according to POLLACK and BIRD's [25], the thymus DNA according to ZAMENHOF's [33] method.

The nuclease activity was measured in a final volume of 0.5 ml in glass tubes (100 × 13 mm), these were kept in an ice bath until the incubation was started. Acid-DNase activity was assayed by a modified test of KOWLESSAR et al. [18] and GEORGATSOS [6] in the following test system:

acetate buffer 0.05 *M* at pH 5.6	0.1 ml
EDTA acid diNa salt 0.05 *M*	0.1 ml
DNA—Na solution 10 mg/ml	0.1 ml
muscle homogenate	0.2 ml

RNase activity was determined in the following test system:

	Alkaline RNase	Acid RNase
Tris-HCl buffer 0.05 *M* at pH 7.5	0.2 ml	—
Acetate buffer 0.05 *M* at pH 5.5	—	0.2 ml
RNA—Na solution (10 mg/ml)	0.1 ml	0.1 ml
Muscle homogenate	0.2 ml	0.2 ml

The test system was incubated at 37 °C for 180 min. Hereafter, the reaction was stopped by the addition of 3 ml ice-cold 10% perchloric acid + 0.1% uranyl acetate and vigorous shaking. The samples were kept in an ice bath for 30 min and centrifuged (1500 *g*, 20 min, 4 °C).

For the assay of DNase activity a 4-fold dilution, for the determination of RNase activity a 10-fold dilution, of the supernatant was made with bis distilled water, the extinctions were read at 260 nm in 10 mm quartz glass cuvettes, in a Beckman DB spectrophotometer. All samples were run duplicate. The enzyme activity was expressed as the difference between the test sample and the blank solution; Δ E260 nm/mg NCP/3 h. STUDENT's *t* test was used for calculating statistical significance [28].

Results

The acid DNase (DNase II) activity was significantly higher in the denervated muscles than in the contralateral control muscles (Table 1). The highest specific enzyme activity was measured on the 14th postoperative day

Table 1

*Activities of acid deoxyribonuclease in intact and denervated skeletal muscles**

Postoperative days	Rats, no.	M. gastrocnemius				M. soleus			
		Control muscle	Denervated muscle	Difference**	P***	Control muscle	Denervated muscle	Difference	P
3	1	0.18	0.32	+ 77.7		0.04	0.07	+ 75.0	
	2	0.17	0.38	+123.5		0.03	0.04	+ 33.3	
	3	0.21	0.35	+ 66.6		0.03	0.04	+ 33.3	
	Mean			+ 89.2				+ 47.2	
7	4	0.41	0.63	+ 53.6		0.11	0.18	+ 63.6	
	5	0.39	0.61	+ 56.4		0.14	0.23	+ 64.2	
	6	0.42	0.65	+ 54.7		0.09	0.14	+ 55.5	
	Mean			+ 54.9	$\leqslant$ 0.001			+ 61.1	
14	7	0.43	0.99	+130.2		0.12	0.25	+108.3	
	8	0.40	0.99	+147.5		0.12	0.20	+ 66.6	
	9	0.41	0.96	+134.1		0.15	0.27	+ 80.0	
	Mean			+137.2				+ 84.9	
28	10	0.39	0.85	+117.9		0.14	0.17	+ 21.4	
	11	0.40	0.89	+122.5		0.13	0.17	+ 30.7	
	12	0.38	0.85	+113.6		0.13	0.18	+ 38.4	
	13	0.39	0.86	+120.0		0.12	0.16	+ 28.0	
	Mean			+118.6				+ 29.6	$\leqslant$ 0.001

* DNase activity is expressed as ΔE 260 nm/mg NCP/3 h
** Percentage difference from contralateral non-denervated muscles
*** Significance of difference

Table 2

*Activities of alkaline ribonuclease in intact and denervated skeletal muscles**

Postoperative days	Rats, no.	M. gastrocnemius				M. soleus			
		Control muscle	Denervated muscle	Difference**	P***	Control muscle	Denervated muscle	Difference	P
3	1	1.26	0.95	− 24.6		1.36	1.36	0	
	2	1.51	1.47	− 2.6		0.53	0.48	− 9.4	
	3	1.19	0.91	− 23.5		0.45	0.49	+ 8.8	
	Mean			− 16.9	< 0.001			− 0.2	> 0.025
7	4	0.81	0.31	− 61.7		1.44	1.08	− 25.0	
	5	0.84	0.38	− 54.7		0.70	0.53	− 24.9	
	6	0.83	0.39	− 53.0		0.80	0.60	− 25.0	
	Mean			− 56.4				− 24.9	
14	7	1.58	1.05	− 33.5		0.91	0.70	− 23.0	
	8	2.51	1.79	− 28.7		0.85	0.65	− 23.5	
	9	1.61	1.01	− 37.2		0.88	0.66	− 25.0	
	Mean			− 33.1				− 23.8	
28	10	2.17	3.36	+ 54.8		1.96	1.64	− 16.3	
	11	2.10	2.38	+ 13.8		2.17	1.37	− 36.8	
	12	2.27	2.80	+ 23.3		2.03	1.75	− 13.8	
	13	2.10	2.40	+ 14.2		2.07	1.87	− 9.6	
	Mean			+ 26.4				− 19.1	$\ll 0.001$

* RNase activity is expressed as ΔE 260 nm/mg NCP/3 h
** Percentage difference from contralateral non-denervated muscles
*** Significance of difference

in both muscle types. At this time, the enzyme activity was by 50% higher in the gastrocnemius muscle compared to the m. soleus.

The alkaline RNase (RNase I) activity was lower in the denervated soleus muscles than in the control during the whole period. The m. gastrocnemius showed a more pronounced relative decrease until the 14th postoperative day (Table 2); on the 28th day the RNase activity was higher in the denervated muscle than in the control.

The acid RNase (RNase II) activity was significantly higher in the denervated muscles than in the controls during the whole period (Table 3). The surplus activity was especially large in the gastrocnemius muscles on postoperative days 7 and 14.

Table 3

*Activities of acid ribonuclease in intact and denervated skeletal muscles**

Postoperative days	Rats, no.	M. gastrocnemius				M. soleus			
		Control muscle	Denervated muscle	Difference**	P***	Control muscle	Denervated muscle	Difference	P
3	1	0.11	0.14	+ 27.2		0.11	0.17	+ 54.4	
	2	0.18	0.28	+ 55.5		0.14	0.18	+ 28.5	
	3	0.17	0.28	+ 64.7		0.14	0.18	+ 28.5	
	Mean			+ o49.1	$\ll 0.001$			+ 37.1	$\ll 0.001$
7	4	0.14	0.49	+ 250.0		0.56	1.16	+ 107.1	
	5	0.14	0.49	+ 250.0		0.56	1.17	+ 112.7	
	6	0.39	1.40	+ 258.9		0.55	1.16	+ 110.9	
	Mea			+ 252.9				+ 110.1	
14	7	0.19	0.61	+ 221.0		0.47	1.02	+ 117.0	
	8	0.15	0.61	+ 280.0		0.49	1.20	+ 144.8	
	9	0.20	0.70	+ 250.0		0.44	1.04	+ 136.3	
	Mean			+ 250.3				+ 132.7	
28	10	0.16	0.36	+ 125.0		0.51	1.24	+ 143.1	
	11	0.19	0.41	+ 115.7		0.46	1.19	+ 158.6	
	12	0.19	0.44	+ 131.5		0.50	1.11	+ 122.0	
	13	0.13	0.29	+ 123.0		—	—	—	
	Mean			+ 123.8				+ 141.2	

* RNase activity is expressed as ΔE 260 nm/mg NCP/3 h
** Percentage difference from contralateral non-denervated muscles
*** Significance of difference

Discussion

The use of an adequate reference base for characterizing biochemical changes in muscular dystrophy is a very substantial and often disputed problem. The decrease of muscular weight, the reduction of fibrillar and sarcoplasmic proteins results in increased enzyme activities calculated per unit of muscle weight or protein quantity. The increased DNA content may cover the enzyme activity increase when DNA is used as reference base. The total enzyme activity of the whole muscle may be a better model, however, this has its disadvantages also [31]. In our present work we used the non-collagenic proteins of the muscle as a reference base, which is proportional, within 5%

of error, to the amount of muscle fibres [20]. ABDULLAH and PENNINGTON [1], furtheron, GOLDSPINK et al. [7, 8] have used the same reference base in similar studies.

Few experiments have been involved with the changes of acid DNase activity in neurogenic muscular dystrophy. WEINSTOCK and LUKÁCS [30] observed an icreased DNase II activity in the limb muscles of rabbits in the early post-denervation period. The same authors reported a 5—10-fold increase of DNase II activity in nutritional muscular dystrophy in rabbits and hereditary muscular dystrophy in mice and chickens [29]. As the DNase II activity already rises significantly on the third postoperative day, furtheron, literary data [2, 24] indicate that in the early period of denervation atrophy macrophage infiltration does not occur, the role of the latter can be disregarded. DNase II, like other acid hydrolases, is a lysosomal enzyme. Assumably, neurotomy induces the increase of lysosomal enzyme synthesis.

Electron-microscopic pictures indicate that as the neurogenic muscular dystrophy pogresses, the number and size of lysosomes increases [25].

The relative and absolute increase of DNA, and the increase of DNase activity following denervation are striking. This phenomenon may be due to the increased regenerative functions of the muscle, which includes an increased DNA synthesis. Detailed investigations into this problem, *viz.*, studying the incorporation of DNA precursors are in progress.

The alkaline RNase is characterized by its extensive localization in animal cells. It occurs both in the particulate and soluble fractions of the cell and is closely related to cytoplasmic RNA [19]. Its activity is inhibited by a specific alkaline RNase inhibitor occurring in the soluble fraction [26]. The physiological role of RNase inhibitor is yet unknown [17]. KRAFT and SHORTMAN [19] presume that an increased inhibitor/RNase ratio indicates an increased RNA synthesis, ensuring a high intracellular RNA concentration, while a low inhibitor/RNase ratio is related to a decreased protein synthesis and to increased catabolic processes.

Literature is poor in data on the effect of neurogenic atrophy on alkaline RNase activity. EPSTEIN [3] observed an RNase activity increase at pH 7.5 in the denervated pectoral muscles of pigeons. We observed an RNase activity increase related to the control, only after the 14th postoperative day in the denervated gastrocnemius muscles of rats, while in the m. soleus a relative decrease was observed during the whole postoperative period. Further studies are necessary for the full understanding of the role of alkaline RNase in the RNA metabolism of atrophic muscles. Such studies may contribute to our knowledge on the role of ribonuclease inhibitors.

The acid RNase is a lysosomal enzyme. Our observations on the increase of RNase II activity in denervated rat muscles are in agreement with literary data [8, 25].

It deserves mentioning that the gastrocnemius muscles displayed an excessive RNase II activity until the 14th postoperative day, however, on the 28th day the RNase II activity was higher in the m. soleus. GOLDSPINK et al. [8] reported a higher RNase II activity in denervated red muscles of rats (m. soleus) compared to the activity in white muscles (m. extensor digitorum longus) on postoperative days 17—21.

The postdenervation increase of acid RNase activity may be due to the increased fragility and size or proliferation of the lysosomes.

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CHANGES IN THE ACTIVITY OF THE EPENDYMA IN THE INFUNDIBULAR RECESS OF THE BRAIN OF *RANA ESCULENTA* L. IN THE ANNUAL CYCLE

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Abstract

Volume of the cell nuclei of the ependyma of the infundibular recess was measured in 30 female and 30 male water frogs (*Rana esculenta* L.) obtained from their natural environment, taking into account the phase of the annual cycle. Karyometry of the ependymal cell nuclei in the infundibular recess in males and females showed statistically significant differences of volume (activity) in the annual cycle. The largest volume of nuclei of the ependymal cells in females and males was observed in the first decade of April (end of hibernation), and the smallest in the first decade of September (end of the period of active life). Activity of nuclei of cells from the infundibular recess clearly correlated with gonadal development.

Introduction

Studies on the ependyma lining the cerebral ventricles in amphibians and other vertebrates have been carried out by many authors including Adam [1], Arnold [4], Braak [5], De Waele and Dierickx [7], Dierickx and De Waele [9], Dierickx et al. [10], Fleischhauer [11, 12], Horstmann [14], Paul [27, 28, 29], and Schimrigk [34]. It has been shown that particularly the ependyma of the 3rd cerebral ventricle shows a high degree of differentiation, and that differences generally pertain to the internal structure of the cells and their metabolism [27, 33]. Differences occur mainly in tanycytes, which are characterized by a special type of contact with the cerebrospinal fluid and circulatory system [22]. According to Paul [27], the role of ependymal tanycytes consists in regulation of the composition of the cerebrospinal fluid and trasportation of some substances from this fluid to the blood and *vice versa*.

Knowles and Kumar [19], Kobayashi and Matsui [20] and Knigge and Scott [18], on the other hand, suggest that tanycytes, indirectly or directly, influence the function of the hypophysis. Of special interest in this respect are the tanycytes described by Adam [1] and Paul [27] in the infundibular recess in *Rana temporaria* L., being, according to Paul [27], modified tanycytes leading to bipolar cell forms and situated in the hypophysiotropic area of the brain [31, 32].

The aim of the present study was to determine the activity of the ependyma in the infundibular recess in the brain in *Rana esculenta* L. during the most important stages in the annual life cycle of this amphibian.

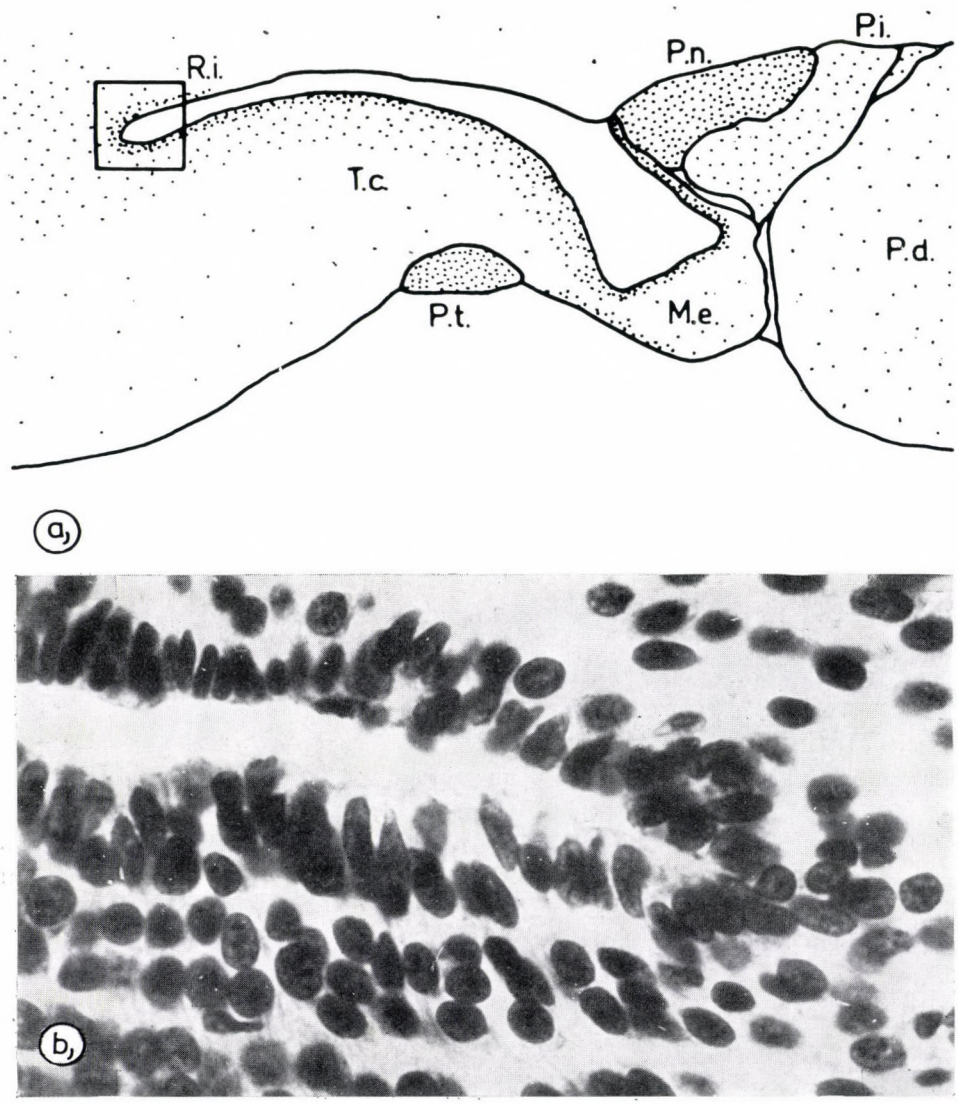

Fig. 1. A. Scheme of the hypothalamo–pituitary system in the water frog (*Rana esculenta* L.) in parasagittal section. Designations: Pd — pars distalis, Pi — pars intermedia, Pn — pars nervosa, Pt — pars tuberalis, Tc — tuber cinereum, Me — median eminence, Ri — recessus infundibularis. B. Photomicrograph of the infundibular recess (enclosed by the square in scheme A). Stained with chrome hematoxylin and floxin. ×760

Materials and methods

Experiments were carried out on 30 sexually mature female and 30 male water frogs (*Rana esculenta* L.) from six periods of the annual cycle which characterize the main stages in the life of this amphibian, *i.e.* 3rd decade of January (middle of the period of hibernation), 1st decade of April (end of hibernation), 3rd decade of May (breeding period), 2nd decade of July (middle of the period of active life), 1st decade of September (end of the phase of active life), and 3rd decade of October (beginning of hibernation). The division of the life cycle into the aforementioned phases was taken from the work of JUSZCZYK [16].

Five female and five male frogs from each phase were captured in the environment of Cracow (50°40' N, 200—220 m above sea level), selecting frogs of suitable body length. In the laboratory, the frogs were killed by decapitation, always at the same time of day to eliminate any influence of diurnal rhythm. The dissected brains were fixed in Bouin fluid and embedded in paraffin, sections 7 μm thick were stained with GOMORI's chrome hematoxylin and floxin in the modification of BARGMANN [30]. In these sections, activity of ependymal cells in the infundibular recess was determined as described by PAUL [27].

Activity was assessed on the basis of the volume of the cell nuclei, which reflects the activity of the cells [2, 3, 6, 21, 25, 35, 36]. The site of the measurements is shown in Figs 1a and 1b. Volume of cell nuclei was calculated after mesauring the long axis (L) and short axis (B) of each cell nucleus and substituting these values in the equation of PALKOVITS [26]: $L \times B^2 \times \pi/6$. One hundred nuclei of ependymal cells from the infundibular recess were evaluated in each brain, making a total of 6000 cell nuclei. Arithmetic means and standard deviations were calculated, and frequency distributions of relative volumes of cell nuclei were plotted. Significance of differences in volume of cell nuclei in females and males in each phase of the life cycle and in neighbouring phases was tested by means of STUDENT and GOSSET's "t" test. Differences were considered significant if the probability of a chance difference was equal to or less than 0.01.

Results

The largest mean volume of nuclei of ependymal cells from the infundibular recess in females (203.370 μm^3) and males (106.314 μm^3) were observed in the first decade of April, after emergence of the frogs from their winter lairs and at the beginning of the period of active life (Table 1, Fig. 2). At this time, in both sexes, a relatively high percentage of ependymal cells contain large nuclei (Tables 3, 4, Figs 3, 4).

In the following periods, the mean volume of the nuclei gradually declines until the 1st decade of September (end of active life), to 65.733 μm^3 in females, and 53.155 μm^3 in males, *i.e.* the lowest mean nuclear volume observed (Table 1, Fig. 2). At the end of the active phase of life, a relatively large proportion of the cell nuclei had small volume (Table 3, Figs 3, 4). The drop in mean volumes of cell nuclei between particular phases is invariably statistically significant (Table 2).

Starting in the 3rd decade of October (beginning of hibernation), the volume of the ependymal cell nuclei from the infundibular recess begins to rise again up to a peak of the mean volumes in the 1st decade of April, statistically significantly in both sexes (Table 2). On the whole, the mean volumes of cell nuclei for females were larger than for males in the same phase (Table 1).

Table 1

Volume of ependymal cell nuclei in the infundibular recess of the brain in male and female water frogs (Rana esculenta L.) in the annual cycle

Period of investigation	Sex	Number of specimens	Volume of ependymal cell nuclei in the infundibular recess in μm^3	
			mean	± SD
January	♀	5	165.266	± 16.35
3rd decade	♂	5	79.105*	± 6.48
April	♀	5	203.370	± 21.83
1st decade	♂	5	106.315*	± 13.16
May	♀	5	125.389	± 11.17
3rd decade	♂	5	75.384*	± 6.97
July	♀	5	99.253	± 8.75
2nd decade	♂	5	63.203*	± 7.94
September	♀	5	65.733	± 10.71
1st decade	♂	5	53.155*	± 3.94
October	♀	5	82.369	± 6.05
3rd decade	♂	5	70.688*	± 7.15

* statistically significant at $P < 0.01$

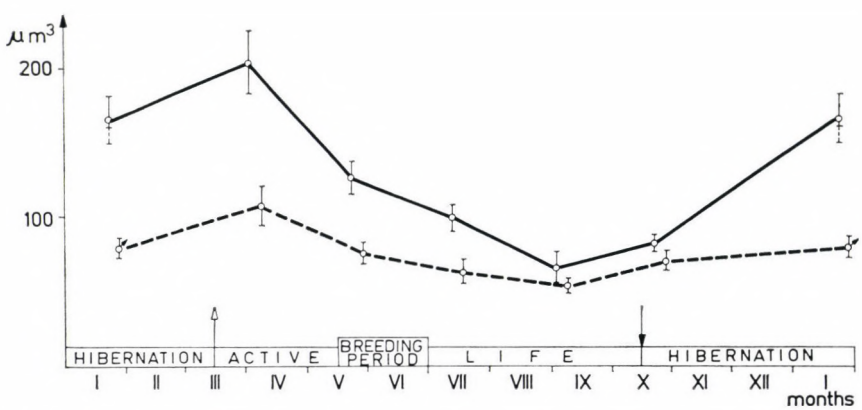

Fig. 2. Mean volume of ependymal cell nuclei in the infundibular recess in the brain of male and female water frogs (*Rana esculenta* L.) in the annual cycle

Table 2

Statistical comparison of results (t) between different phases investigated in the infundibular recess in the water frog (Rana esculenta L.)

Period of investigation	Infundibular recess	
	"t"	
	females	males
January 3rd decade		
	6.99*	9.29*
April 1st decade		
	15.91*	10.41*
May 3rd decade		
	9.20*	5.77*
July 2nd decade		
	12.10*	5.68*
September 1st decade		
	6.76*	10.75*
October 3rd decade		
	23.82*	4.36*
January 3rd decade		

* statistically significant at $P < 0.01$

Table 3

Percentage distribution of ependymal cell nuclei in the infundibular recess in the brains of female Rana esculenta L. in the annual cycle

Period of investigation	Class intervals in μm^3							
	0—50	51—100	101—150	151—200	201—250	251—300	301—350	351—
January 3rd decade	—	7.40	34.20	33.20	20.80	3.80	0.60	
April 1st decade	—	0.71	15.31	35.40	30.14	12.20	4.54	1.67
May 3rd decade	—	25.35	50.70	20.68	2.43	0.60	0.20	
July 2nd decade	1.20	55.71	38.87	4.20				
September 1st decade	27.27	68.98	3.20	0.53				
October 3rd decade	5.26	70.44	23.88	0.40				

Table 4

Percentage distribution of ependymal cell nuclei in the infundibular recess in the brains of male Rana esculenta L. in the annual cycle

Period of investigation	Class intervals in μm^3			
	0—50	51—100	101—150	151—200
January 3rd decade	8.43	70.68	18.47	2.40
April 1st decade	1.15	57.80	28.32	10.98
May 3rd decade	13.06	74.37	12.56	
July 2nd decade	27.04	64.28	8.67	
September 1st decade	46.46	48.98	4.54	
October 3rd decade	15.72	73.79	10.08	0.40

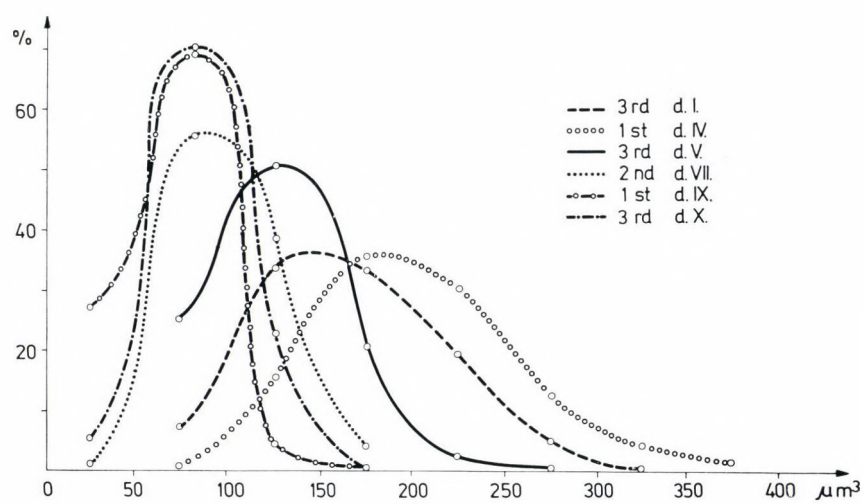

Fig. 3. Percentage distribution volume of ependymal cell nuclei in the brain of female *Rana esculenta* L. in the annual cycle

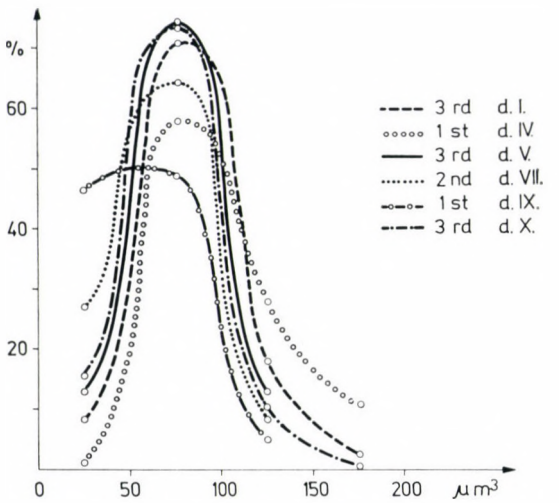

Fig. 4. Percentage distribution of ependymal cell nuclei in the brain of male *Rana esculenta* L. in the annual cycle

Discussion

Analysis of the results indicates that the mean volume of ependymal cell nuclei from the infundibular recess of the brain in *Rana esculenta* L. undergoes cyclic variation in the course of the year. A marked increase in the mean nuclear volumes in ependymal cells was found at the beginning of the period of active life, before ovulation. After that, activity of the cells declines, attaining a minimum in the terminal period of active life. From this time and throughout the period of hibernation, the activity of the ependyma increases until it attains a maximum at the beginning of the period of active life.

It has been proved experimentally that synthesis and release of gonadotropic hormones in the adenohypophysis of amphibians depends, among others, on the release of factors produced mainly by neurocytes of the pars ventralis tuberis cinerei [8, 10, 31]. According to LEVEQUE et al. [24], in some mammals releasing factors may also stem from ependymal cells of the hypophysiotropic area, which includes the ependyma of the infundibular recess. It lies in the direct neighbourhood of the pars ventralis tuber cinereum, which is the main part of the hypophysiotropic area in the amphibian brain [31, 32].

Participation of ependymal cells of the hypophysiotropic area in production of releasing factors is confirmed by changes in these cells in relation to reproductive function [13, 15, 23]. It has been suggested also that releasing factors produced by the ependyma may act indirectly or directly on the function of the hypophysis [18, 20]. It is also very interesting that activity of the

ependyma in the infundibular recess of the brain in *Rana esculenta* L. attains a maximum before ovulation and laying of eggs. JUSZCZYK and ZAMACHOWSKI [17] also observed the highest development of the gonads in this amphibian at this time. The correlation between the highest activity of ependymal cells of the infundibular recess and the highest gonadal development, dependent, as it is known, on the activity of the distal part of the hypophysis, is striking, and the results of the authors cited above suggest that participation of this ependyma in reproductive processes in *Rana esculenta* L. is very likely.

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CELLULAR AUTOPHAGOCYTOSIS IN MOUSE SEMINAL VESICLE CELLS *IN VITRO*

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(Received 1977—07—14)

Abstract

Cellular autophagocytosis was observed in mouse seminal vesicle cells incubated *in vitro* up to 8 h in medium 199 or Krebs–Ringer bicarbonate buffer. During the first 2 h of incubation, early forms of autophagic vacuoles were seen in the cells, advanced forms containing degraded material began to cumulate later. After 6—8 h, early vacuoles occurred sparsely, while advanced forms were detected in a great number. During the first 2 h of incubation, we often observed smooth surfaced membrane pairs between the cisternae of rough surfaced endoplasmic reticulum resembling isolating membranes of autophagic vacuoles. They varied in size and shape from short, straight cisternae to long, curved ones, almost completely encircling areas of the cytoplasm. Based on these observations, we propose a tentative scheme of the formation of autophagic vacuoles, *viz.*, the short, straight cisternae would represent the first stage in the development of an autophagic vacuole, while the curved sack-like forms are interpreted as successive steps leading to the complete sequestration of an area of the cytoplasm.

Introduction

Autophagocytosis is a process during which cells sequester and digest portions of their own cytoplasm. In many cell types, it occurs under physiological conditions or may be easily induced by toxic agents (for review see: [3, 11, 13]). Since its discovery, a great number of papers have been published on this phenomenon, largerly based on experiments conducted *in vivo*. Under these conditions, however, autophagocytosis is influenced by the whole complexity of changes in the metabolism of the organism. *In vitro* experiments offer the possibility to investigate the various factors implicated in regulating of the process separately. In spite of this advantage, *in vitro* studies are rare in this field [1, 4, 10, 14]. This fact prompted us to conduct experiments to elucidate morphological and quantitative features of the autophagocytosis induced in mouse seminal vesicle cells *in vitro*. The cell type was chosen because the details of autophagic process induced in them *in vivo* has already been reported by us [6, 7, 8] and these observations provided a basis for the evaluation of the *in vitro* results. This paper deals with the morphology of the process.

Materials and methods

Male CFLP mice of 26—30 g body weight were used in the experiments. After decapitation, the upper parts of the seminal vesicles of each animal were cut into small pieces. One of them was fixed immediately, while the others were incubated in medium 199 saturated by a gas mixture of 95% O_2 and 5% CO_2 at 37 °C. Some of the experiments were carried out in Krebs–Ringer bicarbonate buffer. The samples taken after 0.5, 1, 2, 4, 6 and 8 h of incubation were fixed in 0.1 *M* cacodylate-buffered glutaraldehyde (5%) containing 7% sucrose, post-fixed in osmium tetroxide, stained in uranyl acetate and embedded in araldite. The ultrathin sections were contrasted in lead citrate.

Results

In seminal vesicle cells fixed immediately after the decapitation of the animals, autophagic vacuoles were usually absent or very seldom seen. However, a considerable amount of them was formed in each region of the cytoplasm during incubation in either M-199 or Krebs–Ringer medium (Fig. 1). Autophagocytosis was a wave-like process in this system: during the first hours of incubation, we mainly observed vacuoles surrounded by smooth membrane pairs (isolating cisternae) and containing rough-surfaced endoplasmic reticulum (RER) fragments without any morphological signs of degradation. We refer to this type of vacuoles as early forms. Later, after 6 h of incubation, they decreased in number. In the same time, vacuoles bordered by single membranes and containing degraded RER fragments accumulated in the cells which may be considered advanced forms of autophagic vacuoles. Their content showed a wide variety of alteration from slightly increased osmiophilia of RER fragments to irregularly arranged dense residual material.

During the first two hours of incubation, we often detected, in the cytoplasm, smooth-surfaced membrane pairs resembling isolating cisterns of autophagic vacuoles. They were always situated between adjacent cisterns of RER, but direct connection of their membranes with those of the RER was not, observed. Some of these smooth membrane pairs run in the cytoplasm straightly while others were curved. Cup-like forms encircling almost completely areas of the cytoplasm were found, too (Figs 2, 3, 4).

One of the characteristic features of early autophagic vacuoles was the presence of tubular elements in the space of the isolating cistern (Figs 5, 6). They always cumulated at one of the poles of the vacuoles and in some cases directly connected the cavity of the sequestered area with the surrounding cytoplasm.

Some of the autophagic vacuoles were encircled by 2 or 3 isolating cisternae. In such cases, small regularly-arranged rodlets, possibly derived from ribosomes, were observed in between them (Fig. 7).

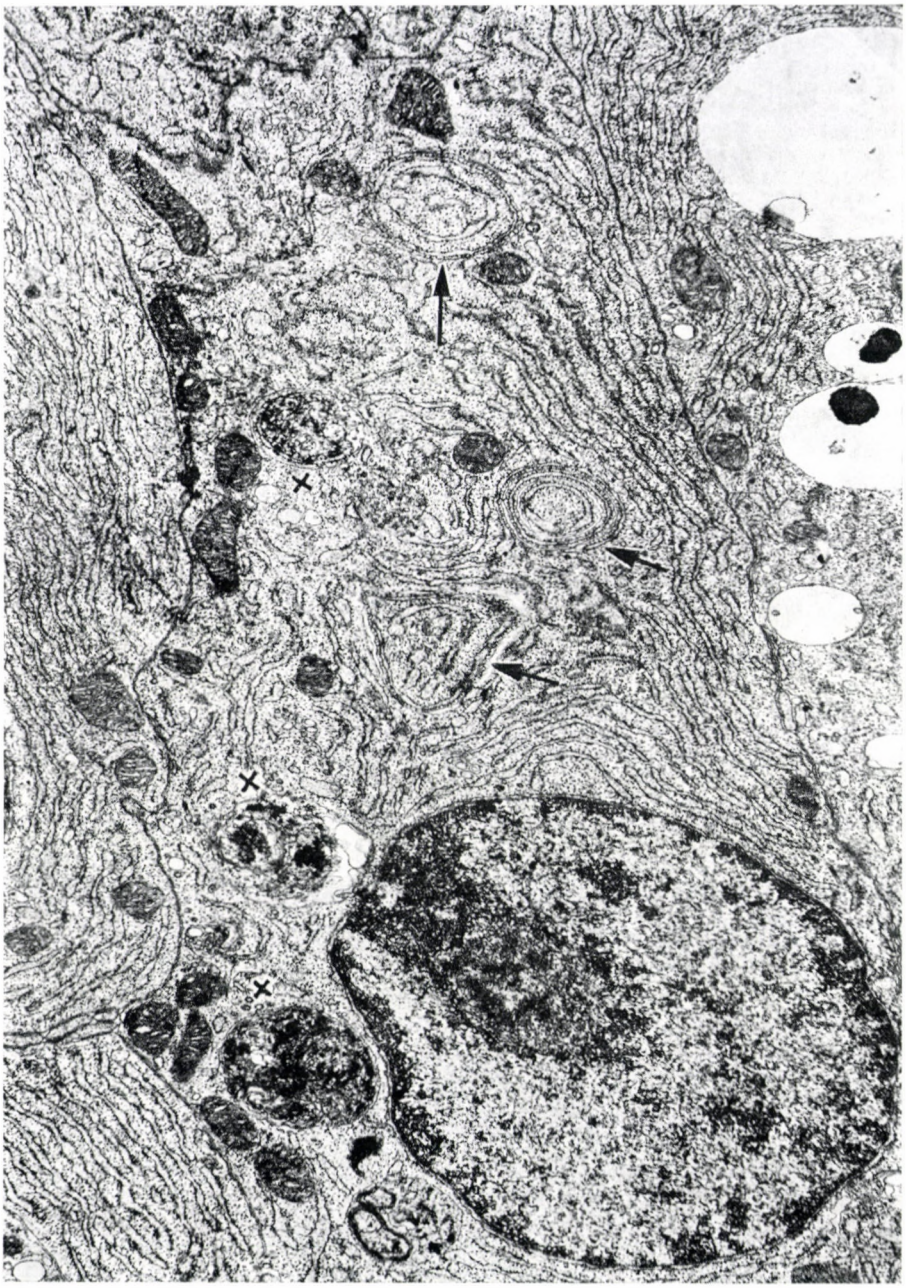
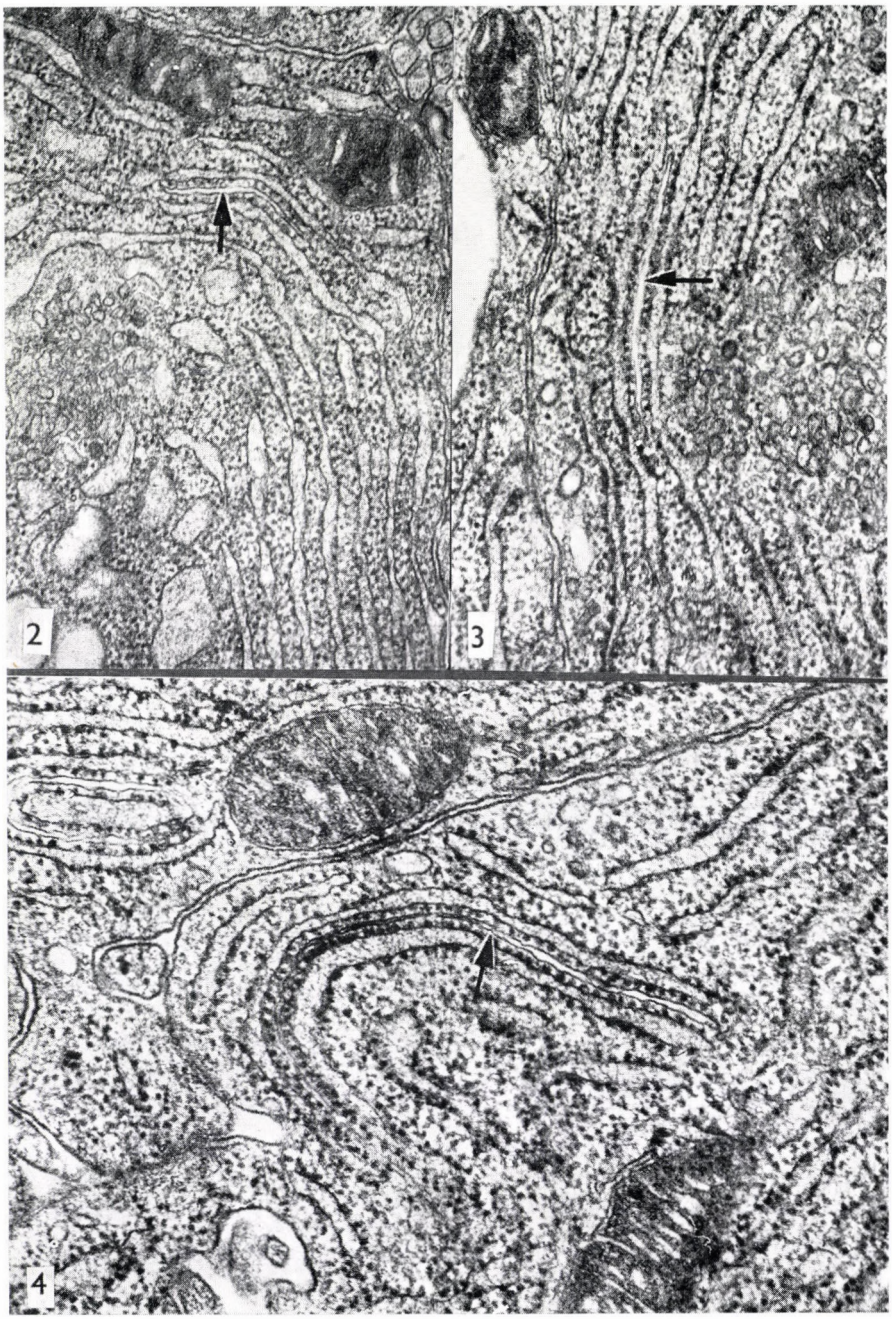
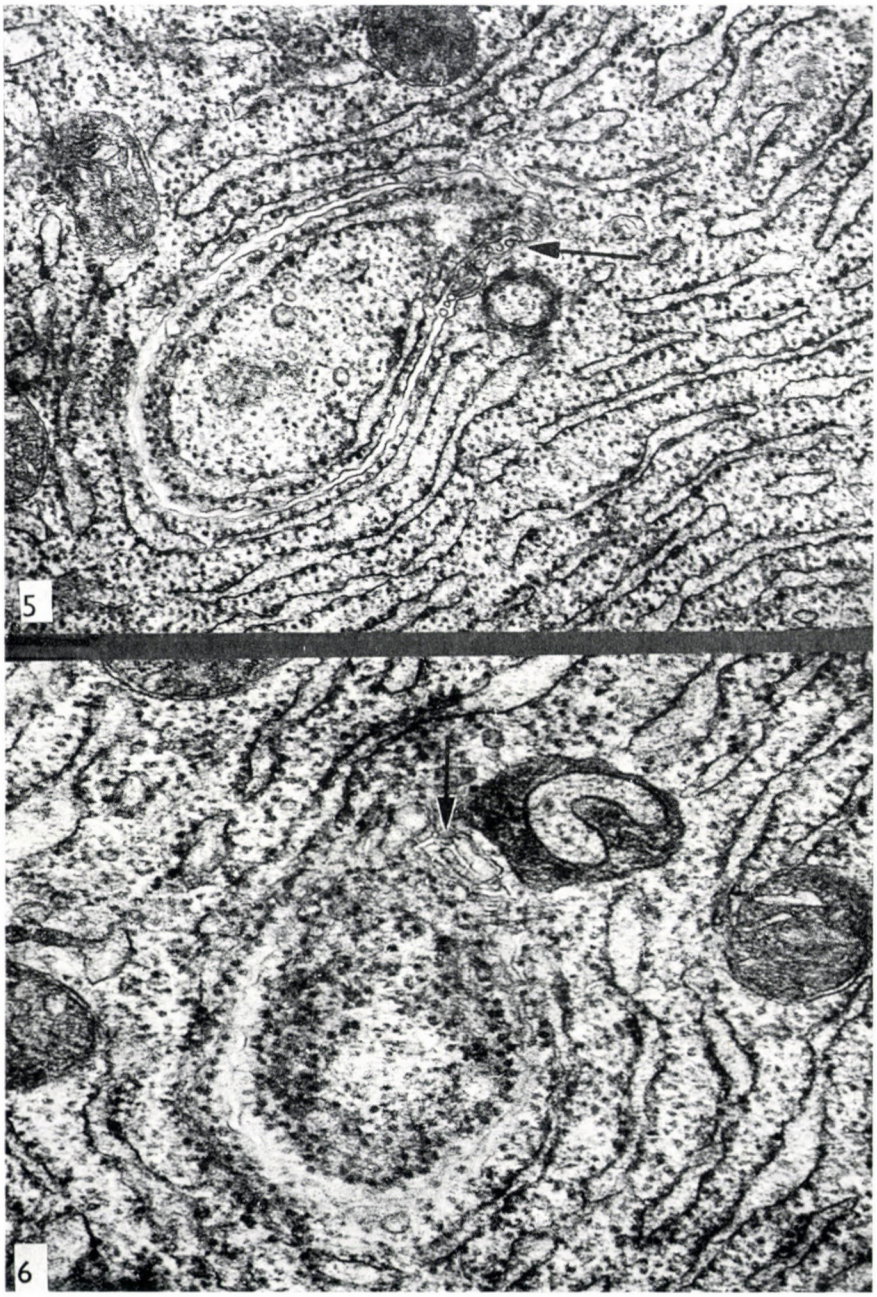


Fig. 1. Seminal vesicle cells incubated for 4 h, early (arrows) and advanced (x) forms of autophagic vacuoles ($\times 9000$)



Figs 2, 3 and *4.* Straight and semicircular smooth surfaced membrane pairs (arrows) between the cisterns of RER; 2 h. In Fig. *4* note that the adjacent RER cisterns closely follow the curvature of the smooth surfaced cistern (×35,000; ×37,000 and ×45,000)



Figs 5 and *6*. Early forms of autophagic vacuoles, 4 h of incubation. Note the tubular elements (arrow) at their poles (×41,000 and ×47,000)

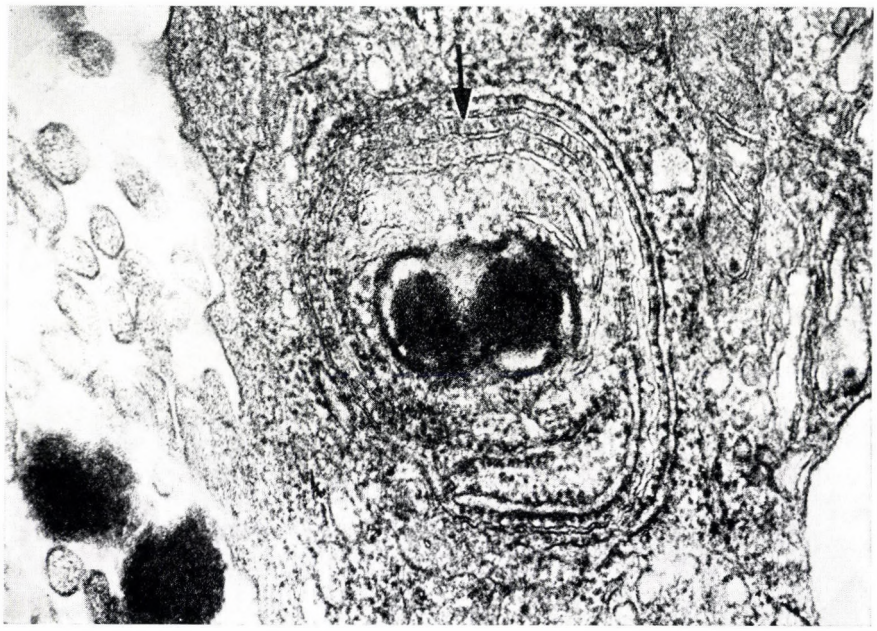


Fig. 7. Autophagic vacuole in the apical cytoplasm of a cell incubated for 2 h. Rod-like particles (arrow) between the two limiting cisterns (×43,000)

Discussion

A comparison of the present data to those of *in vivo* experiments reported earlier [6, 7, 8] reveals that the morphology of the sequestration process is similar in both cases. Therefore, we conclude that the early and advanced forms of vacuoles observed *in vitro* represent different stages of autophagocytosis. Similar autophagic process was described in other cells using various *in vitro* systems [1, 4, 5, 10, 12, 14]. The factors responsible for the induction of autophagocytosis in the seminal vesicle cells have yet to be elucidated. Based on literary data [10] we suppose that the nutritional conditions and/or the lack of appropriate hormonal environment in the medium may trigger the onset of the process.

In the course of autophagocytosis induced *in vivo*, early and advanced forms of autophagic vacuoles were simultaneously detected in the seminal vesicle cells shortly after the application of the inducer [6, 8]. Their development *in vitro* had a different course: in the first hours of incubation early vacuoles were present in the cells and the degrading forms followed them with a distinct delay. After prolonged incubation newly-formed vacuoles could be recognized very sparsely. The wave-like character of the autophagic process

with two separate peaks for early and advanced autophagic vacuoles was proved by quantitative analysis, too. (See the following paper in this volume). This property of the system offers good opportunity for studies designed to investigate the factors involved in the formation and development of early

Fig. 8. Hypothetical representation of how an autophagic vacuole might be formed by a smooth surfaced cistern

and degrading autophagic vacuoles separately. The delay in the development of advanced autophagic vacuoles *in vitro* may be interpreted as a sign of slowing down of the degradation process as compared to *in vivo* conditions.

One of the characteristic events occurring in the cells during the first hours of incubation *in vitro* is the formation of straight or curved smooth-surfaced cisternae between the membranes of RER. Their morphology (see Figs 2, 3, 4) promts us to propose that autophagic vacuoles arise in the way illustrated diagrammatically in Fig. 8. According to this tentative scheme,

the formation of short, straight cisternae represents the first step in the development of an autophagic vacuole, while vacuoles bearing tortuous tubular elements on one of their poles are interpreted as stages just before the complete closing over of the cistern around the sequestered area. The results of *in vivo* investigations bring some support to this hypothesis. In these experiments, flattened vesicles and cisternae bordering parts of the cytoplasm and finger-like projections between the cavity of autophagic vacuoles and the ground substance were occasionally seen in the seminal vesicle cells during autophagocytosis induced *in vivo* [7].

The source of formation of the limiting membranes of the autophagic vacuoles has remained unknown. In many cell types, they originate from the pre-existing membranes of Golgi complex, dense bodies and ER [2, 3, 5, 9], while in others they are believed to arise *de novo* by an assembly of pre-existing precursors [11]. In the seminal vesicle cells, we could not demonstrate their morphological continuity with any pre-existing membranes. It is noteworthy, however, that the early forms of autophagic vacuoles were often surrounded by cisternae of RER, closely following the curvature of their limiting membranes (see Figs 4, 5, 6). The simplest explanation of this observation is that some cisterns of RER participate in the sequestration process together with the isolating membranes. This speculation is further strengthened by the results of experiments in which the effect of protein synthesis inhibitors on autophagocytosis was investigated [13]. Autophagocytosis was prevented by inhibitors preserving the structural integrity of RER but was induced by those agents causing the alteration of the RER membranes and the detachment of membrane-bound ribosomes.

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QUANTITATIVE CHARACTERIZATION OF THE AUTOPHAGIC PROCESS IN MOUSE SEMINAL VESICLE CELLS *IN VITRO*

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Abstract

Pieces of mouse seminal vesicles were incubated *in vitro* for 0.5, 1, 2, 4, 6 or 8 h and the autophagic vacuoles formed in the epithelial cells during incubation were counted. The vacuoles were defined as early or advanced forms by morphological criteria and the autophagic vacuole/cell ratios were determined for the early and the advanced autophagic vacuoles separately. The number of early autophagic vacuoles significantly increased in the first hours of incubation, it was the greatest in the 4th h and rapidly declined thereafter. The number of advanced vacuoles began to rise about 1.5 h later as compared to the early ones, reached its maximum in the 6th h and remained high even in the 8th h. It may be concluded that a wave-like process took place in these cells during the experiment, characterized by a short transient rise in the formation of autophagic vacuoles, and their subsequent transformation into advanced forms filled with degrading cytoplasmic fragments.

Introduction

Most of the works devoted to the study of autophagocytosis deal with the morphological aspects of the phenomenon. Attempts to describe the process in quantitative terms are rare in literature [2, 3, 7, 8, 9]. In spite of their limited number, these reports have demonstrated the usefulness of quantitative methods as means for solving of such diverse problems of the autophagic process as diurnal rhythm [9], the effect of inducers [8], nutrient deprivation [7] and the interaction of lysosomes with autophagic vacuoles [3].

In the course of an ultrastructural study of the autophagocytosis in seminal vesicle cells *in vitro*, we noted that the number of autophagic vacuoles, and especially the proportion of early and advanced forms, markedly changed during the incubation [6]. Here we present the quantitative description of these changes based on counting of autophagic vacuoles in mouse seminal vesicle cells incubated *in vitro* up to 8 h.

Materials and methods

Seminal vesicles of male CFLP mice weighing 26—30 g were used throughout the experiments. After decapitation of the animals the organ was cut into small pieces in a drop of medium 199. One of the fragments was fixed immediately, while the others were incubated for 0.5, 1, 2, 4, 6 or 8 h at 37 °C in medium 199 gassed with a mixture of O_2 and CO_2 (95 : 5). The samples were fixed in 0.1 *M* cacodylate buffered glutaraldehyde, postfixed in osmium tetroxide, stained in uranyl acetate and embedded in araldite.

The autophagic reactivity of the cells was similar in the samples under the experimental conditions used. This was probably due to the fact that the inner surface of the seminal vesicle is covered by a single layer of columnal epithelial cells and therefore all of them have an equal access to the medium.

For quantitative evaluation, early and advanced forms of autophagic vacuoles were counted in samples containing 20—30 cells sectioned along the long axis. For each sample the number of autophagic vacuoles per cell (AV/cell ratio) was determined. For each time of incubation 15 samples, each from different tissue blocks, taken from 3 mice were examined, therefore, the resulting mean ($\bar{X}$) of the AV/cell ratio of a group represents data from 300—450 cells. The difference between two mean values was considered significant at $P < 0.01$. The total number of autophagic vacuoles equals to the sum of early and advanced forms. Autophagic vacuoles were considered early when bordered by double limiting membrane and the content of the vacuole seemed morphologically intact. The content of advanced autophagic vacuoles showed signs of degradation including increased osmiophilia of the sequestered ribosomes and accumulation of membrane whorls and dense material [6].

The reliability of the method was tested in preliminary experiments. For this purpose, the AV/cell ratio was determined in 4 groups of mice, each consisting of 8 animals, the seminal vesicles of which were fixed immediately after killing. The seminal vesicles of other 32 mice, divided into 4 groups were incubated for 2 h before fixation. Totally, about 3000 cells were examined in the preliminary experiments. There was no significant difference between the mean values of AV/cell ratio of identically treated groups, but the 0-time controls significantly differed from the groups incubated for 2 h.

Results and conclusions

The results summarized in Table 1 and Fig. 1 show that the number of early autophagic vacuoles significantly increased in the cells during the first hours of incubation and reached its maximum in the 4th h. This was then followed by a sharp decrease and the 8 h value of young AV/cell ratio approached to the 0-time level. The number of advanced forms began to rise later as compared to the early ones, reached its maximum in the 6th h of incubation and later remained at this level with a small, nonsignificant decrease. This was probably due to fusion of vacuoles which was frequently observed in these cells.

These observations permit us to conclude that a wave of autophagic activity occurred in the cells during the incubation. The formation of advanced vacuoles followed that of the young ones with a distinct delay. This may be explained by the assumption that the emergence of autophagic vacuoles and the degradation of their content are consecutive steps in the course of the autophagic process. This assumption is in harmony with the evidence available from morphological observations [1, 4]. Consequently, one of the factors determining the number of advanced autophagic vacuoles was the amount of early ones available for degradation. That was why the AV/cell ratio for

Table 1

Effect of incubation on the number of autophagic vacuoles per cell

Time of incubation (hours)	Early AV/cell			Advanced AV/cell			Total AV/cell		
	$\bar{X}$	S.E.M.	P	$\bar{X}$	S.E.M.	P	$\bar{X}$	S.E.M.	P
0	0.008	± 0.005		0.03	± 0.007		0.04	± 0.015	
			< 0.01			> 0.01			< 0.01
0.5	0.20	± 0.02		0.08	± 0.05		0.28	± 0.02	
			< 0.01			> 0.01			< 0.01
1	0.36	± 0.04		0.16	± 0.02		0.59	± 0.05	
			> 0.01			< 0.01			> 0.01
2	0.59	± 0.08		0.37	± 0.06		0.96	± 0.13	
			> 0.01			< 0.01			> 0.01
4	0.70	± 0.13		0.78	± 0.07		1.48	± 0.19	
			< 0.01			> 0.01			> 0.01
6	0.25	± 0.05		1.02	± 0.10		1.38	± 0.10	
			> 0.01			> 0.01			> 0.01
8	0.15	± 0.04		0.87	± 0.04		1.09	± 0.05	

The data presented are mean values and their standard errors. For details see Materials and methods

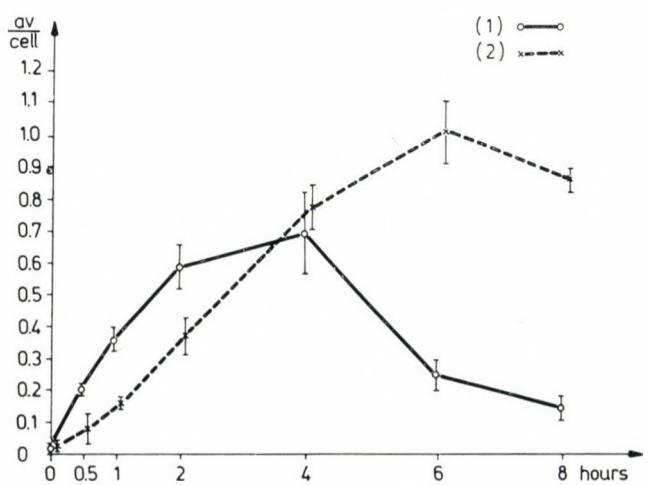

Fig. 1. Changes in the number of early (1) and advanced (2) autophagic vacuoles per cell during incubation. Vertical lines represent standard errors of the means

advanced vacuoles stopped to rise soon after the formation of new vacuoles had begun to fall.

The curve for advanced vacuoles was shifted right to that of the young ones by about 1.5 hours (Fig. 1). This may roughly represent the time necessary for the degradation of the sequestered fragments to the extent detectable on the basis of morphological criteria used here.

This estimation is close to the data given by GLAUMANN et al. [5] for the digestion of endoplasmic reticulum fragments in Kupffer cells.

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ULTRASTRUCTURAL ORGANIZATION OF GIANT NEURONES OF THE MOLLUSC *LYMNAEA STAGNALIS* UNDER DIFFERENT ENVIRONMENTAL TEMPERATURES

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Abstract

The ultrastructure of identified giant neurones of the visceral ring of *Lymnaea stagnalis* ganglion alters with the seasonal change of the animal and, experimentally, from the inactive physiological state (winter time or at +4 °C) to an active one (spring–summer time or at +18 °C). The ultrastructural organization of the active animal's neurones is characterized by morphological alteration pointing to an increased metabolic activity, *viz.*, an increased number of nucleoli, an enlarged surface of nuclear membrane and an increase in the nuclear membrane pores, appearance of a zone of free ribosomes near the nuclear membrane, changing the structure of cytosomes, abundant granular endoplasmic reticulum, increase in the number of mitochondria.

Introduction

The giant neurones of molluscs are of particular interest to neurophysiologists as they are readily available for experiment. Their functional organization is intensively investigated in many laboratories.

Thus, identified giant molluscan neurones have been successfully employed to study the regulatory mechanism of RNA synthesis after the stimulation of their electric activity [4, 19, 22]. These neurones are very convenient as well for a study of changes in general metabolism at the ultrastructural level under various mediators and pharmacological agents [5, 16, 25]. Such studies are directly related to those of the structural organization of neuronal systems, of individual nerve cells and of various cytoplasmic structures of cells.

This paper gives a description of various aspects of the ultrastructural organization of identified giant neurones of the mollusc *Lymnaea stagnalis*. The main objects of the study are to supplement the existing information on the ultrastructural organization of these neurones [6, 7, 24] and to investigate the ultrastructural changes appearing under the active (spring and summer) and passive (winter) physiological state of animals.

Materials and methods

Giant neurones of the left parietal, visceral and right parietal ganglia of *Lymnaea stagnalis* were used (according to the scheme of mapping of giant neurones proposed by DYAKONOVA and VEPRINTSEV [10]).

Ganglia were fixed in 1% osmium tetroxide, dehydrated in alcohol or in acetone and embedded in "Epon-812". Ultrathin sections (Ultratome LKB-III) were stained with an aqueous solution of uranyl acetate at 37 °C for 30 to 90 min and post-stained with lead. The sections were viewed in JEM-7 and UMV-100 (Made in USSR) electron microscopes.

In order to find identifiable cells during dissecting, the arrangement of giant neurones in the ganglion was sketched, then sections about 3—4 μm thick were prepared with the ultratome and the location of the cells was determined under the light microscope.

The ultrastructural organization of *Lymnaea stagnalis* neurones was studied in winter and in spring–summer time. In some cases the animals were kept for a long time in a cooling chamber at +4 °C or at room temperature (18—22 °C). In the last case the animals were active, fed intensively and spawed irrespective of season. At +4 °C all animals were inactive.

Results and discussion

Giant neurones

Figure 1 presents the map of the arrangement of neurones studied in the visceral ring of the ganglion. A detailed cytochemical and physiological characterization of these cells has been presented elsewhere [10, 14].

The giant neurones vary in size between 100 and 250 μm; their location in the ganglion is nearly uniform. All the giant neurones studied were ultrastructurally similar in all ganglia. The cells are surrounded by glial cells. Glial cell processes penetrate the neuronal cytoplasm along the neuronal periphery, and the cytoplasm of the processes often contains a fine granular material.

The actual observation on the different number of glial processes penetrated into the perikaryon of "active" and "inactive" neurones allows to conclude that the degree of penetration depends on the functional state of the neurone.

Like in other molluscs [1, 9, 22], a regular zonal distribution of cytoplasmic structures from the cell periphery toward the nucleus could be noted in all the giant neurones. This distribution became apparent when neurones were developing into giant neurones. Figure 2 shows a schematic representation of a generalized ultrastructural organization of the giant neurone in *Lymnaea stagnalis*.

Nucleus

The greater part (up to 75% of the neuronal soma) is occupied by the nucleus, which contains numerous nucleoli more or less evenly distributed in the nucleus, though, large nucleoli are more often found in the centre of the nucleus. In some cases so-called ring-shaped nucleoli were found. Within

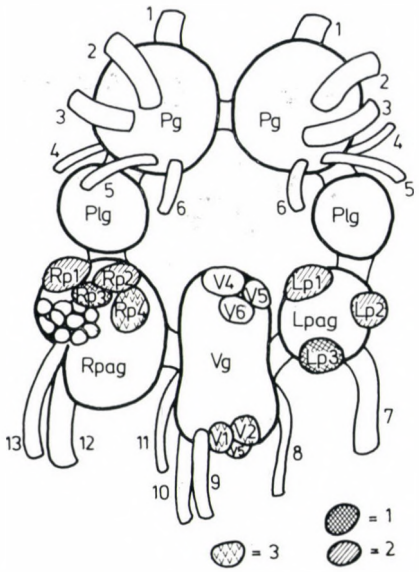


Fig. 1. Schematic arrangement of giant neurones in the visceral ring of molluscan ganglion; (1) spontaneously active neurones, (2) silent neurones (3) secretory neurones

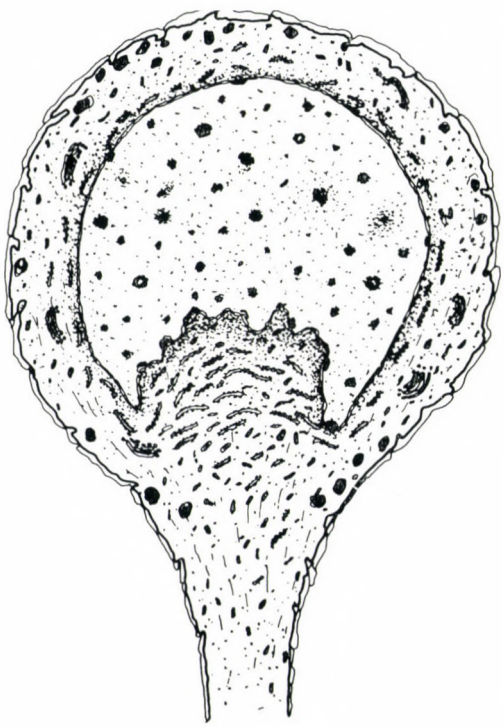


Fig. 2. Schematic representation of ultrastructural organization of giant molluscan nerve cells

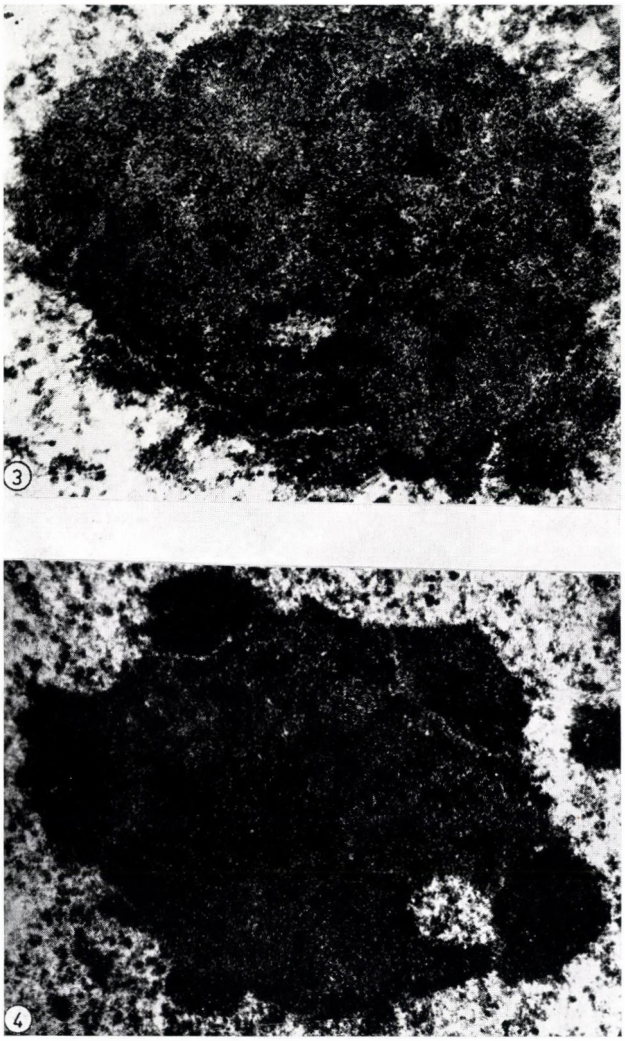


Fig. 3. Nucleolar ultrastructure of giant neurone 3MP. Granular and fibrillar components of the nucleolus are clearly defined. Granular component is predominant. ×30,000
Fig. 4. Nucleolus fibrillar type with chromatin plaques. Note the vacuole inside the nucleolus. ×30,000

the nucleoli, the granular and the fibrillar elements are well defined. Compact round granules about 150 Å in diameter prevail in former structures, while thin fibrils entwined with tightly-packed granules predominate in the latter (Figs 3 and 4). Dense homogeneous nucleoli with chromatin plaques are most numerous. The main components of such nucleoli are fibrillar structures (Fig. 4). Besides, there occur nucleoli showing a predominance of distinct ribosome-like particles (Fig. 3). The amount of fibrillar material in such

nucleoli is insignificant. The nuclear membrane forms numerous processes and invaginations, thus considerably increasing the nucleus–cytoplasm interface, especially in the region of the axon hillock. In the region of invaginations, pores increase in number as compared with the other parts of the nuclear membrane.

Cytoplasm

The perikaryon of giant neurones is extremely rich in structural elements. The qualitative and quantitative composition of these elements is inhomogeneous in different parts of a neurone. The network of rough endoplasmic reticulum (RER) is well developed and presents a typical element of the cytoplasm of large and small neurones of *Lymnaea stagnalis*. This very labile structure reflects the level of neuronal metabolism. The network of RER is developed at different degree in different regions of the cytoplasm. The region most abundant in RER is the axon hillock, where a dense meshwork of long RER cisterns is present, elongated in the direction of the nuclear membrane with ribosomes very densely packed along membranes (Figs 10, 11, 12). At the periphery of the cytoplasm, RER is found in small amounts in the form of separate closed vesicles studded with ribosomes (Fig. 7). Neurones of active animals are characterized by a great number of mitochondria 0.2 to 2 μm long which are evenly distributed in the cytoplasm (Fig. 11). These organelles are, however, practicularly abundant near the axon hillock (Fig. 2) and, morhologically, two types of mitochondria are recognizable: those with a light matrix and those with a dense dark one. Such differences in matrix structure appear to correspond to different functional state of the mitochondria [11].

The Golgi structures in giant neurones occur throughout the middle zone as well as at the periphery of the cell. They are less frequent near the nuclear membrane (Fig. 6). Probable successive formations of secretory granules can rather frequently be observed in Golgi structures; dense osmiophilic material inside Golgi structures, with granules having a dense core budding from the ends of cisterns, side by side with the formation of secretory granules (Fig. 5).

In the cytosomes, pigment granules containing phospholipids, carotenoids, haemoproteins and a variety of enzymes, including those of the electron transport chain [3, 15, 16, 26], can be demonstrated.

These are rather large inclusions ranging in size between 2 and 5—8 μm. They mainly occur at the periphery of the perikaryon (Fig. 7), but they are also often found in the central regions of the cytoplasm. Large clusters of cytosomes can be observed in the apical region of the perikaryon and at the place where a process is formed from the soma. The ultrastructural organization of cytosomes is fairly labile and changes with the functional state of

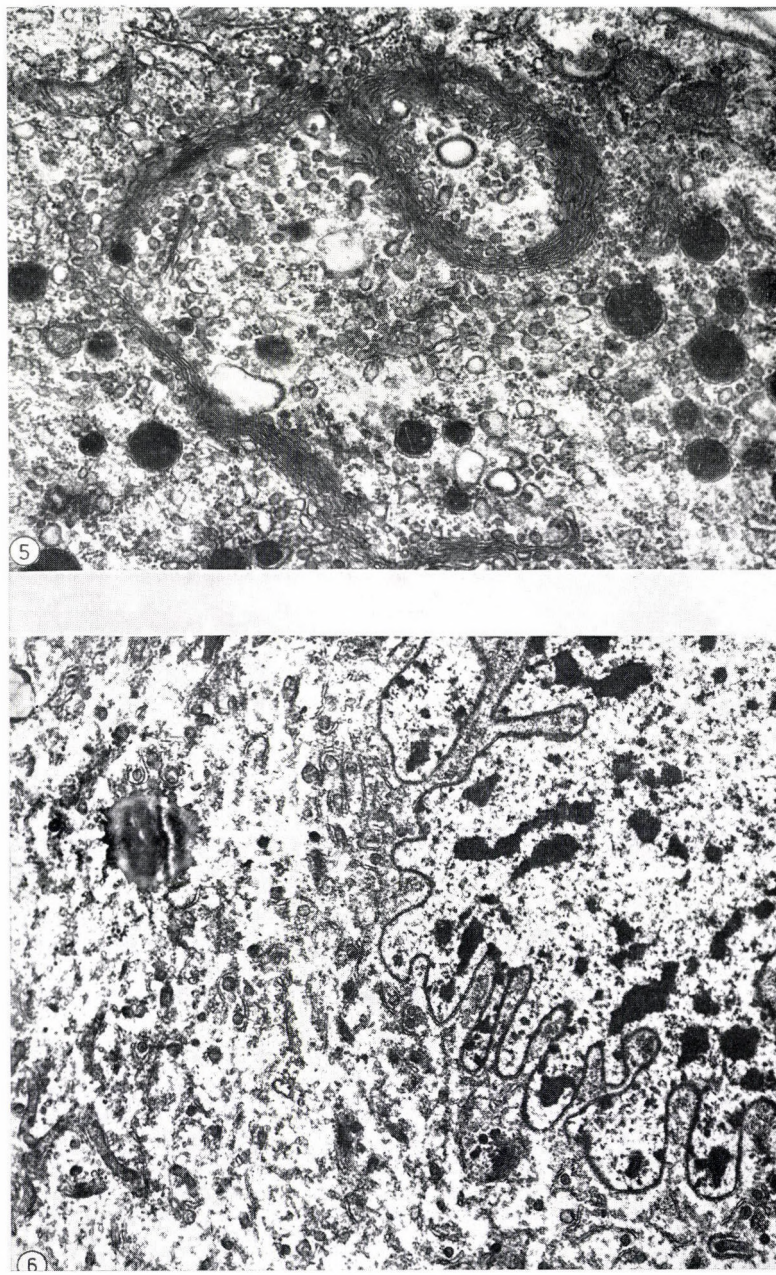


Fig. 5. Golgi structure. Localization of osmiophilic components, inside cisterns and dyctiosomes are clearly seen. Osmiophilic granules detaching from the cisternal ends and "mature" osmiophilic granules are also seen. ×18,600
Fig. 6. Perinuclear region of neurone BP-1. Nuclear membrane folds, a few RER profiles and mitochondria ×5000

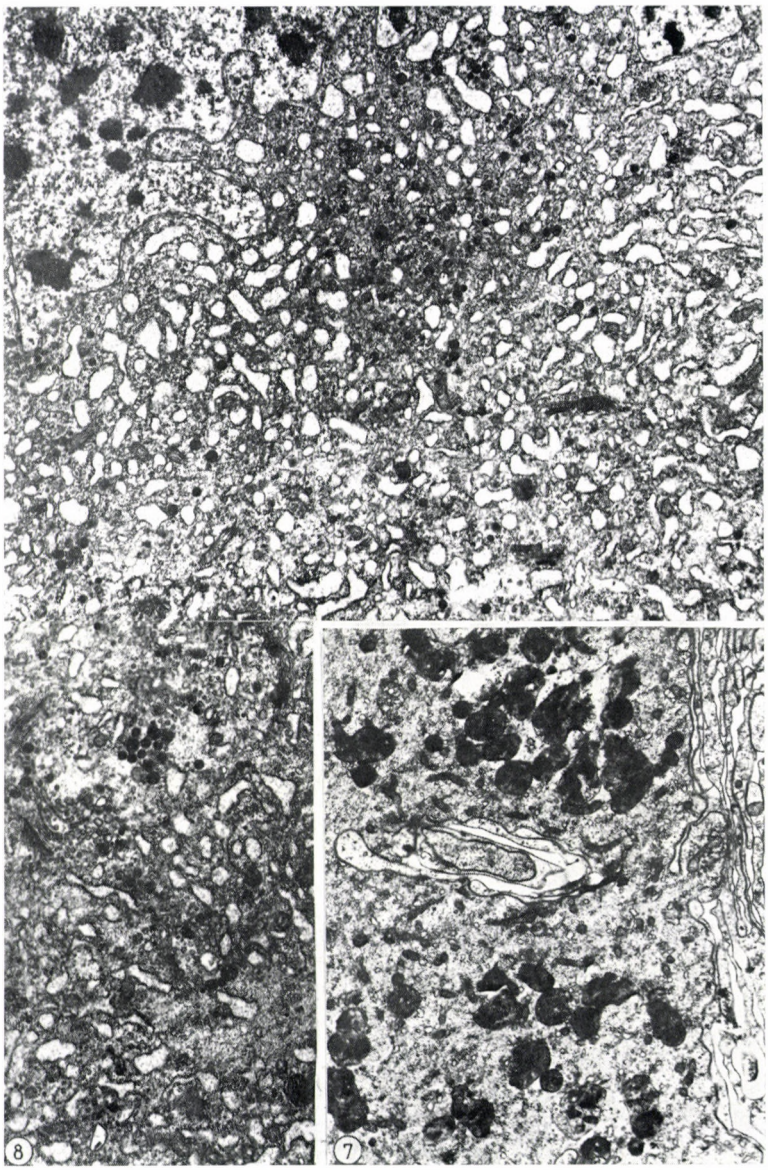


Fig. 7. Periphery of cytoplasm of neurone 3MP, showing numerous cytosomes and glial cel processes. Winter time animal at +4 °C. ×5000

Fig. 8. A region of cytoplasm of neurone V-1 at +18 °C, spring time animal. Numerous endoplasmic reticulum cisterns with widened bands can be seen. At the periphery, these cisterns grow and appear to fuse forming lacunae. ×6000

the animal. As it has been described for cytosomes of *Anodonta* neurones [25] a large number of lamellar (membraneous) structures were formed inside cytosomes when the animal was kept under anoxic conditions [25, 26]. We have

observed similar changes in the structural organization of cytosomes of *Lymnaea stagnalis* neurones at the transition from an inactive (winter time, at $t = +4$ °C) to an active state of animals (spring, summer time, at $t = +22$ °C [7]).

The cytosomes appear to be involved in the energy supply for general neuronal metabolism when the tissue is deficient in oxygen [15] or upon a sharp increase of general neuronal metabolism associated with the transition into an active state of animal.

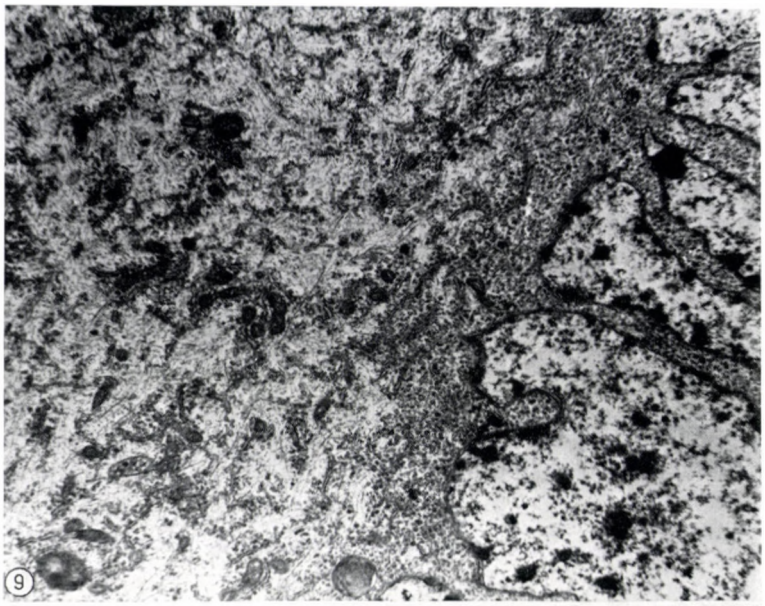
Secretory neurones

The structural organization of secretory neurones, which include neurones V-1, V-2 and RP-4, is substantially different from the scheme presented in Fig. 2. In fresh preparation, these cells are of a whitish colour, different from the bright yellow colour of other neurones. The structural organization of these cells is different from that of neurones 1LP, 2LP, 3LP and 1RP by the presence of a great number of endoplasmic reticulum cisterns (Fig. 8). RER cisterns are not so enlarged in the perinuclear zone as at the cell periphery. It appears that RER membranes subsequently lose their ribosomes, and their individual small cisterns fuse to form "lacunae" along the cell periphery. The cytoplasm of these neurones shows no distinct zones in the distribution of cytoplasmic structures. Moreover, large osmiophilic granules about 1000 Å in diameter are seen in the cytoplasm of neurones V-1 and V-2 (Fig. 8).

Characteristics of spontaneously active and silent giant neurones

Among the giant neurones of *Lymnaea stagnalis* two groups of cells are recognizable depending on the character of the background electrical activity: spontaneously active neurones and neurones showing no spontaneous activity (silent cells [14]).

The cytoplasm of spontaneously active neurones (3LP) is much richer in structural components than of silent ones (1RP, 1LP; Figs 12 and 6) in animals exposed to the same ambient temperature. The activity of these neurones usually increases with rising ambient temperature [8, 18], resulting in a change in their ultrastructural organization. A comparison of the ultrastructural features of identical neurones taken from *Lymnaea stagnalis* individuals exposed to temperatures of +4 °C and +18—22 °C indicates that the neuronal cytoplasm of more active animals (18—22 °C) is richer in cytoplasmic structures (Figs 9, 10, 11, 12), the number of free ribosomes mainly located in the perinuclear zone increases, the RER network is more developed, mitochondria are more numerous, cytosomes are more osmiophilic, granular-type nucleoli increase in number/nucleus, and many invaginations appear in the nuclear membrane. All these structural features are indicative of a higher



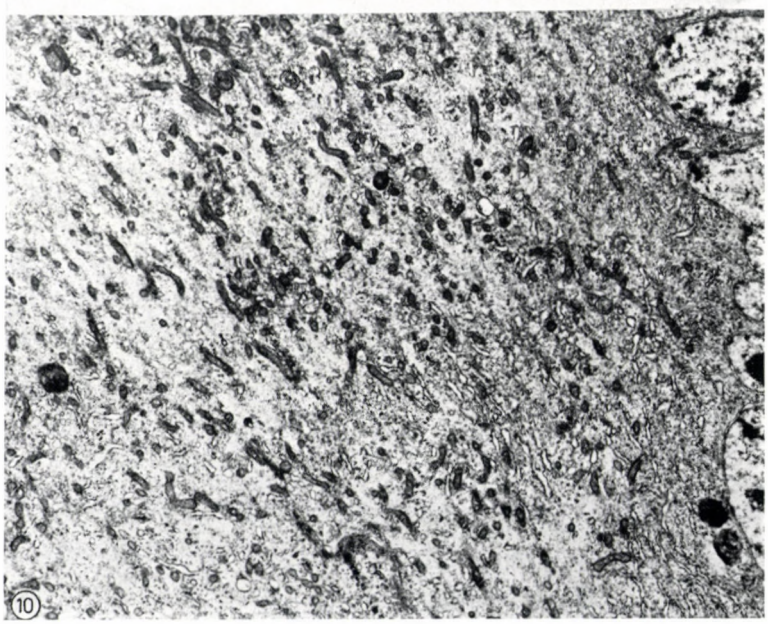
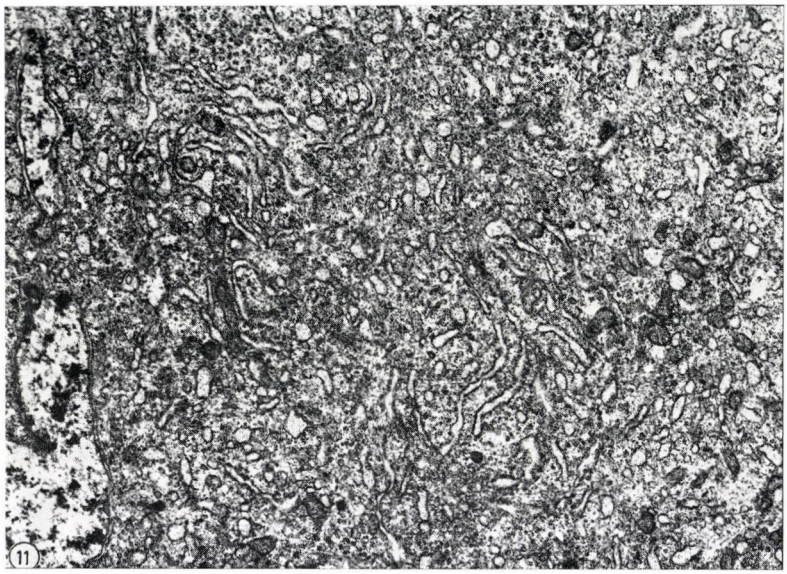


Fig. 9. Perinuclear region of cytoplasm of neurone 1MP, at +4 °C, winter time. Deep nuclear membrane invaginations, perinuclear zone of polyribosomes, a few mitochondria and RER profiles. Numerous neurofibrils are clearly seen. ×7000

Fig. 10. A region of cytoplasm of neurone 2LP, at +22 °C, spring time. Ribosomes and RER cisterns are greatly increased. ×50,000



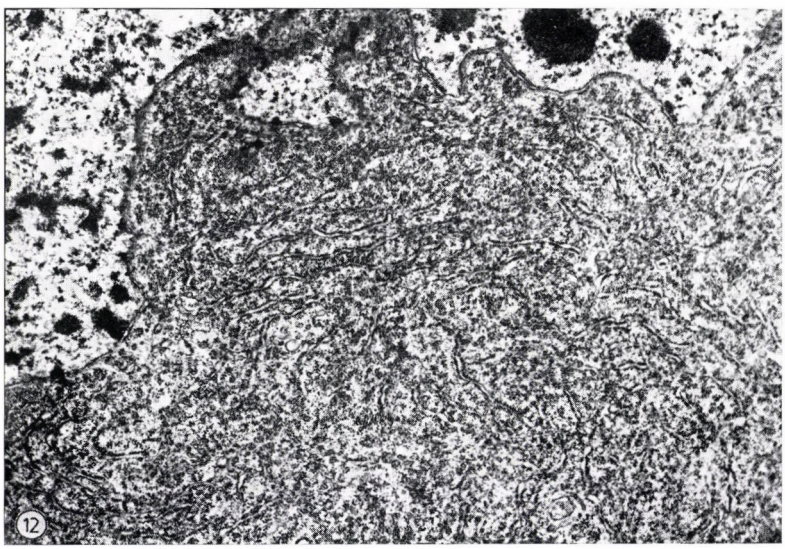


Fig. 11. Axon hillock of neurone 3LP, at +4 °C, winter time. A large number of mitochondria are visible. ×7000

Fig. 12. A region of cytoplasm of neurone 3LP, at +22 °C, spring time. A well developed RER network and numerous free and membrane-bound ribosomes are seen. ×7000

metabolic level in the neurones of active animals. Yet, these structural distinctions are not the same for different cells. The changes in ultrastructural organization of spontaneously active neurones occurring after the transition from +4 °C to +18—22 °C are smaller than those in silent neurones. Thus, for

example, neurones 3LP, 3RP (spontaneously active) still have a granular reticulum in the form of short cisterns with a small number of ribosomes at +4 °C, while the cytoplasm of neurones 1LP, 2LP and 1RP (silent neurones) is poorly differentiated at the same temperature. A zone of free ribosomes is found in these cells only in the region of the axon hillock, while the network of the granular reticulum is almost absent. The cytoplasm of the same neurones is considerably richer in structures at +18—22 °C, than at +4 °C (Figs 9, 11). However, in some cases, the silent neurones (1LP, 2LP, 1RP) show no ultrastructural manifestations of any significant increase in functional activity even at +18—22 °C.

In studying the seasonal changes in the ultrastructure of *Lymnaea stagnalis* giant neurones, we have come to the conclusion that these changes are analogous to those caused by temperature. Winter animals kept at +18—22 °C become active and can even spawn. The ultrastructural organization of their neurones becomes similar to that of spring or summer-time molluscs. Conversely, if animals are kept in a cooling chamber at +4 °C during the summer time for a period of two to three weeks, the ultrastructural organization of these cells becomes similar to that of neurones of winter time animals.

Conclusion

The structural features of giant neurones of the left parietal, right parietal and visceral ganglia are similar and do not differ essentially from the structure of giant neurones of other molluscs described in the literature [1, 9, 22].

All giant neurones are distinguished by a high degree of ploidy reaching, *e.g.*, 1000 p in *Tritonia* [23]. The appearance and persistence of giant cells seems to be conditioned by an increase in DNA content. The nuclei of *Lymnaea stagnalis* giant neurones are also polyploid. The amount of DNA in the cell of cerebral ganglion attains 256 p [20]. Their nucleus can be considered actively synthesizing. Persistent incorporation of H^3-uridine and of labelled methionine into the nuclei suggested an intense ribosomal RNA and protein synthesis in these nuclei [21]. The comparison of ultrastructural organization of nucleoli of winter and spring/summer time animals also suggests an increased synthetic capacity of neurones. The increase of the nucleus–cytoplasm interface due to processes and invaginations in the nuclear membrane is associated with an enhancement of the exchange between the actively synthesizing nucleus and the cytoplasm. The structural characteristics of the cytoplasm also indicate an intensive cell metabolism. The most metabolically active regions are those probably located near the axon hillock where more intensive synthesis of different proteins appears to take place. It is possible that such a localization

of the synthetic apparatus is convenient for further transport of metabolic products along the axon.

The intensity of protein synthesis in the neurone may vary considerably. Thus, for example, autoradiography has shown that electric stimulation promotes the incorporation of H^3-uridine into the neuronal nucleus [5, 6]. Electron microscopy has demonstrated an increased number of nuclear membrane processes, nuclear pores and granular-type nucleoli accompanied by an increase of the RER network, and free ribosomes. We have here shown analogous, though less pronounced, changes occurring also in the neurones of animals after a change in the ambient temperature from +4 °C to +18—22 °C.

The apparent difference in the fine structure of the nuclei and the RER between spring, summer- and winter-time neurones may be indicative of certain differences in the functional activity of the protein synthetizing apparatus. More detailed information on this activity requires further investigation.

However, the structural studies of the giant neurones of *Lymnaea stagnalis* at different functional state of the animal allow to conclude that the ultrastructural organization of neurones of the central nervous system directly reflects changes in their metabolism.

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IDENTIFICATION AND ESTIMATION OF AN ACETYLCHOLINE-LIKE SUBSTANCE IN THE VENOM OF *DENDROASPIS JAMESONI*

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Abstract

An acetylcholine-like substance was isolated from desiccated Jameson's mamba venom by one-dimensional ascending paper chromatography. The migratory and staining properties of the substance were identical with those of standard acetylcholine. Pharmacological identification on various *in vitro* and *in vivo* biological test objects showed that the substance was acetylcholine. It was further confirmed by high-voltage paper electrophoresis. The acetylcholine content was 4.35—4.36 mg/g of desiccated venom as shown by two different biological assay methods, and the index of discrimination was found to be 1.1, further confirming that substance in snake venom was acetylcholine.

Introduction

Pharmacologically active substances are widely distributed in various biological systems of both plant and animal origin. Acetylcholine, 5-hydroxytryptamine, histamine and a kinin have been identified in the venom of the common wasp *Vespa vulgaris* [7, 6]. The venom of the European hornet, *V. crabro*, contains 5-hydroxytryptamine, histamine, with higher concentrations of acetylcholine and a kinin different from that found in the common wasp venom [2]. Acetylcholine-like substances have been detected in hypobranchial glands of certain gastropods [3, 9] and in the three species of African mambas *Dendroaspis angusticeps*, *Dendroaspis polylepis* and *Dendroaspis jamesoni* [12, 13]. The present work sets out to isolate, identify and measure the amount of the acetylcholine-like substance in the whole venom of *D. jamesoni* by biochemical and biological methods.

Materials and methods

One-dimensional ascending paper chromatography

Desiccated venom (20.0 mg) and acetylcholine standard (5 mg) were dissolved in 0,5 ml ethanol (50%) and 50 μl of each sample (equivalent to 2000 μg of crude snake venom and 500 μg of standard acetylcholine hydrochloride) were carefully spotted on Whatman no. 1 paper

with a microlitre syringe (SGE Type A-RN). One-dimensional ascending chromatograms were run for a period of 46 h at room temperature (20—30 °C), using two solvent systems; n-propanol: formic acid: water: (8 : 1 : 1 : v/v) [13] and n-butanol: ethanol: acetic acid: water (8 : 2 : 1 : 3 v/v) [1]. Development of chromatograms was carried out in iodine vapour. Two chromatograms of the test acetylcholine-like substance were pooled by extracting in 5 ml of Tyrode solution or saline.

Identification and assay of acetylcholine-like substance on biological test objects

The pooled eluates were tested on guinea pig ileum (n = 5), rat ileum (n = 13), frog rectus abdominis (n = 4) and cat blood pressure (n = 8). The response of ileum to standard acetylcholine and test eluate from chromatograms were studied before and after addition of atropine (2 ng/ml) to the bath (hexamethonium bromide 1×10^{-6} *M* was added to the Tyrode solution). Enzymatic and alkaline hydrolysis was carried out by addition of either *N* NaOH (few drops) or 1 ml of guinea pig serum to 1 ml each of standard acetylcholine and test eluate; the mixtures were incubated at 37 °C for 3 h.

Contractions of the frog rectus abdominis muscle were recorded after acetylcholine or test eluate had been added before and after addition of either eserine sulphate (8.0 μg/ml) or d-tubocurarine (20 μg/ml). The period of incubation of eserine or d-tubocurarine was 10 min.

In blood pressure studies on cats, equipotent doses of acetylcholine and test eluate were selected for eliciting the vasodepressor response after intravenous administration. These doses were repeated 45 min later, after intravenous injection of mepyramine maleate (5 mg/kg) and atropine sulphate (2 mg/kg).

High-voltage electrophoresis

Ascending paper chromatography was carried out on crude desiccated venom (16 mg) and standard acetylcholine (10 mg) dissolved in 1 ml of 50% ethanol as described above. The eluted standard acetylcholine and test substance together with freshly-made acetylcholine solution (10 mg/ml) and snake venom (40 mg/ml) were streaked on Whatman paper 3MM; deionized water was used as solvent. High-voltage electrophoresis (Savant Instrument Inc.) was carried out at 60 V/cm for 30 min, using acetic acid buffer (acetic acid: pyridine: water: 9 : 1 : 190 v/v) of pH 3.6. After drying, the paper was sprayed with ethanolic hydroxylamine and ferric chloride solution [13].

Bioassay

Estimation of acetylcholine-like substance in the test eluate was carried out on rat ileum (four-point assay) and cat blood pressure (bracketing). The values of acetylcholine are expressed in terms of the base. The index of discrimination was determined by the method of GADDUM [5].

Drugs used: acetylcholine chloride (E. Merck, Darmstadt), atropine sulphate (Sigma, London), hexamethonium bromide (Sigma, London), neostigmine bromide (Sigma, London), d-tubocurarine chloride (Sigma, London), histamine diphosphate (Sigma, London) mepyramine maleate (May & Baker, England). Desiccated whole venom was obtained from Baringo Snake Farm, Nakuru, Kenya and kept in the refrigerator at 4 °C prior to use. Suitable quantities of drugs and snake venom were dissolved in deionized water or in saline. Concentrations of drugs refer to their salts unless stated otherwise.

Results

One-dimensional ascending paper chromatography

The R_F values of standard acetylcholine and test substance in the crude snake venom are summarized in Table 1.

Table 1

R_F values of standard acetylcholine and test substance from snake venom after single-dimensional ascending paper chromatography

Chromatogram solvent system	R_F values	
	Standard acetylcholine	Test substance
n-propanol : formic acid : water 8 : 1 : 1 (v/v)	0.73	0.75
n-butanol : ethanol : acetic acid : water 8 : 2 : 1 : 3 (v/v)	0.45	0.45

Biological test objects

The spasmogenic effect of the test substance (0.25 ml in 5 ml bath) and standard acetylcholine (0.2 μg/ml) were tested on isolated guinea pig ileum before and after addition of atropine sulphate (2 ng/ml). The contractions elicited by test eluate and acetylcholine were considerably reduced by the atropine (Fig. 1 A). Similarly, the contractile responses to test eluate and standard acetylcholine were virtually abolished after enzyme and alkaline hydrolysis of the samples. However, unhydrolysed acetylcholine and test eluate after incubation at 37 °C for 3 h did not produce any diminution of the contractile response of guinea pig ileum at the same dose levels (Fig. 1 B).

The standard acetylcholine (0.2 μg/ml) and test eluate (0.1 ml in 5 ml bath) produced sustained contractions of frog rectus abdominis muscle. The contractions were markedly potentiated by eserine (8 μg/ml) and completely abolished by d-tubocurarine (20 μg/ml) when they were added to the bath 10 min before samples were tested (Fig. 2).

The hypotensive response of cat blood pressure induced by intravenous administration of 0.2 μg of standard acetylcholine and 0.1 ml of the test eluate was not blocked by prior intravenous administration of mepyramine maleate (5 mg/kg), but was completely blocked after intravenous injection of atropine sulphate (2 mg/kg) (Fig. 3).

High-voltage electrophoresis

The migration of standard acetylcholine chromatography eluate S(ChE), freshly prepared standard acetylcholine S(ACh), test substance from chromatography eluate T(ChE) and desiccated snake venom (SV) by high-voltage electrophoresis are shown in Fig. 4.

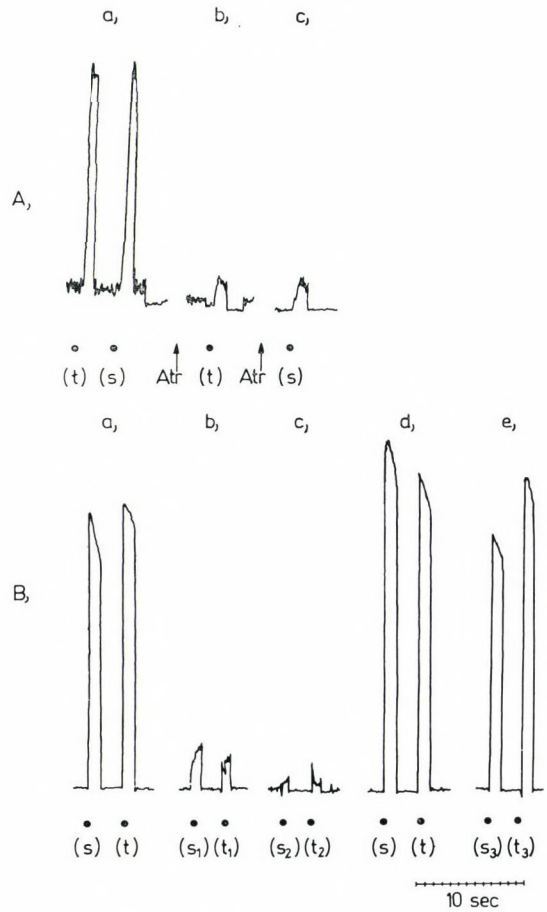

Fig. 1. Effect of acetylcholine and test eluate on the contraction of guinea pig ileum (volume of bath 10 ml). *A: a.* normal responses of ileum to standard acetylcholine (s) 20 ng/ml and test eluate (t) 0.1 ml; atropine sulphate (Atr) 2 ng/ml was added to the bath, between *a* and *b*, and between *b* and *c*; almost complete absence of response *b* and *c*, to the same dose of (s) and (t). *B: a.* contraction of ilexm to standard acetylcholine (s), 20 ng/ml, and test eluate (t), 0.25 ml; *b* and *c.* absence of contractile response after enzyme (s_1 and t_1) or alkaline (s_2 and t_2) hydrolysis; *d.* responses to the same dose of (s) and (t); *e.* Responses to unhydrolysed (s_3) and (t_3) after incubation for 3 h

Biological assay

Quantitative estimations of the acetylcholine-like substance in the test eluate on two biological systems are shown in Table 2. The index of discrimination was found to be 1.1.

Table 2

Amount of acetylcholine-like substance in the venom (assay on two biological system)

Biological test system	No. of experiments	Amount of test substance in the snake venom (mg/g) + S.E.M.	P
Rat ileum	13	4.63 ± 0.44	> 0.05
Cat blood pressure	8	4.35 ± 0.54	

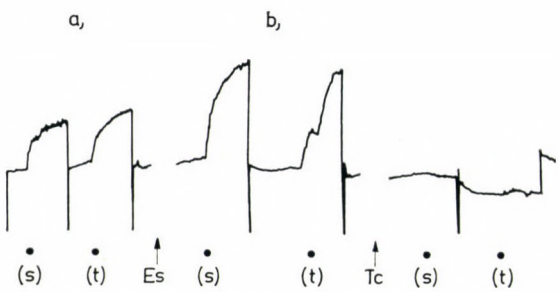

Fig. 2. Effects of acetylcholine and test eluate on the contractile responses of frog rectus abdominis (volume of bath 5 ml) *a*. Contractile response of frog rectus abdominis to standard acetylcholine (s) 0.2 μg/ml and test eluate (t) 0.1 ml; between *a* and *b*, eserine (Es), 8 μg/ml was added to the bath; *b*. potentiation of the response to (s) and (t) 10 min later; between *b* and *c*, d-tubocurarine (Tc) 20 μg/ml was added; *c*. complete abolition of the response to (s) and (t) 10 min later

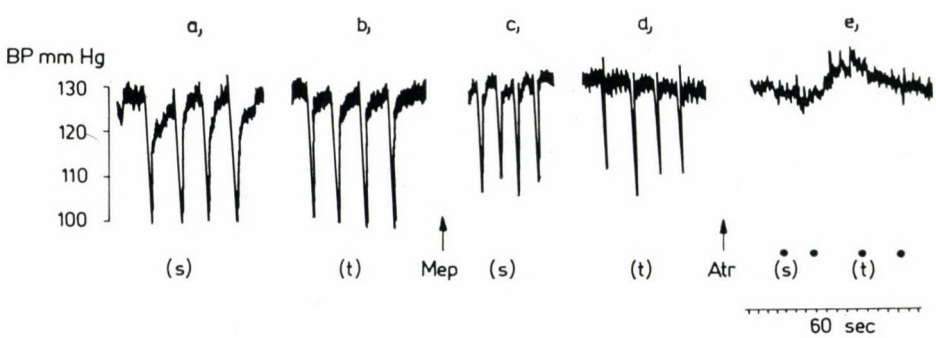

Fig. 3. Effect of acetylcholine and test eluate on the blood pressure of cat 3.3 kg. Anesthesia: ether and chloralose 80 mg/kg intravenously; *a* and *b*. vasopressor effects of 0.2 μg standard acetylcholine (s) and 0.1 ml of test eluate (t). Between *b* and *c* mepyramine maleate (Mep), 5 mg/kg, was administered intravenously; *c* and *d*. repeated doses of (s) and (t) 45 min after mepyramine maleate; between *d* and *e*, atropine sulphate (Atr), 2 mg/kg, was administered intravenously; *e*. responses to (s) and (t) were completely blocked 45 min after atropine sulphate

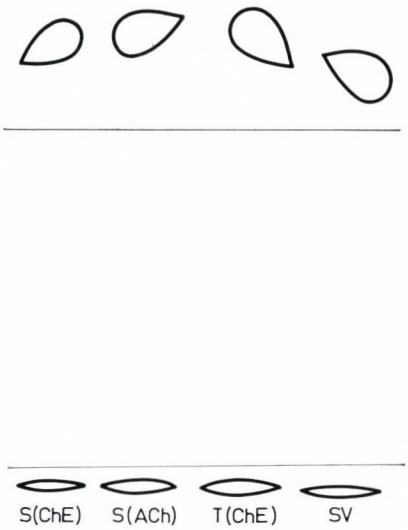

Fig. 4. High-voltage electrophoresis of standard acetylcholine S(Ach), standard acetylcholine from chromatography eluate S(ChE) test substance from chromatography eluate T(ChE) and snake venom SV. Parameters for electrophoresis: 60 V/cm for 30 min on Whatman paper 3MM. The spots were made visible by spraying with ethanolic hydroxylamine ferric chloride

Discussion

The presence of an acetylcholine-like substance in the venom of *Dendroaspis jamesoni* has been demonstrated by WELSH [13] using paper chromatography alone. WANGAI et al. [12] have identified, by both chemical and biological methods, our acetylcholine-like substance in the venom of *Dendroaspis angusticeps*. In the present work, paper chromatography demonstrated that the test substance in the venom had R_F values corresponding to those of standard acetylcholine in two solvent systems, *viz.*, n-propanol: formic acid: water and n-butanol: ethanol: acetic acid: water (See Table 1).

The test substance elicited spasmogenic responses in guinea pig ileum which were blocked by atropine sulphate, suggesting that the substance under investigation had muscarinic properties like acetylcholine. This spasmogenic activity of the substance was markedly reduced by enzymatic and alkaline hydrolysis. The residual activity could be attributed to the presence of choline in the hydrolysate. Further evidence of the muscarinic properties of the substance was shown, when the hypotensive response produced by the test substance and standard acetylcholine was completly blocked after atropinization (Fig. 3). The acetylcholine-like substance in venom contracted frog rectus abdominis muscle as did standard acetylcholine. This effect was completly blocked by d-tubocurarine and potentiated by eserine, thus suggesting that the substance has nicotinic properties as well.

The results of high-voltage electrophoresis provide further evidence that the test substance in *D. jamesoni* venom is acetylcholine, with slight reduction in mobility during electrophoresis, presumably because of strong binding of the test substance to the venom.

TELANG et al. [11] demonstrated the presence of an acetylcholine-like substance in the venom of *Dendroaspis jamesoni* by its effects on the contractions of nictitating membrane of cat after close intra-arterial injection of lyophilized venom into the superior cervical ganglion. There was no significant difference between the values obtained by us using the two assay methods, but were higher than those published by TELANG et al. [11]. The variation in assay results can be attributed to a number of factors, like the age of the snake, the season of the year, variation in the method of obtaining and preparation of dry venom.

The physiological or toxicological role of acetylcholine in the venom of *D. jamesoni* has not been established. KEELE and ARMSTRONG [8] suggested that acetylcholine commonly found in snake and other venoms may play a defensive role as one of the pain-producing tissue hormons. Furthermore the local vasodilator effect of acetylcholine may promote the absorption of other toxic constituents of the snake venom [13]. A bite from *D. angusticeps* yields a total of 60—95 mg of dry venom [10]. It is assumed that *D. jamesoni* bite yields about the same amount of dry venom. From the results of the bioassays, the amount of acetylcholine injected in a single bite of *D. jamesoni* would be about 0.27—0.43 mg. According to GOODMAN and GILMAN [4] 90—140 mg of acetylcholine is required to produce vasodilation and hypotension in an adult human being. It is therefore concluded that the acetylcholine in the *D. jamesoni* venom is far too little to account for the shock produced by the snake venom in man.

Acknowledgements

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ELECTRON MICROSCOPIC INVESTIGATION OF A GIANT NEURON IDENTIFIED IN THE RIGHT PARIETAL GANGLION OF *LYMNAEA STAGNALIS* (L)

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Abstract

The ultrastructure of a giant neuron (GRP_1) identified in the right parietal ganglion of *Lymnaea stagnalis* was examined. The results suggest that GRP_1 is a neurosecretory cell. The perikaryon is characterized by many neurosecretory-like granules of variable electron density. The average diameter (longer axis) of the granules was 1850 Å. Furthermore highly-developed rough endoplasmic reticulum and Golgi complex were observed in the cytoplasm. Synapse-like structures were often found on the somatic membrane, but typical, true synaptic endings were not seen.

Introduction

A number of giant neurons have been identified in the central nervous system (CNS) of *Lymnaea stagnalis* by electrophysiological methods [8, 12, 17]. In spite of this practically no data are available concerning the ultrastructure of giant neurons. There are some ultrastructural data on small neurons, especially neuroseretory ones [2, 13, 16].

A giant neuron A10 in the visceral ganglion has been investigated by both electrophysiological and morphological methods [6]. This neuron is electrotonically connected with another giant neuron (P1) located in the right parietal ganglion [5]. Both neurons (A10 and P1) have a whitish colour under light microscope, which is known to be characteristic of neurosecretory cells [16, 17]. According to ultrastructural observations [6], A10 proved to be a neurosecretory cell.

Our present investigations were aimed at clearing up the fine-structural characteristics of the giant right parietal P1 neuron (GRP_1) and comparing the results with data available for some other neurons. The question arose whether the ultrastructure of GRP_1 is similar to that of A10, that is, they are symmetrical giant neurons like those described in several gastropods [3, 15].

Materials and methods

The fine structure of the isolated GRP_1 of *Lymnaea stagnalis* previously identified electrophysiologically was investigated.

Double fixation method was employed: after fixation in 2.5% glutaraldehyde buffered with 0.1 *M* cacodylate (pH 7.2) for 1 h at room temperature, the materials were washed three times for 2—3 min in the same buffer enriched with 7.5% sucrose. Isolated cells were then placed into 8% solution of agar-agar. Post-fixation was performed in ice-cold 2% OsO_4 buffered with s-collidin (pH 7.2), for 1 h. After dehydration in graded alcohol and propylene oxide (uranyl acetate block staining was done in 75% alcohol), the materials were embedded in Araldite (Durcupan, ACM, Fluka). Ultrathin sections were cut on an LKB III ultratome, stained with lead citrate and examined in "Tesla-BS-413-A" and "Tesla-BS-513-A" electron microscopes. Ultrathin sections were cut after identification of the cell in semithin sections (1—2 μm) stained by 1% toluidine blue.

Results

GRP_1 neuron is situated in the right parietal ganglion (Fig. 1).

Light microscopic investigations of GRP_1 showed that it was characterized by an ovoid form and an average diameter of 140 μm.

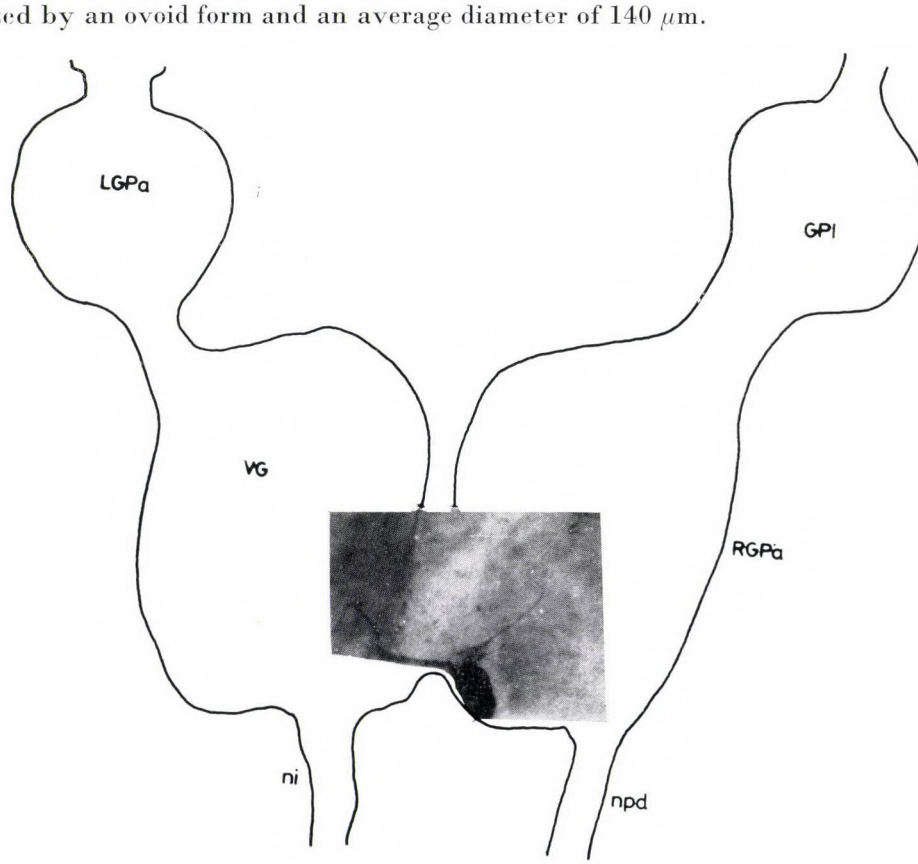

Fig. 1, Position of GRP_1 in the CNS. LGPa — left parietal ganglion; RGPa — right parietal ganglion; VG — visceral ganglion; GPl — pleural ganglion; ni — nervus intestinalis; npd — nervus pallialis dexter

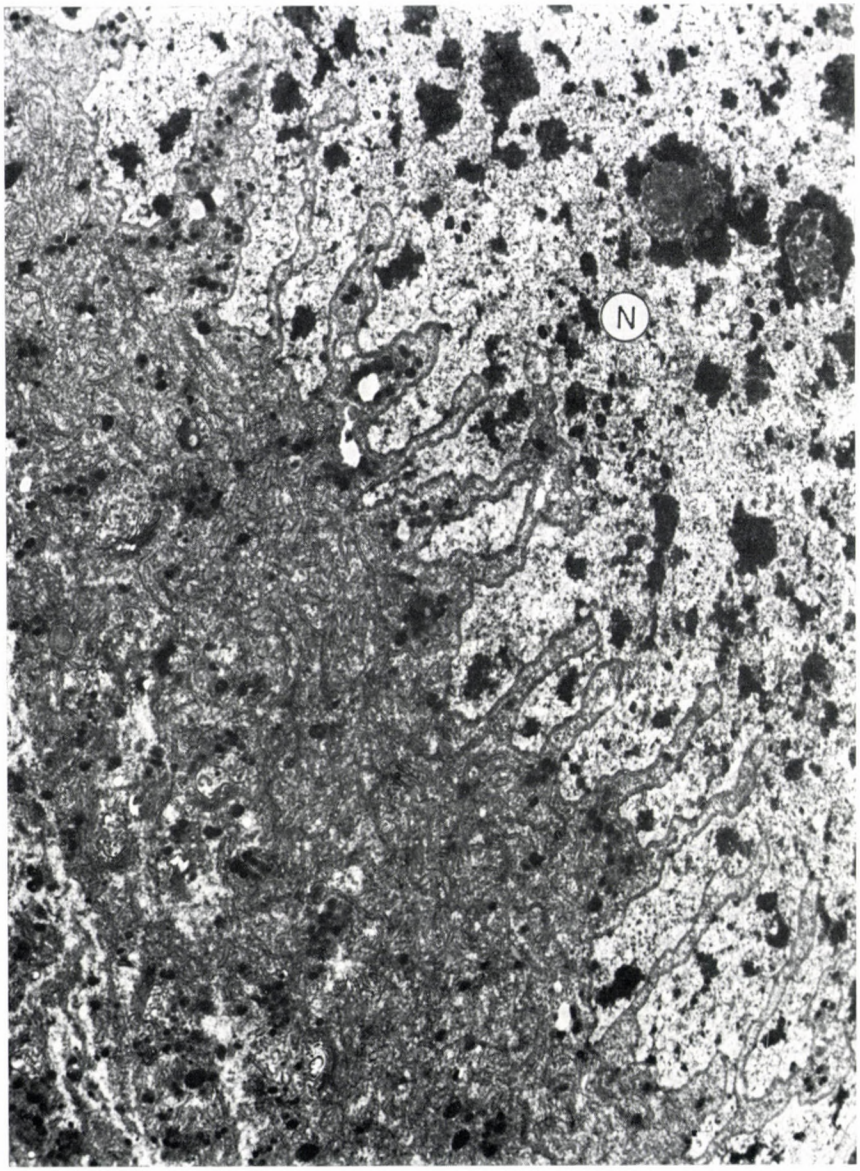

Fig. 2. Electron micrograph taken from the soma of GRP_1. N — nucleus ×10,000

Electron microscopic analysis showed that the soma of the cell is surrounded by a high number of satellite glial processes. One of the most characteristic fine-structural properties of the nuclear membrane was the numerous invaginations (Fig. 2), and chromatin was distributed in the immediate vicinity of this membrane (Fig. 3). In the cytoplasm of GRP_1 soma, highly-developed

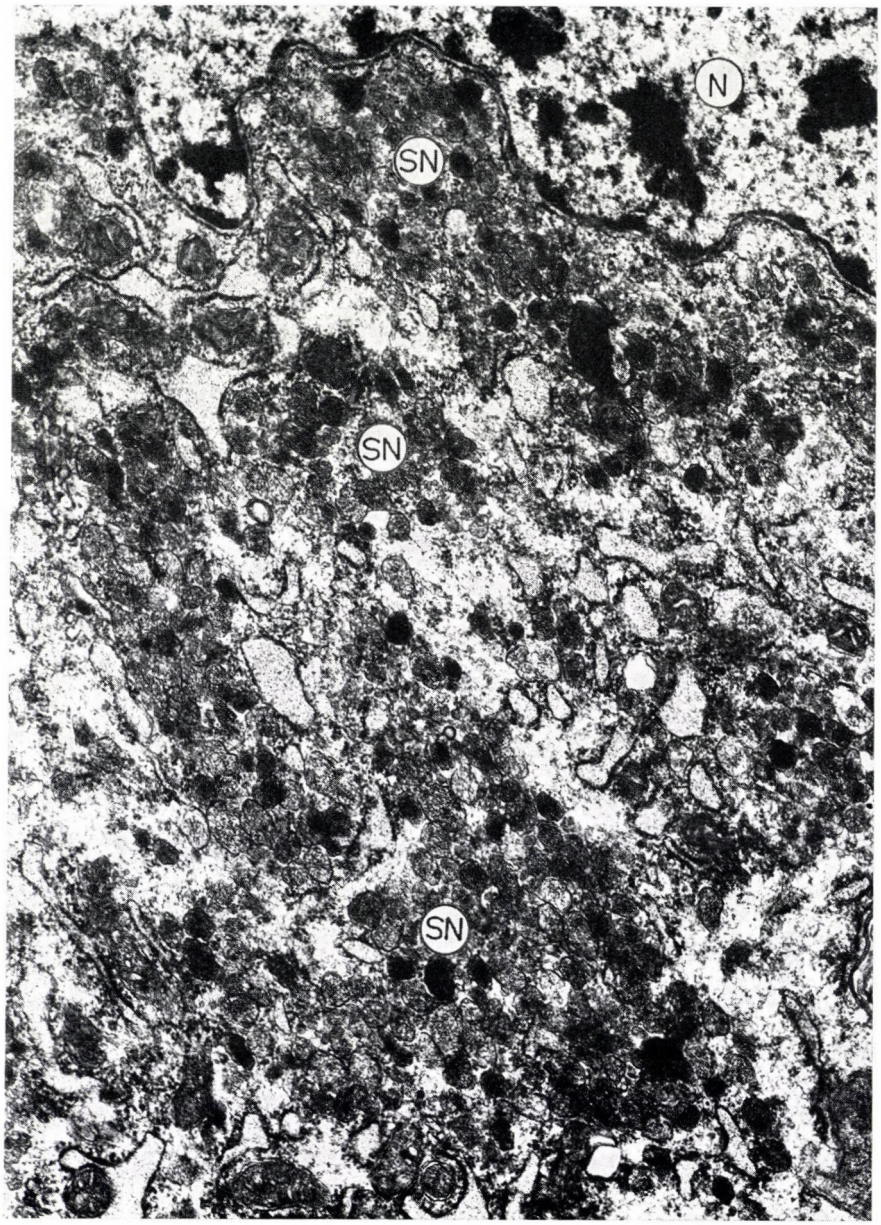

Fig. 3. Neurosecretory-like granules (NS) with dense or finely granulated material in the soma of GRP_1. N — nucleus ×22,500

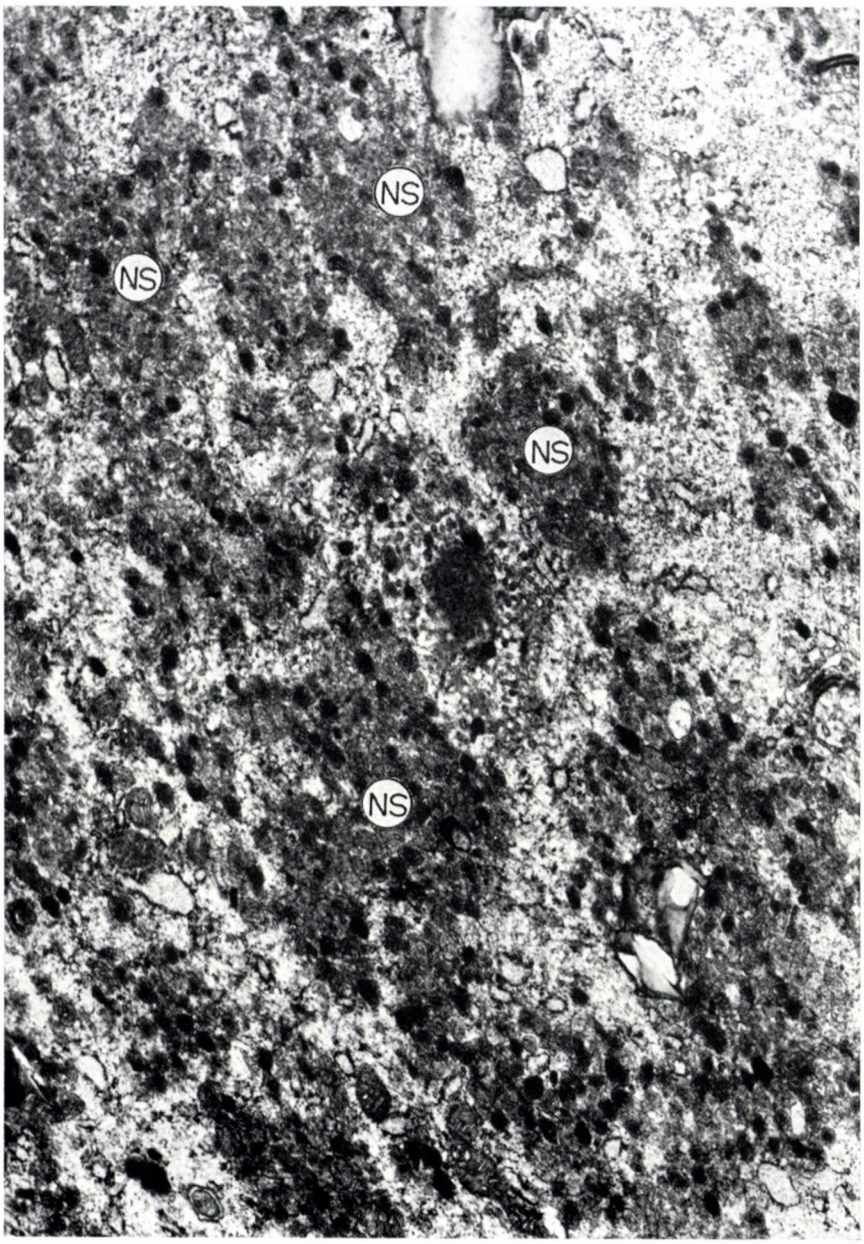

Fig. 4. Aggregations of the neurosecretory-like granules (NS) in the perikaryon ×18,000

rough endoplasmic reticulum (RER) was observed. Free ribosomes were often found in the cytoplasm. Sometimes rosettes of glycogen granules and lysosomes were seen. The Golgi complex was well-developed and frequently contained electrondense material. Electrondense material was commonly present at the internal surface of the tubular membrane, cisternae and vesicles of the Golgi complex. Occasionally budding off of electrondense granules was observed at the cisternae of the Golgi elements.

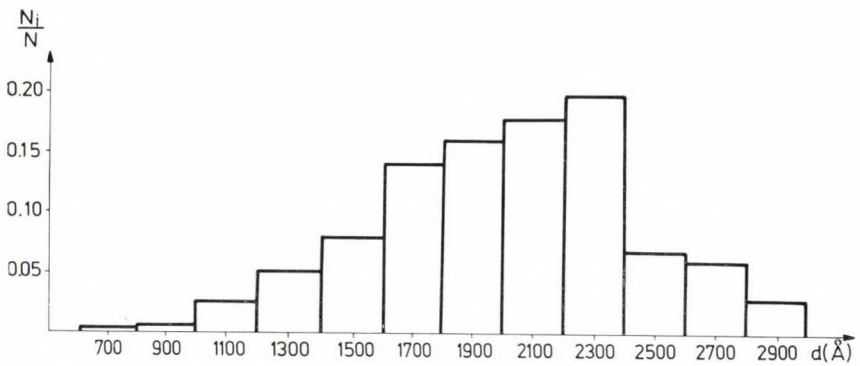

Fig. 5. Frequency distribution histogram of the granules

The soma of GRP_1 was characterized by a great number of neurosecretory-like granules of variable electron density (Figs 3, 4). Some of them contained electrondense material surrounded by unit membrane, others had lower electron density and contained a finely-granulated material (Figs 3, 4, 7). Since the shape of the granules in the soma was in general ovoid, both axes were measured, and the longer ones are represented in frequency distribution histogram (Fig. 5). The average size of the large axis is 1850 Å and that of the smaller one is 1310 Å. The neurosecretory-like granules often formed typical aggregations (Fig. 4). The density of the aggregations was much higher as compared with other parts of the cytoplasm. These populations of the granules were found all over the cytoplasm, both in the perinuclear and peripheral regions of the soma.

When investigating the ultrastructure of GRP_1 neurons prepared and fixed in different seasons some seasonal variation was observed. In autumn, the RER was well-developed and the cytoplasm was poor in granules. In other seasons, swelling of RER elements and an increase in the number of granules were demonstrated.

Synapse-like structures were often found on the surface of the soma, which invaginated into the membrane (Figs 6, 7), but no typical, true synaptic ending was shown among them. The synapse-like axon profiles contain various

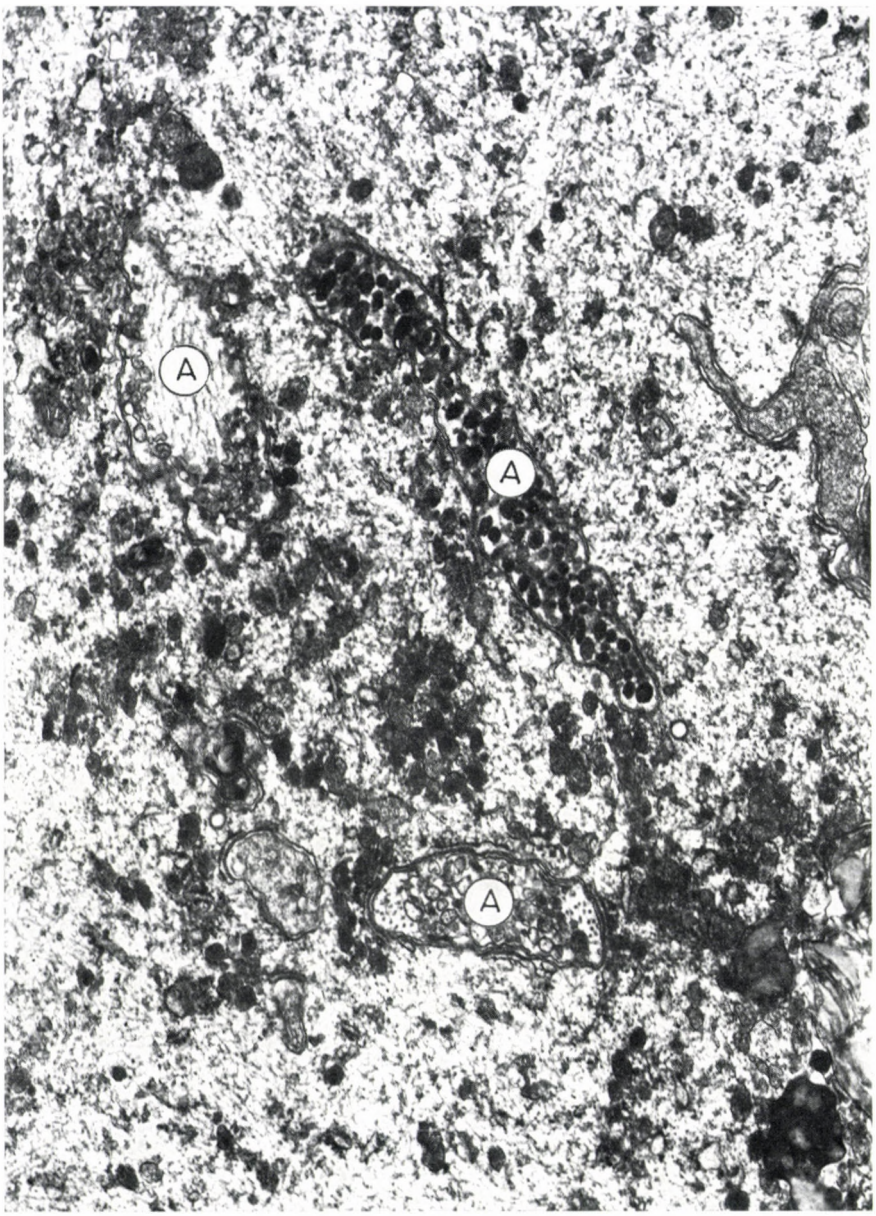

Fig. 6. Axons (A) invaginating the soma of GRP_1 containing dense-core clear vesicles and dense granules

types of vesicles (Fig. 6): dense-core and clear synaptic vesicles as well as highly electron-dense granules. The synapse-like structures were often surrounded by cytoplasmic cisternae and RER elements (Figs 6, 7).

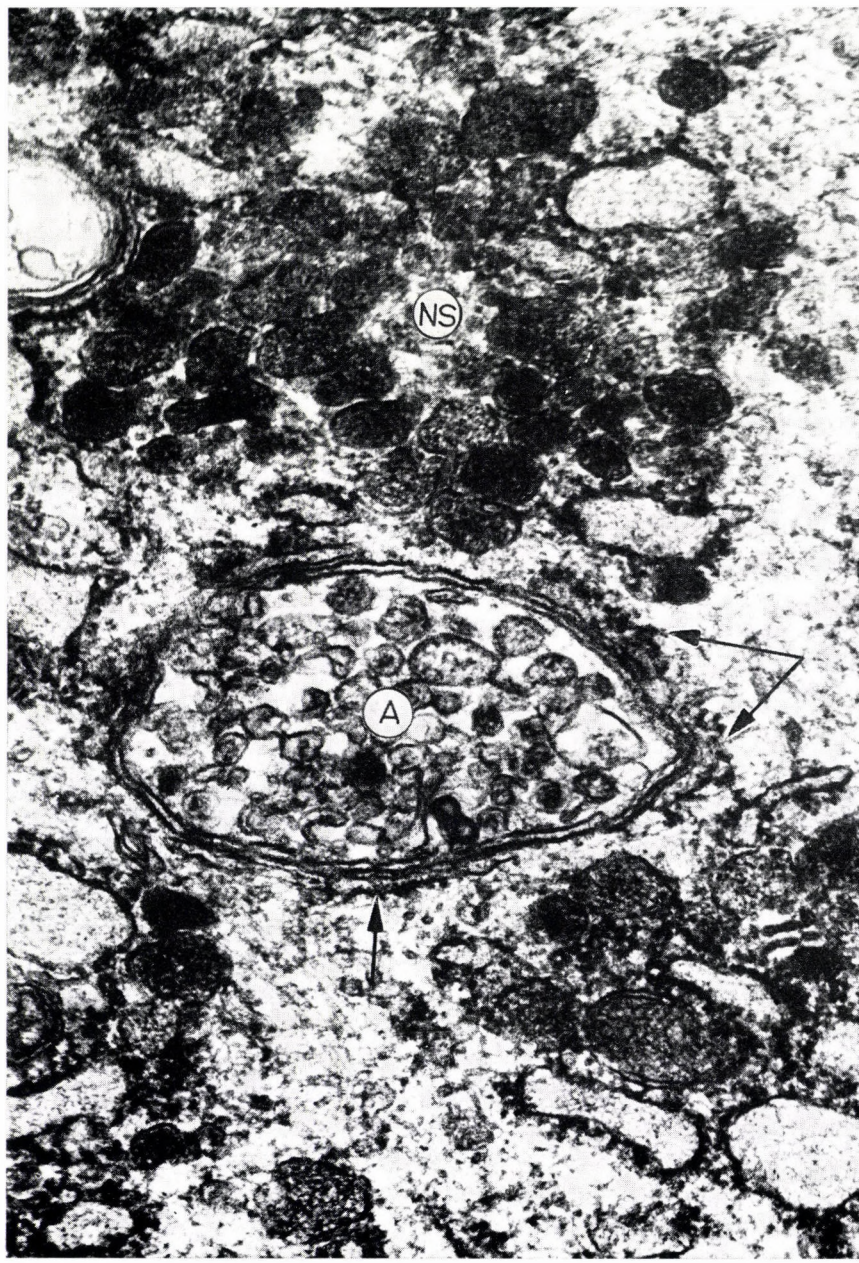

Fig. 7. Invagination of a synapse-like axon (A) into the surface of the soma. Note the population of neurosecretory-like granules (NS) with dense or granulated material in the perikaryon. (Arrows point to the RER elements in the vicinity of the synapse-like axon) ×54,000

Discussion

According to a new concept of neurosecretion, the results obtained with classical staining methods are more and more being questioned (cit. in TOMBES [14]). The electron microscopic demonstration of the elementary neurosecretory granules as well as determination of their shape and size have become predominated [9]. In the present work only the ultrastructural characteristics of the neuron were investigated. The appearance and the size of the granules in the cytoplasm suggested that GRP_1 is a neurosecretory cell. Comparing the fine structure of the granules with that described in the small neurosecretory cells of *Lymnaea* [10, 13, 16], we found no similarity. On the other hand, BOER et al. [2] described a giant "dark green" cell (GDGC) in the right parietal ganglion of another fresh-water snail *Bulinus truncatus* in the very same position as GRP_1. The fine structure of the elementary secretory granules in GDGC seems to be similar to that of most of the granules observed in the present work: they show a granular inner content of variable density. Accordingly GRP_1 might be regarded as a giant "dark green" cell being homologous to GDGC. Surprisingly, this cell has not been described by any of the authors who have examined the right parietal ganglion of *Lymnaea stagnalis* [2, 13, 16] by either classical histochemical methods or electron microscopy. The ultrastructure of GRP_1 is also similar to that of the giant neurosecretory cell A10 examined by KISS and BENEDECZKY [6], as concerns the morphology of granules and their arrangement in the cytoplasm.

The seasonal differences in the ultrastructure of GRP_1 (*e.g.* in the number and fine structure of the elementary neurosecretory granules, in the morphology of RER elements) suggest the possibility of seasonal change in the secretory activity of the cell.

It was demonstrated by intracellular recording from the soma of GRP_1 [7, 8] that different types of synaptic inputs converge onto the cell. In the present work synapse-like structures without any synaptic specialization containing various vesicles were shown on the cell surface. Similar structures were described by ROUBOS [10] and BOCHAROVA et al. [1] in *Lymnaea*. It may be supposed that these structures are the morphological basis of the above physiological results. More detailed ultrastructural examinations are required to clear up if there are also axo-somatic synapses or axo-axonal connections in the neuropile.

On the basis of the electrophysiological results [8], GRP_1 has been considered to be a secondary sensory neuron being in direct axonal connection with the intestinal nerve. Neurosecretory cells with sensory function have also been described in *Aplysia* [4]. The present results confirm that there are neurons in the central nervous system of gastropods which may have both neurosecretory and sensory functions.

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THE DORSOMEDIAL NUCLEAR GROUP OF CRANIAL NERVES IN THE FROG

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(Received 1977—10—24)

Abstract

The dorsomedial motor nuclei were demonstrated by the cobalt-labeling technique applied to the so-called somatic motor cranial nerves. The motoneurons constituting these nuclei are oval-shaped and smaller than the motoneurons in the ventrolateral motor nuclei. They give rise to ventral and dorsal dendrite groups which have extensive arborization areas. A dorsolateral cell group in the rostral three quarters of the oculomotorius nucleus innervates ipsilateral eye muscles (m.obl.inf., m.rect.inf., m.rect.med.) and a ventromedial cell group innervates the contralateral m. rectus superior. Ipsilateral axons originate from ventral dendrites, contralateral axons emerge from the medial aspect of cell bodies, or from dorsal dendrites, and form a "knee" as they turn around the nucleus on their way to join the ipsilateral axons. A few labeled small cells found dorsal and lateral to the main nucleus in the central gray matter are regarded as representing the nucleus of Edinger-Westphal. The trochlearis nucleus is continuous with the ventromedial cell group of the oculomotorius nucleus. The axons originate in dorsal dendrites, run dorsally along the border of the gray matter and pierce the velum medullare on the contralateral side. A compact dendritic bundle of oculomotorius neurons traverse the nucleus, and side branches appear to be in close apposition to the trochlearis neurons. A dorsomedial and a ventrolateral cell group becomes labeled *via* the abducens nerve. The former supplies the m. rectus lateralis, while the latter corresponds to the accessorius abducens nucleus which innervates the mm. retractores. Neurons in this latter nucleus are large and multipolar, resembling the neurons in the ventrolateral motor nuclei. Their axons originate from dorsal dendrites and form a "knee" around the dorsomedial aspect of the abducens nucleus. Cobalt applied to the hypoglossus nerve reaches a dorsomedial cell group (the nucleus proper), spinal motoneurons and sympathetic preganglionic neurons. Of the dorsomedial motor cells, the hypoglossus neurons are the largest, and a branch of their ventral dendrites terminates on the contralateral side. Some functional and developmental biological aspects of the morphological findings, such as the crossing axons and the peculiar morphology of the accessory abducens nucleus, are discussed.

Introduction

Groups of motoneurons supplying the external ocular muscles and the tongue muscles are called the somatic motor column, or the dorsomedial motor column, as opposed to the special visceral or branchiomotor column occupying a ventrolateral position in the brain stem. Due to their distinct Nissl staining properties, the position and topography of motor nuclei have been correctly recognized from the early literature to date [6, 11]. A number of questions

concerning finer details, on the other hand, still remain unresolved. For example, nothing is known about the representation of individual eye muscles in the oculomotor nucleus, or to what extent oculomotor fibres cross in *Amphibia*, although both questions have been thoroughly investigated in the mammalian brain [4, 10, 20, 25]. The abducens nuclear group presents another puzzle, in as much as ADDENS [1] distinguished an accessory nucleus for the innervation of the eye retractor muscles, while others [11] consider this nucleus to be part of the nucleus reticularis medius. BARNARD [2] and SENN [16] described a dorsal and a ventral division in the hypoglossus nucleus, but this distinction is denied by NIEUWENHUYS and OPDAM [11]. Visceral motoneurons of the oculomotor nucleus are mentioned only by SENN [16], who states that some small neurons among the oculomotor neurons may represent the nucleus of Edinger-Westphal.

From the application of the cobaltous sulphide technique to the cranial nerves in the frog, we have obtained a body of data on the neuronal organization and fibre connections of the branchial cranial nuclei [9]. In the present paper, we give an account of a series of similar investigations made on the dorsomedial nuclear group of cranial nerves, including the nuclei of the oculomotorius, trochlearis, abducens and hypoglossus nerves.

Materials and methods

The investigations were performed on the common water frog, *Rana esculenta*. In anaesthetized animals (tricain methanesulphonate) the cranial nerve used for cobalt filling was exposed through a pharyngeal approach. Through an incision of the mucous membrane overlying the roof of the mouth, the cranial cavity was opened by removal of a portion of the parasphenoidal bone. The oculomotorius and trochlearis nerves were found in the dural sac medial to the trigeminus nerve. The abducens nerve was found in a common bundle with the trigeminus and facialis nerves. For a complete exposure of the hypoglossus nerve, the lateral occipital bone had to be removed. In many cases, the trigeminus nerve and the ganglion prooticum were also excised in order to make room for the plastic tube containing cobaltous chloride solution. In order to study the representation of the four external ocular muscles innervated by the oculomotorius nucleus, branches of the oculomotorius nerve were dissected in the orbit and filled separately with cobalt, each branch in two animals. Nerves to the m. rectus medialis, m. rectus inferior and m. obliquus inferior were dissected in supine frogs through the oral cavity, and the nerve to the m. rectus superior was located from a dorsal approach after removal of the superior eyelid. We failed to discern an elevator muscle to the eyelid in the frog. With a similar technique, and for the same reason, branches of the abducens nerve supplying the m. rectus lateralis and the mm. retractores were also filled separately with cobalt, each branch in two frogs. Altogether 43 frogs were used in the present study.

The axonal filling with $CoCl_2$ was performed according to the technique described previously [18]. Briefly, the proximal stump of the dissected nerve was introduced into a small polyethylene tube which contained $CoCl_2$ solution (0.11 *M*). To facilitate transport, 0.013 g bovine serum albumin was added to 100 ml of $CoCl_2$ solution [17]. The cobalt filling ran for two days, with the animal being kept in a shallow pool of diluted (1 : 5000) anaesthetic at 4 °C. Cobalt ions taken up by the nervous tissue were precipitated with a 0.11 *M* solution of sodium orthophosphate saturated with H_2S. The brain was fixed in 70% ethanol, quickly dehydrated and embedded in paraffin. Serial sections of 20 μm thickness were made, and the CoS precipitate was intensified with a modified TIMM's [24] procedure. As suggested by GALLYAS [5], the original TIMM's developer was replaced by the following mixture: Solution A: 4% gelatine and 40% ammonium nitrate, solution B: 1% silver nitrate, solution C: 10% hidroquinone.

The developer was freshly prepared by combining 8 volumes of solution A to 1 volume of solution B, and adding 0.5 volume of solution C. The sections were kept in the developer for 10 min, then counterstained with methylene blue.

Results

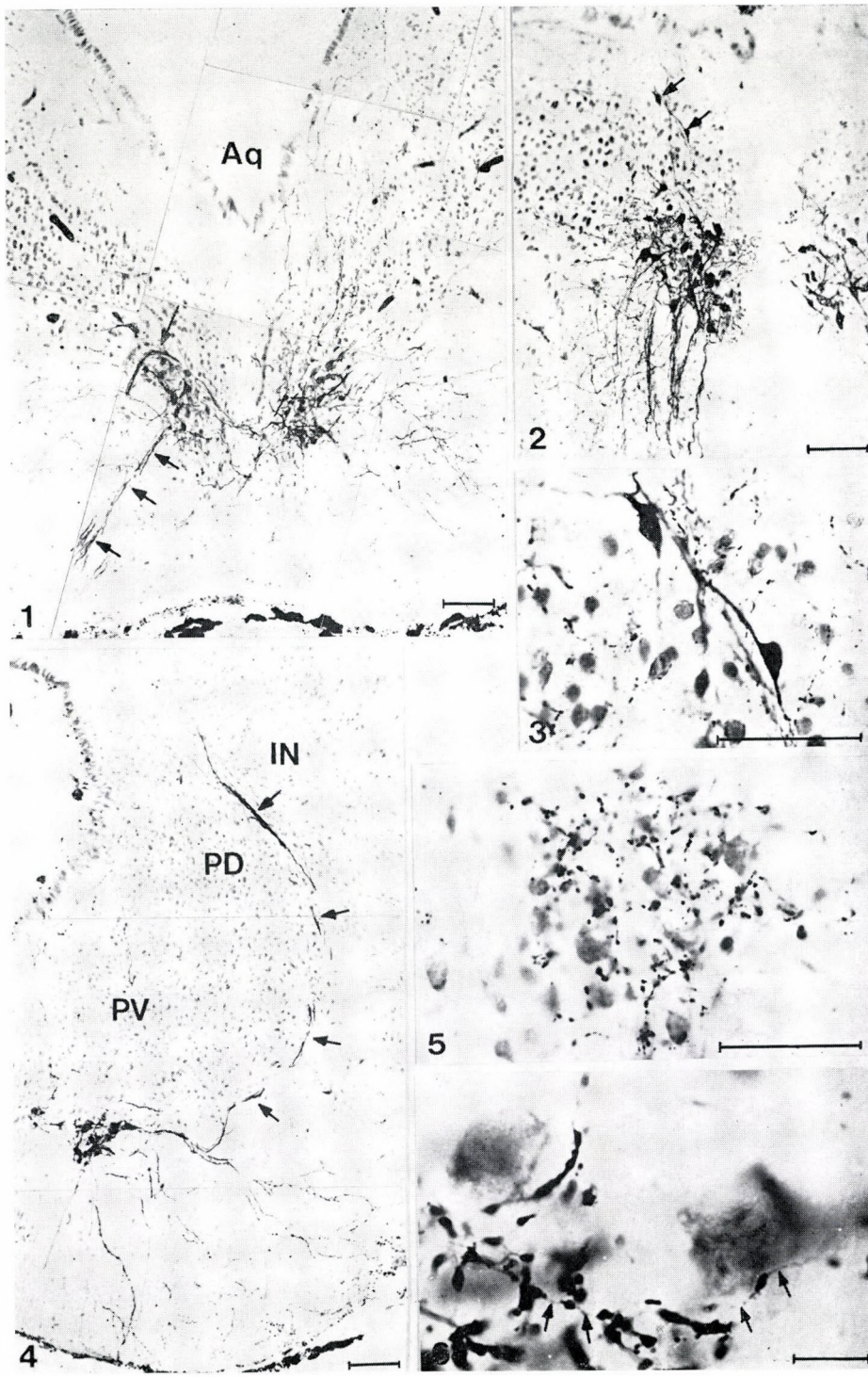
Nucleus oculomotorius

The nucleus oculomotorius is an elongated accumulation of motoneurons in the tegmentum mesencephali. The oral pole reaches the pretectal region, while the caudal pole is continuous with the trochlearis nucleus (Fig. 7). The longitudinal extent of the nucleus is about 800 μm. The ventromedial aspects of the two nuclei fuse in the caudal part.

The oval-shaped and polygonal motoneurons are relatively small, measuring 17—25 μm in the longest diameter. The dendritic arbor spreads out in a fan-like manner with the main branches oriented in the dorsal and in the ventrolateral direction. With its several side-branches directed medially and laterally, the dendritic arbor covers a semicircular area extending from the floor of the mesencephalic aqueduct to the interpeduncular nucleus (Figs 1, 2, 14). This area includes the subependymal zone, the anteroventral and posteroventral tegmental nuclei of POTTER [12], the fasciculi tegmentales, the nucleus opticus tegmenti in the oral part of the dendritic arbor, and a number of unidentified tracts and structures in the basis of the mesencephalon. The dendrites extend rostrally into the posterior third of the diencephalon, where the fine end-twigs terminate in the subependymal zone and in the hypothalamic white matter. In the caudal direction, the dorsal and ventrolateral dendrites cease fairly abruptly at the caudal pole of the nucleus; then a narrow and compact bundle of dendrites stretches caudally among the neurons of the trochlearis nucleus (Fig. 5). Side branches of this caudal dendritic bundle closely follow the proximal dendrites of trochlearis motoneurons, and dendritic beads appear to be in close apposition to motoneuron somata (Fig. 6). There is a distinct impression that some kind of contacts are established between oculomotorius dendrites and trochlearis neurons. This caudal dendritic bundle terminates exactly at the caudal pole of the trochlearis nucleus.

Axonal cobalt filling of the oculomotorius nerve on one side results in bilateral labeling of both nuclei (Figs 1, 2). On the contralateral side, most of the labeled neurons are localized in the ventromedial aspect in the caudal three-quarters of the nucleus. Neurons in the rostral ipsilateral pole are not labeled from the contralateral side.

As a rule, the ipsilateral axons emerge from a ventrally directed, stronger dendritic stem, and form several small fasciculi which converge at the site of the nerve exit. Axons of the contralateral neurons originate either from the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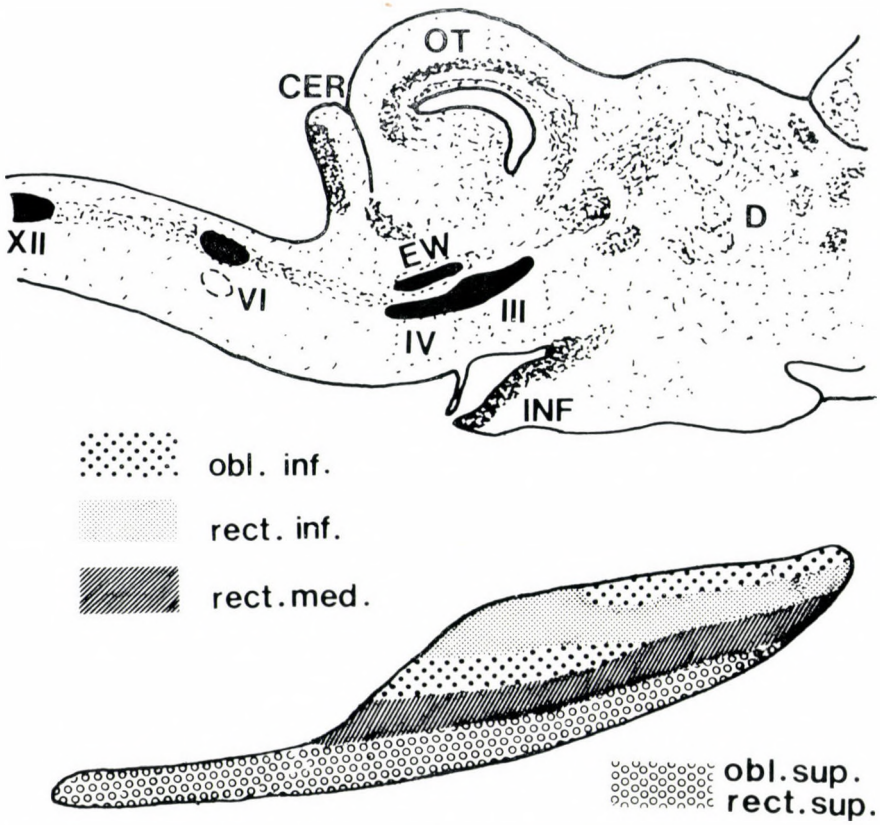


Fig. 7. Drawing of a paramedian section of the brain stem showing the location of the dorso medial motor nuclei. D = diencephalon, OT = optic tectum, CER = cerebellum, INF = infundibulum, EW = nucleus of Edinger-Westphal. Roman numerals indicate the respective cranial nerve nuclei. Broken line at VI outlines the accessorius abducens nucleus, which lies laterally to this level of sectioning. In the bottom drawing, the representation of the individual eye muscles is shown in an enlarged scheme of the oculomotorius–trochlearis nuclei. Symbols of the eye muscles are indicated on the left

←

Fig. 1. Photomontage of the oculomotorius nucleus. The left oculomotorius nerve was filled with cobalt. Small arrows point at the root of the ipsilateral axons, the large arrow indicates the dorsal "knee". Aq = mesencephalic aqueduct

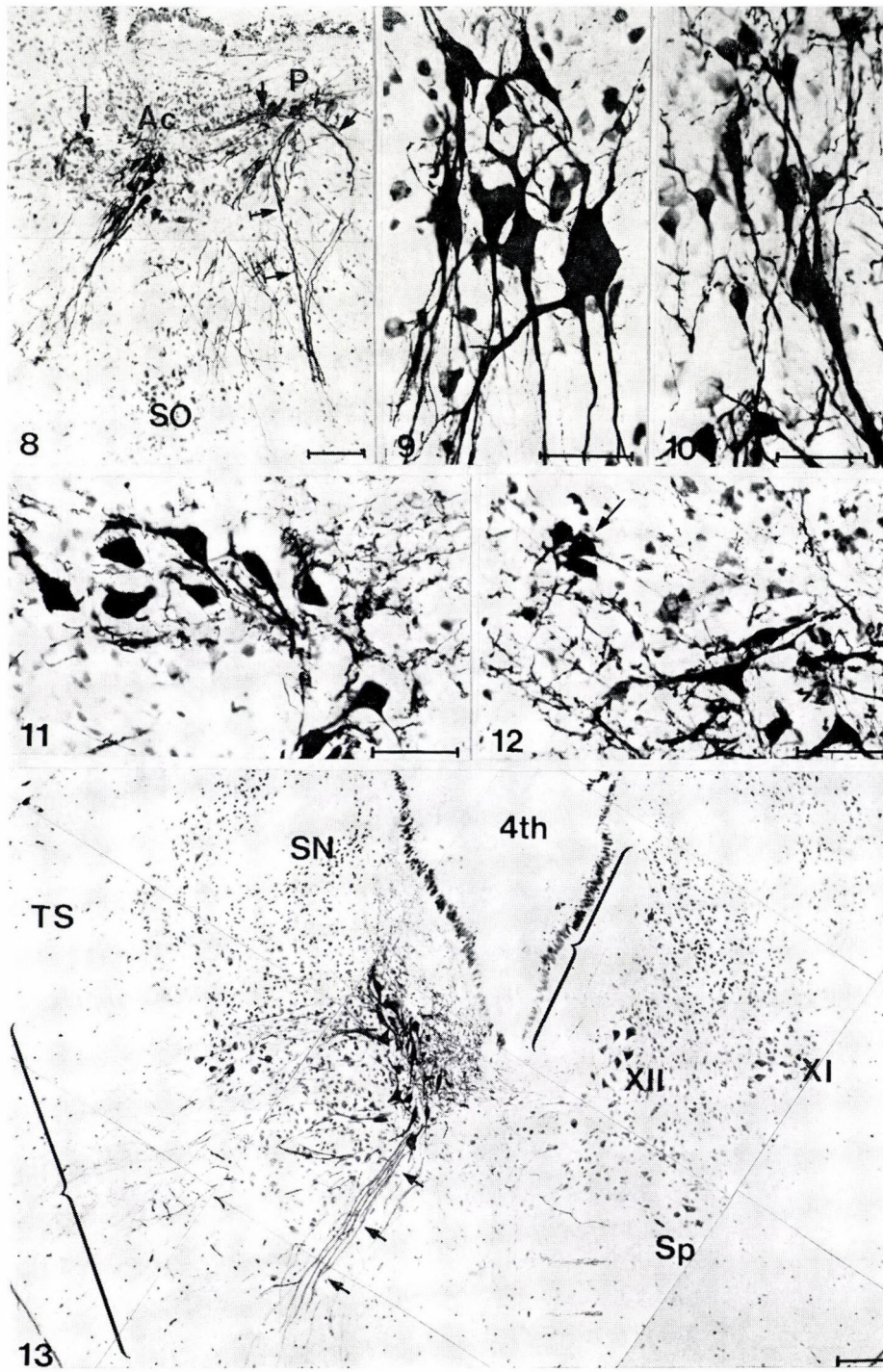
Fig. 2. The oculomotorius nucleus is shown from another specimen. The nerve on the left side was filled with cobalt. Arrows point at the visceromotor neurons representing the nucleus of Edinger-Westphal. Note the floor of the mesencephalic aqueduct at the top of the picture

Fig. 3. The same two visceromotor neurons shown at higher magnification

Fig. 4. Photomontage showing the trochlearis nucleus at a level just rostral to the nucleus isthmi. Arrows point at the axons. PD = nucleus posterodorsalis, PV = nucleus posteroventralis of the mesencephalic tegmentum, IN = the most rostral aspect of the nucleus isthmi

Fig. 5. The caudal dendritic bundle of the oculomotorius is shown among the neurons of the trochlearis nucleus

Fig. 6. The same with higher power. Note the finely-beaded dendritic branches (arrows) meandering among trochlearis neurons. The calibration bar is 100 μm in *Figs 1, 2, 4*, 50 μm in *Figs 3, 5* and 10 μm in *Fig. 6*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medial aspects of the perikarya or from a dorsal dendrite. Crossing axons form a dorsal and a ventral bundle (Figs 1, 14). The first bundle bends dorsally at the midline and forms a distinct "knee" around the dorsal aspect of the nucleus. The ventral bundle arrives at the ventral aspect of the nucleus, where the axons turn sharply ventrally, also forming a "knee" when joining ipsilateral axons. A few axons may follow a route between the two "knees", especially at the caudal pole of the nucleus.

With separate axonal filling of the 4 branches of the oculomotorius nerve, it was shown that the m. rectus superior is innervated by contralateral neurons which are predominantly found in the ventromedial part of the caudal three-quarters of the nucleus (Fig. 7). A few neurons, however, can also be encountered in a dorsolateral position. The areas of representation of the three ipsilateral muscles greatly overlap in the rostral three-quarters of the nucleus. The most rostral pole is occupied by neurons which innervate the m. obliquus inferior. They are closely followed by neurons innervating the m. rectus inferior, then by neurons of the m. rectus medialis. The neurons of these three muscles are located mainly in the dorsolateral part of the nucleus.

In specimens with the entire oculomotorius nerve filled with cobalt, a few labeled neurons appear dorsal and lateral to the nucleus in the periventricular gray. They possess spindle-shaped perikarya with two larger dendrites, which moderately arborize in the gray matter (Figs 2, 3). Both in their shape and their size (12—15 μm), they closely resemble the neurons in the nucleus salivatorius, and may therefore represent the visceromotor (Edinger-Westphal) nucleus innervating the internal ocular muscles through the ganglion ciliare. The nucleus is small, containing an average of two cells in each section of 20 μm in the caudal half of the oculomotorius nucleus.

Fig. 8. Photomontage showing the abducens nuclear complex. P = principal abducens nucleus, Ac = accessory abducens nucleus, SO = superior olive. Small arrows point to the "knee" formed by the accessory abducens axons, arrows with bar show the principal abducens axons. Large arrow points to two small neurons which represent the salivatory nucleus and which were labeled through the facialis nerve. Note the floor of the 4th ventricle at the top

Fig. 9. Neurons of the accessory abducens nucleus at high magnification

Fig. 10. Neurons of the principal abducens nucleus at the same magnification

Fig. 11. Neurons of the hypoglossus nucleus at the same magnification. The dorsal dendrites are seen in the right upper corner of the picture

Fig. 12. Spinal motoneurons and sympathetic preganglionic neurons (arrow) in the first spinal cord segment labeled through the hypoglossus nerve

Fig. 13. Photomontage from the obex showing the hypoglossus nucleus. Brackets show the dendritic arborization area in the ipsilateral white matter and in the contralateral subependymal zone. Axons (arrows) are retouched. SN = nucleus solitarius, 4th = fourth ventricle, Sp = spinal motor nucleus, TS = spinal tract of the trigeminus, XI, XII = accessorius and hypoglossus nuclei on the side contralateral to labeling. The calibration bar is 100 μm in *Figs* 8, 13 and 50 μm in *Figs 9, 10, 11, 12*

Nucleus trochlearis

This nucleus is smaller in cross sectional extent and contains fewer neurons than the oculomotorius nucleus. However, in the longitudinal direction it is not much shorter than the oculomotorius nucleus (approximately 600 μm), and appears to be the direct continuation of the ventromedial part of this nucleus innervating the m. rectus superior (Fig. 7). At the transition zone of these two nuclei, there is an anatomical overlap, since labeled and unlabeled cells can be found side by side for a distance of about 100 μm in the rostrocaudal direction. The caudal pole terminates at the middle level of the nucleus isthmi.

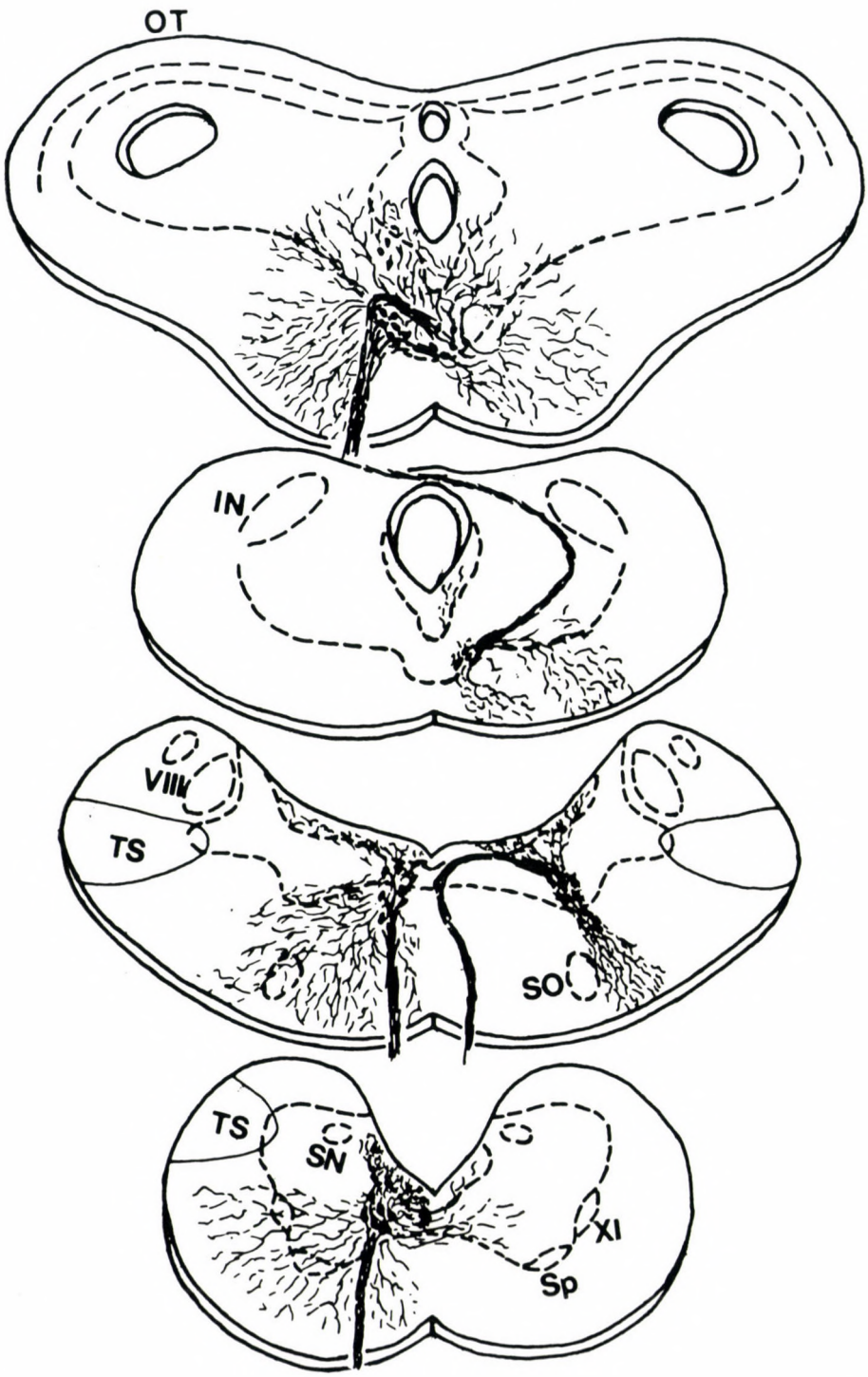
In the overlapping part of the two nuclei, the shape of the oculomotorius and trochlearis neurons is similar. They have relatively weak dorsal dendritic processes, with most of the dendrites being oriented laterally and ventrally. Somewhat more caudalward, the cell bodies assume a spindle shape and lie at the ventrolateral margin of the gray matter (Figs 4, 14). The two tips of the spindle give origin to the dorsolateral and ventrolateral dendrites. These emit collaterals which meander among the bundles of the fasciculi tegmentales and terminate in the ventrolateral white matter, leaving free the interpeduncular nucleus. Not infrequently one finds round "monopolar" neurons with a strong dendritic stem oriented in the lateral direction, dividing into a dorsolateral and a ventrolateral branch. These results give one the impression that the greatly enlarged nucleus posteroventralis of the mesencephalic tegmentum pushes the dorsal dendrites laterally, simultaneously compressing the dendritic arbor in the ventrolateral white matter. The rostrocaudal extension of the dendrites is much shorter than in the oculomotorius nucleus.

Trochlearis axons originate from the dorsolateral dendrites, following a gently-arched course dorsalward and slightly caudalward along the border of the gray matter. At the ventromedial aspect of the nucleus isthmi they become interposed between this nucleus and the nucleus posterodorsalis. In their further course, they pierce the velum medullare and emerge on the contralateral side.

Nucleus abducens

Filling the abducens nerve with cobalt reveals two distinct groups of motoneurons in the middle of the rhombencephalon (Figs 8, 14). One group, with its dorsomedial location, corresponds to the conventional abducens nucleus,

Fig. 14. Schematic drawings showing the dorsomedial motor nuclei at the corresponding cross sectional levels of the brain stem. The first section is at the level of the oculomotorius nucleus. Note the crossing axons. On the left side, dorsal to the nucleus, three black dots represent the nucleus of Edinger-Westphal. — The second section shows the right trochlearis nucleus filled from the left nerve. — In the third section the primary abducens nucleus is filled on the left side and the accessory abducens nucleus on the right side. — Bottom section shows the hypoglossus nucleus at the level of the obex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whose neurons can be selectively labeled through the nerve which innervates the m. rectus lateralis. The second group is located ventrally and laterally to the first, and obviously corresponds to the nucleus abducens accessorius of ADDENS [1], since its neurons can be selectively labeled from the nerve to the mm. retractores. Both nuclei are at the same rostrocaudal level as the nucleus salivatorius, between the facialis and glossopharyngeus motor nuclei.

The neurons constituting the principal abducens nucleus resemble the oculomotorius neurons both in size and shape (Fig. 10). A few larger polygonal cells can be found only in the caudal part of the nucleus. The perikaryon gives rise to a dorsal and to a ventral dendrite group. The dorsal dendrites branch in the subependymal zone and extend laterally into the region of the vestibulocochlearis nuclei. The ventral dendrites cover a wide area which includes the oliva superior laterally. The end-twigs of the long dendrite branches reach the subpial surface. The dendrites also have a significant rostro-caudal extension. The axons originate from the ventral dendrites and run straight toward the point of exit.

The accessory abducens nucleus is composed of polygonal neurons which are larger (23—30 μm) and are more densely packed than the cells in the principal nucleus (Fig. 9). The perikarya emit dorsal and ventral dendrites, of which the dorsal dendrites arborize in a manner similar to that in the principal nucleus. The ventral dendrites cover a much narrower area, in this they are reminiscent of the dendritic tree of branchiomotor nuclei [9]. Accessory abducens axons originate from the dorsal dendrites; take a dorsomedial course, and turn around the dorsomedial aspect of the principal nucleus, thus forming a well-pronounced "knee" under the floor of the 4th ventricle. They leave the rhombencephalon together with the axons of the principal nucleus.

Nucleus hypoglossus

At the exit from the medulla oblongata, the hypoglossus nerve merges with the first spinal nerve. In addition to motor fibres to the tongue, this nerve trunk carries fibres to the supra- and infra-hyoid muscles, to the deep trunk muscles, and to the ganglion sympathicum II [6]. Cobalt treatment of the whole nerve trunk labels the corresponding centres: a dorsomedial cell group in the obex region, motoneurons in the ventral horn of the first spinal cord segment, neurons in an intermediate position between these two groups and small, presumably preganglionic, sympathetic neurons (Figs 12, 13). Filling the nerve close to the tongue, where the branches to the m. hypoglossus leave the nerve, results in a labeling confined to the dorsomedial cell group, which may then be regarded as the nucleus proper of the hypoglossus nerve.

It begins in the upper obex region and runs a few micra caudalwards to the closure of the 4th ventricle, measuring about 600 μm in the rostrocaudal direction.

The hypoglossus neurons have oval-shaped perikarya which are definitely larger (30—35 μm) than the perikarya in the other dorsomedial nuclei (Fig. 11). A dorsal and a ventrolateral dendrite group originate from these neurons (Figs 13, 14). The dorsal dendrites invade the subependymal zone and form a dense plexus as far dorsally as the solitary nucleus. The ventrolateral dendrites arborize extensively in the gray and white matter, covering the area which lies between the motor root and the tractus spinalis trigemini. The branches traverse the vagus and accessorius motor nuclei as well, and terminate before reaching the subpial zone. A number of crossing branches can be followed to the contralateral side, where they arborize in the region of the hypoglossus nucleus and in the adjacent gray matter. The axons originate from the ventral dendrites, descend to the caudal pole of the nucleus, and unite with the axons of spinal motor cells.

Discussion

The findings presented in this study corroborate earlier descriptions on the location of the dorsomedial motor column, and extend our knowledge of the internal organization of these nuclei.

The innervation of individual ocular muscles corresponds to what has been found in the mammalian brain [4, 10, 20, 25]. Motoneurons innervating the m. obliquus inferior, m. rectus inferior and m. rectus medialis predominantly occupy a dorsolateral position in the rostral three-quarters of the nucleus. The m. rectus superior is innervated by motoneurons which are mainly found ventromedially in the caudal three-quarters of the contralateral nucleus. The caudal part, which contains only the ventromedial neurons, continues into the trochlearis nucleus, where the motoneurons which innervate the m. rectus superior and m. obliquus superior, constitute a common column. It is characteristic of the trochlearis and m. rectus superior motoneurons that their axons emerge from the dorsal aspect of the neurons and cross to the contralateral side.

The crossing axons have an interesting functional significance in the light of synergism in eye muscle actions vividly shown and discussed by SZENTÁGOTHAI [21]. The m. rectus superior contracts simultaneously with the contralateral m. obliquus inferior, and the m. obliquus superior with the contralateral m. rectus inferior in response to stimuli of the superior and posterior semicircular canal, respectively. Due to the crossing of axons to the upper eye muscles, the activation of ipsilateral motoneurons results in this pattern of parallel contraction of contralateral muscles. The functional significance of

the caudal dendritic bundle of oculomotorius neurons, which trails down among the trochlearis neurons, is less clear. Direct interaction between motoneurons is known to exist in the frog's spinal cord. Physiological experiments suggest electrotonic coupling [3, 7, 15], and the cobalt-labeling technique in the electron microscope studies indicates dendro-dendritic contacts [19] as the probable substrate in these interactions. It would be interesting to investigate whether the close appositions between oculomotorius dendrites and trochlearis cell bodies — as seen in the light microscope — may have a similar functional meaning.

The morphology and functional significance of the accessory abducens nucleus was first recognized by TERNI [23] and PREZIUSO [13] in reptiles, birds and mammals. In the frog, ADDENS [1] gave the first description of this nucleus, but he could not follow the axons, and did not regard this observation as definitive. These authors believed that the accessory abducens nucleus was a "trigemino-abducens reflex center", with the neurons migrating ventrolaterally towards the descending trigeminus root from the principal abducens nucleus in response to the influence of neurobiotaxis.

From the present observations, the existence of an accessory abducens nucleus is clear, though no morphological relationship can be found between the two abducens nuclei. The form and the dendritic arborization of neurons in the principal nucleus are like those in the oculomotorius nucleus, and the neurons of the accessory abducens nucleus resemble those in the branchiomotor nuclei. In the horizontal plane the accessory nucleus is located closer to the ventrolateral (branchiomotor) than to the dorsomedial (somatomotor) nuclei, while in the rostrocaudal direction it is almost the direct continuation of the facialis nucleus in the frog. Although it innervates a group of external ocular muscles, the morphological characteristics classify the accessory abducens nucleus into the branchiomotor, rather than into the somatomotor, group. This situation suggests that the branchiomotor and somatomotor classification should not be regarded as representing two discrete groups of motoneurons, but rather the two extremes of a continuum. The fact that in the frog the facialis nucleus does not have a knee, but the accessory abducens does, calls for a comprehensive comparative study on the question of whether the latter nucleus could not be regarded as the primordium of the true (mammalian-type) facialis nucleus with a knee around the abducens nucleus.

A short reference may be made to the unusual courses of axons (crossing and knee formation) of the m. rectus superior, trochlearis and accessory abducens neurons. It is common for these neurons to have their axons originate in the dorsal part of the perikarya, unlike in the rest of the cranial nerve nuclei. If one assumes that the parent cells were in the same position at the time of axonal outgrowth, then the initial orientation of growing axons appears to determine their course, at least in the initial section. A similar mechanism

has already been suggested to explain the projection pattern of sensory dermatomes upon the dorsal horn in the spinal cord [22]. This mechanism could explain why the m. rectus superior axons (oriented dorsomedially) cross the midline, the trochlearis axons (oriented dorsolaterally) take a dorsal course, and the accessory abducens axons (oriented dorsomedially) turn around the abducens nucleus. Another explanation for the decussation of the m. rectus superior axons may be that the parent cells migrated from the contralateral side, as claimed from a number of observations made on embryonic material [8, 14]. While this explanation may seem more plausible at first glance, it leaves open the question (suggested by the position of the oculomotorius knee) of why the most laterally and dorsally situated neurons "decided" upon a contralateral migration. The extension of this kind of investigation to embryonic material may throw more light on the mechanisms that govern directed axonal growth.

The first account of cranial vegetative nuclei in *Amphibia* was given in our earlier paper [9], where we described the exact positions of the nucleus salivatorius and of the preganglionic neurons in the glossopharyngeus-vagus nuclei. In the present work, we have succeeded in locating the Edinger-Westphal nucleus. Taken together, these data indicate that the frog's brain stem has the same number of vegetative nuclei, in the same positions, as the mammalian brain stem. The observation of sympathetic preganglionic neurons following cobalt treatment of the hypoglossus and the first spinal nerve shows that the spinal vegetative column begins in the most rostral part of the spinal cord. This finding concurs with GAUPP's [6] classic description of a ramus communicans originating from the hypoglossus nerve and joining the second sympathetic ganglion.

Acknowledgement

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RECENSIONES

Contemporary Topics in Immunobiology Vol. 5. Ed. W. O. WEIGLE, Plenum Press, New York and London (1976), pp. 341, Figures 47, Tables 39, US $35.40.

According to the Editor the aim of the fifths volume of the series containing nine chapters is to bring together some of the most exciting areas of immunology and thereby to act as a cross-stimulation to investigators in their particular areas of interest.

In the first chapter K. SHORTMAN and co-workers describe separation techniques for isolation of B-cell subsets and present a model of antigen-dependent By lymphocyte differentiation. In the second chapter A. S. ROSENTHAL and E. M. SHEVACH summarize the role of macrophages in the regulation of antigen recognition by the T lymphocyte. C. W. PIERCE and J. A. KAPP draw together the information from those systems in which suppressor T cells have been thoroughly characterized (Chapter 3). H. N. CLAMAN and J. W. MOORHEAD analyse specific acquired unresponsiveness ("tolerance") in contact hypersensitivity and support the concept that development of tolerance is an active process involving suppressor cells (Chapter 6). J. W. STOCKER and C. J. V. NOSSAL emphasize that one major element of discrimination between immune induction and tolerance in most physiological situations depends on the stage of maturation of immunocytes encountering with antigen. They favour the "clonal abortion" hypothesis of self-tolerance induction. (Chapter 5). H. D. ENGERS and H. R. MACDONALD describe *in vitro* system used for generation of cytotoxic T lymphocytes and discuss their significance *in vivo* (Chapter 4). Focusing on the latter D. D. MCGREGOR and A. A. I. KOSTIALA review the role of activated T cells in infection immunity (Chapter 7). Chapter 8 by R. T. KUBO and H. M. GREY gives up-to-date information on the structure and function of microglobulin. In the last chapter J. KLEIN attempts to interpret the mouse H-2 complex offering a unifying hypothesis.

The book is useful for readers interested in modern immunology.

I. FÖLDES (Budapest)

Ergebnisse der Mikrobiologie und Immunitätsforschung Vol. 75. Springer-Verlag, Berlin—Heidelberg—New York (1976), pp. 202, Figs 22, US $37.80

In this book there are five articles of current interest. *In vitro* Approach to Development to Immune Reactivity by A. GLOBERSON; Blocking and Unblocking Serum Factors in Neoplasia by S. C. BANSAL, B. R. BANSAL, and J. P. BOLAND; Bacteriophage T7 Genetics by R. HAUSMANN; IS Elements in Microorganisms by P. STARLINGER and H. SAEDLER and Structure and Molecular Biology of Rabies Virus. This volume contains also the Cumulative Author and Subject Index of Volumes 40—75 of the series. Very different fields of immunology and microbiology are covered; all the topics are of importance.

It is one of the most important methodological requirements for immunology of today to create *in vitro* systems for studies of differentiation of isolated tissues of cells. The critical review of the information so far obtained about culture techniques, cell interactions, differentiation, etc., may help further work on this field. The great interest in the possible role of serum

factors in neoplasia and the possibility that some components of the tumour tissue may disturb the immunological response of the host are easy to understand. The results and the hypothesis described may prove valuable for further research.

The central role of *E. coli* to develop basic genetics is flourishing once again though some authors forecast the end of the "*coli* era". The discovery of the significance of IS elements in *E. coli* and in its plasmids are important facts for geneticists studying any kind of organisms. The knowledge obtained in the last 10 years about the virion structure and molecular biology of rabies virus is clearly described and provides a good summary for those who are interested in rabies either from pathogenetical or epidemiological aspect.

The active role of the authors in the research of the relevant topics make the reviews critical and worth for reading.

G. Szabó (Debrecen)

Enviromental Mutagens

Proceedings of the 6th Annual Meeting of the European Enviromental Mutagen Society. Abhandungen Der Akademie Der Wissenschaften Der DDR Akademie-Verlag Berlin (1977), pp. 182, M 26.0

Three symposia with invited lecturers were arranged. The first symposium dealt with the very important problems involved in modifications by endogenous and exogenous factors of the mutagenic action of chemical agents. W. Klinger's interesting paper concludes that chemicals can influence drug metabolism and the mutagenicity of a compound in different ways. Inhibition as well as stimulation lead to an increase or a decrease of activation or inactivation, inhibition and stimulation can act in the same or in the opposite direction. This depends on dosage, time-response relationship and finally, on the properties of the metabolizing test system.

The topic of the second symposium was: the dose-effect relationships, threshold effects and the question of chronic *vs.* acute exposure.

Third symposium: effects of mutagenic chemicals in risked populations. This topic was of special interest with respect to the Seveso calamity. In addition, N. Loprieno's paper deals with occupational hazards: mutagenic activity of industrial compounds. Mammalian metabolic systems have been employed in the mutagenic analysis, stressing therefore the importance of the metabolic conversion of these compounds (stryrene, vinylchloride, chlorophene, trichlorethylene).

Two panel discussions took place: "Problems with bacterial screening tests incorporating metabolic activation" and "Testing chemical compounds in a multicomponent test system". It would be very useful for all the biologists, toxicologists and scientific researchers interested in environmental protection.

Kornélia Lehoczky (Budapest)

Coppel H. C., Martins J. W.: **Biological Insect Pest Suppression** Berlin—Heidelberg—New York. Springer Verlag (1977)
In: *Advenced Series in Agricultural Sciences. Vol. 4.* Co-ordinating Editor: Yaron B. Figs 49, Table 1, XIII., pp. 314, Cloth US $29.60

The book is divided into five parts as follows.

Glossary; Historical, theoretical and philosophical bases of biological insect pest suppression; Organisms used in classical biological insect pest suppression; Manipulation of the biological environment for insect pest suppression; A fusion of ideas

The book includes a list of References and a detailed Subject index.

This is a successful attempt to encompass the multifarious biologically based methods of insect pest population reduction. The glossary is valuable for understanding the subject. The reader finds, besides the historical, theoretical and philosophical bases of biological insect pest suppression, information regarding human influence on insects. The book contains the full scale of biological pest suppression including the use of parasitoids, nematods, insect, invertebrate and vertebrate predators, pathogenic microorganism, and co-ordinated pest suppression. Chapter 4 discusses the questions of resistance, autocidal control and genetic manipulation, as well as modulation of the metamorphosis and behaviour or different species. The last chapter provides useful information on the integrated pest suppression and the future directions.

The book is undoubtedly the most successful attempt to cover the field of insect pest suppression. It can be recommended for students and research workers alike. It will give valuable help to the practice and theoretical works as well. It can be used by beginners and trained workers as a book containing the necessary information in the field of insect pest suppression.

KATALIN S.-RÓZSA (Tihany)

Water and Plant Life Problems and Modern Approaches Ed. LANGE, O. L.—KAPPEN, L.—SCHULZE, E. D. Springer Verlag, Berlin—Heidelberg—New York (1976), pp. 536, Figs 178, DM 120, $49.20.

The book produced with the collaboration of 47 authors is the 19th volumes of the series: *Ecological Studies, Analysis and Synthesis* (Edited by W. D. BILLINGS, F. GOLLEY, O. L. LANGE, J. S. OLSON.)

The book handles one of the most fundamental problems of the economy of animate nature. One of its main characteristics is that starting from numerous aspects it presents the role of water in plant ecology on a very broad basis. Another main asset of the volume lies in the fact that it treats each subject in great detail, points out theoretical and practical problems of general interest and provides recommendations for future tasks and for their solution. Emphasis is moreover to be laid on the mostly successful endeavour to furnish a comprehensive picture of the significance of water for plant life from the molecular-ultrastructural level and from the entire plant organism to ecosystems and vegetation zones.

The book is organized into the seven major parts, namely, *(1)* The fundamentals of water relations, with special attention to water status within the plant tissues and within the soil-plant-atmosphere continuum; *(2)* to a number of characteristic types of roots and ecosystems; *(3)* Transpiration and its regulation and in this connexion stomatal response; *(4)* Direct and indirect water stress in relation to its effect on metabolism and special respect to ultrastructural, biochemical and hormonal relations; *(5)* Water relations and CO_2 fixation types as adaptive biochemical phenomena; *(6)* Water relations and productivity; water status as a determinant in the productivity of plants and plant communities, including aspects of artificial irrigation of plant cultures; *(7)* The importance of the water factor for the formation of vegetation patterns and the development of vegetation types.

The significance of the subject and of the book is enhanced by the fact that investigation of biological organic matter production is a task of world-wide interest both in theory and practice.

L. FRIDVALSZKY (Alsógöd)

ATHER ALI M. and ANCTIL M.: **Retinas of Fishes. An atlas.** Springer-Verlag, Berlin—Heidelberg—New York (1976), pp. 284, Figs 364, US $59.90

A collection of short texts and photomicrographs, this atlas is intended to present in a phylogenetic order data on fish retinal structure. Besides, it helps the visual physiologist or biochemist to select a particular fish species which has morphological characteristics compatible with his specific requirements.

The first part of the atlas is an introduction to the classical morphology of the retina in fishes. A short list of the main histological techniques used for studying fish retinal structure as well as a chapter about preparation of the retina is also included. The second part is a catalogue of retinal morphology of fish groups in a phylogenetic order. The material is presented in the usual atlas pattern. A text page on the left side describes the phylogenetic position of the fish group with a line diagram and a short paragraph of the group. There is a paragraph about the most striking morphological features of the retina, a collection of references and data about the visual pigments. The illustration page on the right contains a few photomicrographs taken from transverse and tangential sections of the retinas. The latters are especially useful to illustrate the oriented pattern of photoreceptor cells in the plane of the retina. The illustration material consists of good quality black-and-white photomicrographs of mostly well-preserved retinas. As the third part of the atlas, the reader finds a detailed bibliography and a synopsis of fish families according to the modern classification. The atlas can be of good use for neuroanatomists, physiologists and biochemists interested in vision biology.

P. RÖHLICH (Budapest)

LEUTHOLD, W.: **African Ungulates**
A Comparative Review of Their Ethology and Behavioral Ecology. Springer-Verlag, Berlin—Heidelberg—New York (1977), pp. 307, Figs 55, $31.70

The book has been issued as the 8th volume of the series *Zoophysiology and Ecology*. The Swiss-born author has been working for ten years as a research zoologist at the Research Center in Tsavo East National Park (Voi Kenya). All of his observations and conclusions are based on personal field observations. And this is of great scientifical importance. It is well-known that, in spite of the many thousandes of safaris undertaken during the last century in the savannas of East Africa and the huge number of ungulates fallen to the hunter's gun, the ethology of these interesting mammals had not been clarified until the last decades. Ethology is in our days one of the most rapidly developing area of the biological sciences and even its advences stress the misleading effects of the investigations carried out only on domesticated and laboratory animals. The observations made by European ethologists on the big mammals and common birds of our continent, however great in number, seem not to be reliables sources for general conclusions. *E.g.* for explaining the acting factors in the social behaviour and the social organization of the groups of even-toed ungulates are our observations made on dense populations of deers and roedeers quite unacceptable.

The present book is of undoubted value for the practical solving of many difficult ecological problems of Africa, called by some authors "The devasting continent".

A further value of this book is the abundant list of references — most of them unfamiliar for zoologists dealing with European ungulates. The photos taken by the author in the East African savannas and the schematic drawings supplement in every respect the carefully written text.

G. STOHL (Budapest)

Growth and Development; Physique.

Edited by

O. G. Eiben

(PROCEEDINGS VOLUME OF THE SYMPOSIUM ON HUMAN BIOLOGY, BALATONFÜRED, HUNGARY, SEPTEMBER 1976.) (SYMPOSIA BIOLOGICA HUNGARICA 20.)

This volume contains 50 papers of research workers from eighteen countries from all over the world. The first section discusses the genetic and environmental factors influencing growth processes. Thus, papers analyze the effect in growth and development and maturity of climate, socio-economical, nutritional, and other factors, the intrauterine growth, and the maturation of girls (age at menarche), as well as the phenomenon of secular trend and the so-called "acceleration" or growth. Some papers give a view about growth studies made in different countries, others deal with the cilinical and/or physical educational aspects of these investigations. The second part of the book presents papers analyzing the variations of physique. Besides the two main methods: somatotyping and factor analysis, there are studies connected with clinical and/or physical educational applications, as well as studies on body composition.

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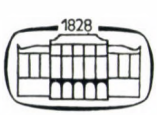

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