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INFLUENCE OF X-RAYS ON THE RATE OF ACCUMULATION OF ^{131}I IN THE THYROID OF GUINEA PIGS IN THE INITIAL STAGE OF RADIATION DISEASE

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(Received 1974—06—18)

Abstract

The influence of single, total body X-ray irradiation with 500 and 1 500 R on ^{131}I uptake in the thyroids of guinea pigs in the initial stage of radiation disease was studied. Radioactivity of the thyroid was measured 1, 3, 6, 24 and 48 h after i. p. injection of ^{131}I, i. e. 69, 71, 74, 92 and 116 h after the and of X-ray irradiation. Sixty-nine and 71 h after the and of X-irradiation, accumulation of ^{131}I was higher in the thyroids of male and female irradiated guinea pigs in comparison with controls. At 75 h after X-irradiation i. e. 6 h after administration of ^{131}I, a drop in the iodine content of the thyroid occurred, which lasted up to 48 h. The drop in ^{131}I uptake by the thyroid was particularly significant at 92 h after irradiation of male and female guinea pigs i. e. at 24 h after introduction of the isotope, in comparison with the controls.

Introduction

Numerous investigators have studied the influence of X-rays and ^{131}I on morphologic changes, induction of tumors and function and activity of the thyroid in humans and animals [1, 9, 11, 12, 14, 17, 21, 23—25].

Thyroid activity after irradiation with X-rays undergoes very distinct changes, as noted by BETZ [6] and MONROE and co-workers [15]. Whole body X-irradiation of rats causes increased accumulation of ^{131}I in the thyroid according to EVANS and co-workers [cit. in 8]. CLOSON and BETZ [8], on the other hand, noted a drop in iodine uptake by the thyroid 48 h after whole body X-irradiation of rats with 800 R.

Changes in activity and function of the thyroid after X-irradiation and in iodine metabolism are of particular interest in the first stage of radiation disease.

This study was concerned with the influence of large, total doses of X-rays on the accumulation of ^{131}I in the thyroid of guinea pigs in the initial period of radiation disease. The relationship between the dosage of X-rays and rate of ^{131}I accumulation, and the possible sex differences were also studied.

Material and methods

Forty sexually mature, 6-month-old guinea pigs weighing 500 g on average were used in the experiments. The animals were kept at an ambient temperature of 18—20 °C on standard feed throughout the experimental period.

The animals were divided into a control group and two experimental groups. The control group numbered 10 guinea pigs (5 males and 5 females).

The first experimental group of 15 guinea pigs (9 females and 6 males) were irradiated on the whole body with 500 R of X-rays from a roentgen apparatus with 200 kV, 15 mA and 1 mm Cu filter. Time of exposure was 8 min and 50 s.

The guinea pigs of the second experimental group were irradiated in a similar manner but with 1 500 R and exposure for 15 min and 50 s. Dosage was determined in air by means of a Universal Dosimesser.

On the first day after irradiation the behaviour of the guinea pigs was normal. On the second day, the animals were somnolent, apathetic and ate little. Bloody extravasations appeared on the mucous membranes. Some animals had diarrhoea. Random blood samples showed leucopenia. These symptoms were considered indicative of the first stage of radiation disease, and measurements of thyroid activity were begun.

At 68 h after irradiation of the animals of the first experimental group (9 females and 6 males) which received a dose of 500 R, each of the 15 guinea pigs was given an intraperitoneal injection of 3 μCi of ^{131}I. The background and impulses in a standard were also measured. The same procedures were followed with the second experimental group irradiated with 1 500 R. At the same time, 3 μCi of ^{131}I was injected in the control guinea pigs. One hour after injection of ^{131}I and 69 h after X-irradiation, impulse counts were made on the thyroids of guinea pigs irradiated with 500 R. For this purpose, the animals were placed in a lead cylinder with a window measuring 4 × 2.5 cm, 5 mm thick. The whole thyroid was exposed in the window to the probe, directly opposite to the collimator. After determining the distance, radioactivity was measured over the thyroid.

In the same way impulse counts were made on the thyroids of the control guinea pigs and group of guinea pigs irradiated with 1 500 R.

Next, measurements were made in the control and experimental animals 3 h later, i.e. 4 h after administration of radioactive iodine and 72 h after termination of irradiation.

At 24 h after administration of ^{131}I and 93 h after X-irradiation, impulse counts were made over the thyroids of guinea pigs of all three groups.

Finally, measurements were made 48 h after administration of ^{131}I and 117 h after termination of X-irradiation.

Injections of ^{131}I were given in the form of a solution of Na I of activity 10 μCi/ml.

Radioactivity was measured with a scintillation counter. From the results, percentage uptake of ^{131}I by the thyroid was calculated in relation to the administered dose according to BEIERWALTES and co-workers [4], allowing for decay of radioactive iodine and background radiation. Statistical analysis of the results included calculation of arithmetic means, standard deviation, mean error, and Student's-test.

Results

Males. Accumulation of ^{131}I in the thyroids of control males 1 h after its administration amounted to 11.73%. Three hours after injection of the isotope iodine uptake of the thyroid rose to 12.43%, and after 6 h to 17.40%. Maximum accumulation of ^{131}I was noted after 24 h, with a mean of 32.09%. Forty-eight hours after injection of the isotope accumulation of ^{131}I in the thyroid dropped on the average to 23.96% (Table 1, Fig. 1).

In males of the first experimental group irradiated with 500 R of X-rays, one hour after administration of ^{131}I and 69 h after termination of irradiation iodine uptake increased statistically significantly to 17.14% in relation to the controls. At 3 h, ^{131}I uptake dropped to 16.00% compared with the first

Table 1

^{131}I uptake by the thyroid of the control and X-irradiated male guinea pigs

Interval after injection of ^{131}I	Group	No. of animals	Mean percentage of ^{131}I in the thyroid	Standard deviation	Mean error	Student's *t*-test
1 h	Control	5	11.726	1.613	0.721	—
	X-ray (500 R)	6	17.144	1.308	0.538	14.03 **
	X-ray (1 500 R)	6	12.506	5.335	2.178	0.29
3 h	Control	5	12.428	1.330	0.595	—
	X-ray (500 R)	6	16.000	2.030	0.829	8.30 **
	X-ray (1 500 R)	6	14.118	5.623	2.296	0.58
6 h	Control	5	17.395	2.945	1.317	—
	X-ray (500 R)	6	18.176	3.135	1.280	0.99
	X-ray (1 500 R)	6	15.888	5.200	2.123	1.15
24 h	Control	5	32.094	6.201	0.980	—
	X-ray (500 R)	6	19.894	4.011	1.637	15.28 **
	X-ray (1 500 R)	5	19.213	2.523	1.128	20.28 **
48 h	Control	5	23.960	6.201	2.773	—
	X-ray (500 R)	6	24.408	2.715	1.108	0.24
	X-ray (1 500 R)	5	22.762	1.544	0.691	0.67

* The animals were injected 68 h after irradiation of the experimental animals
** Statistically significant for $t_{0.001}$

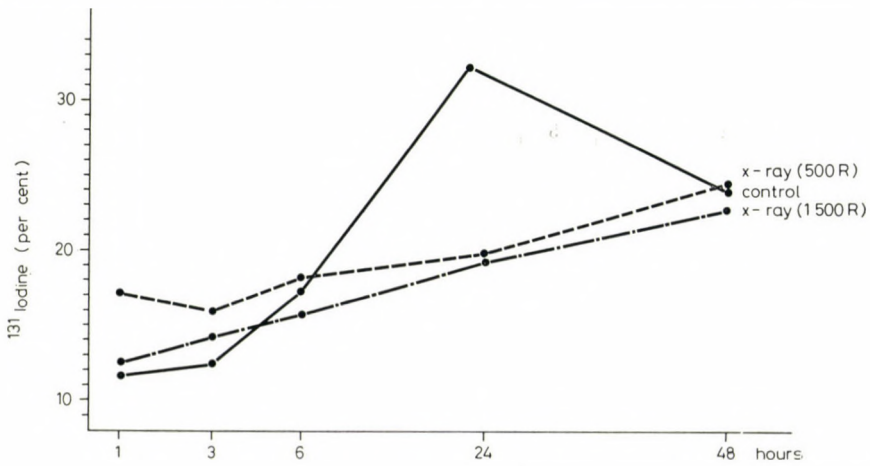

Fig. 1. ^{131}I uptake in the thyroid of the male guinea pigs of the control group and groups X-irradiated with 500 and 1 500 R

hour, but was much higher (statistically significantly) than in the control males. At 6 h ^{131}I uptake was 18.18%, close to control values. A slight increase in iodine uptake was noted after 24 h compared with the measurements after 1.3 and 6 h, but a statistically significant drop of thyroid radioactivity in comparison with controls at the same time. Forty-eight hours after administration of the isotope, i. e. 117 h after termination of exposure to X-rays, the level of ^{131}I was 24.40%, about the same as in control males (Table 1, Fig. 1).

The thyroid of the male guinea pigs of the second experimental group irradiated with 1 500 R began to accumulate ^{131}I already in the first hour after injection of the isotope, to a level of 12.51%. Three hours after injection of the isotope the level of ^{131}I in the thyroid rose to 14.12%, and remained above the control values, but not statistically significantly. Six hours after injection of the isotope a slight increase in ^{131}I to 15.89% took place compared with the values of measurements after 3 h, but a distinct drop of radioactivity of the

Table 2

^{131}I uptake in the thyroid of control and X-irradiated female guinea pigs

Interval after injection of ^{131}I	Group	No. of animals	Mean percentage of ^{131}I in the thyroid	Standard deviation	Mean error	Student's *t*-test
1 h	Control	5	9.876	1.740	0.778	—
	X-ray (500 R)	9	16.902	1.533	0.511	16.46 **
	X-ray (1 500 R)	9	15.028	3.334	1.111	10.14 **
3 h	Control	5	12.334	1.265	0.566	—
	X-ray (500 R)	9	17.167	1.280	0.427	16.60 **
	X-ray (1 500 R)	9	16.227	3.415	1.138	8.53 **
6 h	Control	5	15.773	2.137	0.056	—
	X-ray (500 R)	9	18.837	1.203	0.401	22.52 **
	X-ray (1 500 R)	9	15.267	3.696	1.323	1.14
24 h	Control	5	33.492	2.125	0.950	—
	X-ray (500 R)	9	19.519	1.630	0.543	30.31 **
	X-ray (1 500 R)	7	17.958	5.402	2.042	17.63 **
48 h	Control	5	20.320	4.332	1.937	—
	X-ray (500 R)	9	22.813	2.409	0.803	1.40
	X-ray (1 500 R)	5	18.560	4.771	2.134	0.61

* The animals were injected 68 h after irradiation of the experimental animals
** Statistically significant for $t_{0.001}$

thyroid was observed in relation to the controls. At 24 h radioactivity of the male thyroids was 19.83%, i.e. by 12.9% less than in the control males, the difference being statistically significant. At 48 h ^{131}I uptake by the thyroid of X-irradiated males with 1 500 R was 22.76%, i.e. below the mean control value (Table 1, Fig. 1).

Females. ^{131}I uptake by the thyroids of control females 1 h after administration of the isotope was 9.88%. After that radioactivity of the thyroid rose steadily up to 24 h with the following values: after 3 h, 12.33%, after 6 h, 15.77%, and after 24 h, 33.49%. Twenty-four hours after injection of ^{131}I a distinct drop occurred in radioactivity of the thyroid, after 48 h the mean radioactivity of the thyroid was 20.32% (Table 2).

The thyroid of female guinea pigs of the first experimental group irradiated with 500 R of X-rays accumulated ^{131}I differently than in the control females. At 1, 3 and 6 h after injection of ^{131}I respectively 69, 72, and 75 h after termination of X-irradiation, uptake of radioactive iodine by the thyroid increased (statistically significantly) compared with the control females. Twenty-four hours after injection of ^{131}I radioactivity of the thyroid increased slightly in comparison with the preceding periods, but a very distinct, statistically significant drop in radioactivity of the thyroid, amounting to about 13.973%, took place in comparison with the female controls. Finally, 48 h after injection of the isotope and 117 h after termination of X-irradiation the thyroid of females accumulated ^{131}I to the level of 22.813% (Table 2).

^{131}I uptake in the thyroids of female guinea pigs of the experimental group irradiated with 1 500 R was similar to that in the females irradiated with 500 R. After 1 and 3 h, respectively 69 and 72 h after irradiation, accumulation of ^{131}I in the thyroid increased statistically significantly. Next, after 6, 24 and 48 h, respectively 75, 95 and 117 h after termination of irradiation, ^{131}I uptake by the thyroid was inhibited in comparison with the controls (Table 2).

Discussion

An analysis of the results clearly shows that the thyroid gland of guinea pigs irradiated with 500 R and 1 500 R of X-rays on the whole body takes up more ^{131}I up to 75 h after exposure, and this is followed by a drop in the radioactivity of the thyroid in comparison with controls. In control animals, accumulation of ^{131}I increased up to 24 h from injection of the isotope, followed by a very significant fall in radioactivity of the thyroid.

The observed increase in accumulation of ^{131}I in the thyroid during the first 75 h after the and of irradiation of the animals with 500 and 1 500 R is in agreement with the generally held opinion that after sublethal doses of X-rays at first thyroid activity increases, followed by a gradual drop [15].

The increased accumulation of ^{131}I in the thyroid of animals X-irradiated in the early period of exposure may be attributed to an increased synthesis and release of TSH from the hypophysis [5, 6].

Changes in thyroid activity after X-irradiation are accompanied by morphologie changes in the hypophysis and in the content of TSH [3, 22].

Since biosynthesis and release of thyroid hormones are strictly related to the blood level of TSH [10], the increase in ^{131}I uptake by the thyroid in the initial period after X-irradiation is probably linked with an increased secretion of thyroxine, and consequently with a greater iodine requirement of the body.

In the early hours after irradiation of the animals with 500 or 1 500 R of X-rays, the thyroid uptake of ^{131}I was somewhat higher than in the controls, and in the later stage the comparative iodine uptake decreased distinctly.

Our observations are in agreement with those of CLOSON and BETZ [8], who noted a drop in iodine uptake by the thyroid in rats 48 h after whole body irradiation with 800 R.

The uptake of radioactive iodine by the thyroid in guinea pigs X-irradiated with 500 R and 1 500 R at 1 to 48 h after administration of the isotope was essentially similar. The threefold higher dose of X-rays had no statistically significant effect on the rate of radioactive iodine uptake by the thyroid.

Although MOSKALEV and co-workers [16] reported that the rate of iodine uptake by the thyroid is proportional to the dose of radiation applied, our observations do not confirm a relationship.

Measurements of radioactivity revealed similar ^{131}I uptake in the thyroids of male and female guinea pigs irradiated with 500 and 1 500 R of X-rays. SIKOV [18] also found no difference in ^{131}I uptake between male and female animals of the same age.

The observed changes in iodine metabolism, especially those concerning the rate of accumulation of ^{131}I in the thyroid of X-irradiated guinea pigs, are related to changes in activity of the hypothalamus and hypophysis. Total irradiation of animals with X-rays leads to changes in activity of the hypothalamus [2, 13].

Changes in hypothalamic activity are reflected by changes in the level of hypothalamic TRF, which in turn affect the content and release of TSH [19, 20]. At the same time, morphologic changes occur in the hypophysis and in the amount of thyreotropic hormone (TSH) released [3, 22] and CLOSON and BETZ [8] reported changes in levels of thyroxine in animals exposed to X-rays.

The facts clearly indicate that changes in activity of the hypothalamus, and indirectly in the hypophysis, are reflected by thyroid function, manifested by changes in iodine uptake by the thyroid gland.

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INFLUENCE OF X-RAYS ON DIURNAL VARIATION IN ^{131}I UPTAKE BY GUINEA PIG THYROID

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(Received 1974—11—25)

Abstract

The ^{131}I uptake by guinea pig thyroid is subject to distinct diurnal variation. In control males, peak values occurred at 12 h, and minima at 24 h. In control females, peak values were also at 12 h, but minima at 6 h. In guinea pigs irradiated with 500 R of X-rays, a rise in ^{131}I uptake began 1 h after termination of irradiation in both sexes and at all intervals, but maximum and minimun values were at the same time as in controls.

Introduction

Changes in thyroid activity in animals following X-irradiation have been described by numerous investigators [5, 8, 10, 11, 20, 26, 27].

Whole-body irradiation of rats with X-rays increases ^{131}I uptake by the thyroid [8]. According to SINGH [17], small doses of X-rays increase, and large doses decrease ^{131}I uptake by the thyroid. CLOSON and BETZ [9] observed, however, a drop in iodine uptake 48 h after whole-body irradiation of rats with 880 R. Similar results were reported by SONG and EVANS [21], who observed lowered ^{131}I uptake by the thyroid of mice 6 h after irradiation.

Although experimental studies have shown that thyroid uptake of ^{131}I changes in animals irradiated with X-rays, there is lack of accurate information about the influence of irradiation on the diurnal rhythm of radioactive iodine uptake. The present study was concerned with the following problems:

(1) Does ^{131}I uptake by the thyroid of male and female guinea pigs vary in the course of the day?

(2) How is ^{131}I uptake by the thyroid influenced by single total doses of X-rays?

(3) At which time of the day is the thyroid most sensitive to X-irradiation?

Material and method

Experiments were carried out on 100 six-month-old guinea pigs of 500 g average weight. The animals were kept at 18—20 °C ambient temperature and fed a standard diet. The animals were divided into a control group (25 male and 25 female guinea pigs) and an

experimental group (25 male and 25 female guinea pigs), i. e. 50 in each group. Each group was subdivided into 5 subgroups of 10 guinea pigs (5 males and 5 females). The groups of experimental guinea pigs were irradiated with a single dose of 500 R at 12, 18, 24, 6, and 12 h. Twentytwo hours after irradiation, i.e. at 10, 16, 22, 4 and 10 h, each group of guinea pigs was injected intraperitoneally with 1 μCi of ^{131}I. The control group of animals also received injections of the isotope.

Radioactivity was measured 2 h after isotope injection, i.e. 24 h after irradiation.

The source of radiation was a Philips roentgen machine for deep therapy, at 200 kV and 15 mA, 1 mm Cu filter. Exposure was for 8 min and 50 s. Air dose was measured with a Universal Dosimeter. Radioactive iodine with 10 μCi/ml activity of ^{131}I-Na was injected.

Thyroid activity was measured with a scintillation counter. When calculating results in per cent of the administered dose in the thyroid, corrections were made for decay of radioactive iodine and background radiation according to BEIERWALTES and co-workers [6].

For statistical analysis, means, standard deviation and Student's t-test were calculated.

Results

Females. Maximum ^{131}I uptake by the thyroids of control females was noted at 12 h, and minimum at 6 h (Table 1, Fig. 1).

In females irradiated with 500 R of X-rays, ^{131}I uptake by the thyroid increased distinctly and the maximum shifted from 12 h to 18 h. Statistically significant differences in thyroid uptake of ^{131}I were noted between control and irradiated mice at 18 h, 6 and 24 h (Table 1, Fig. 1).

Males. In control males, ^{131}I uptake also varied at different times of the day. Highest activity of the thyroid was noted at 12 h, and lowest at 24 h (Table 1, Fig. 1).

Table 1

Percentage ^{131}I uptake 2 h after i.p. injection of ^{131}I various times of the day by the thyroid of control guinea pigs and guinea pigs irradiated with 500 R of X-rays

Time of measurements	Groups of animals	Dose of X-rays in R (24 h before measurements)	Mean percentage ^{131}I uptake by the thyroid	
			male	female
Noon	Control	—	12.320 ± 1.408	11.990 ± 1.204
	X-irradiated	500	13.117 ± 1.495	12.786 ± 1.584
6 p.m.	Control	—	9.600 ± 0.596	10.350 ± 0.620
	X-irradiated	500	11.473 ± 1.166*	13.020 ± 2.363*
Midnight	Control	—	8.200 ± 1.934	9.253 ± 2.312
	X-irradiated	500	11.382 ± 0.993*	12.540 ± 1.244*
6 a. m.	Control	—	10.340 ± 1.626	9.000 ± 1.553
	X-irradiated	500	12.690 ± 0.819*	12.183 ± 1.844*
Noon	Control	—	13.400 ± 0.698	13.070 ± 0.698
	X-irradiated	500	13.297 ± 0.528	13.017 ± 0.852

* Significant at a level of 0.001

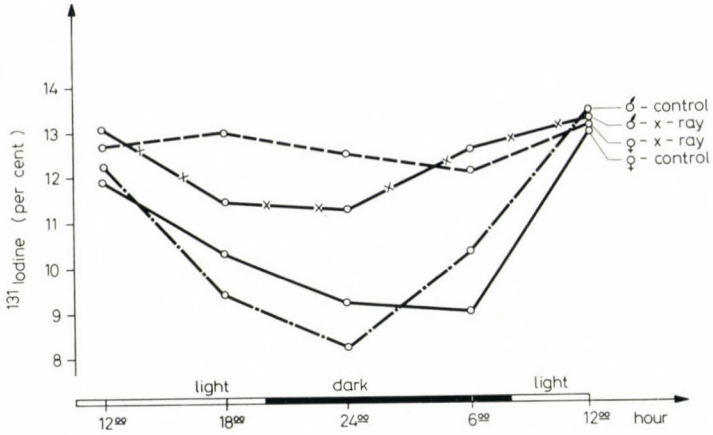

Fig. 1. Diurnal rhythm of ^{131}I uptake by the thyroid of male and female control guinea pigs and guinea pigs irradiated with 500 R of X-rays

Measurements in male guinea pigs irradiated with a single dose of 500 R showed much higher values at various times of the day compared with control males. Maxima and minima were at the same hours, but at higher levels. Statistically significant differences in accumulation of radioactive iodine in the thyroid were noted at 18, 24 and 6 h (Table 1, Fig. 1).

Discussion

Analysis of the findings shows that ^{131}I uptake by the thyroid of control male and female guinea pigs varies very distinctly in the course of the day. Maximum peak of ^{131}I uptake in control guinea pigs was at 12 h (noon), in both sexes. Minimum uptake was at 24 h in males, and at 6 h in females.

The results of this study are consistent with the findings of SINGH and co-workers [18], who noted that in rats plasma concentrations of TSH rise in the morning hours, attain a peak at 15 h, and then fall to the minimum level at 3 h in the night.

It seems likely that the rise in plasma TSH in the morning hours continuing until 15 h, causes an increase in thyroid activity and consequently also in ^{131}I uptake by the thyroid. On the other hand, low levels of TSH in the nighttime are associated with a decrease in ^{131}I uptake.

According to BAN [3], in euthyroid subjects diurnal variation in triiodothyronine are characterized by a maximum during sleep, accompanied by a slight rise in thyroxine. However, a distinct rhythm was not apparent unless the hormone was calculated in relation to total protein. In this author's opinion, the rise in triiodothyronine during sleep is probably mainly due to blood

dilution. It is also probable, however, that the elevation of thyroxine levels is partly responsible for the rise in triiodothyronine.

NICOLOFF (cit. [25]), observed fluctuation in release of iodine from the thyroid with a maximum at 4 h in the morning and minimum at 17 h in the afternoon. These data suggest acceleration of intrathyroid kinetics of iodine and enhanced secretion of hormones by the thyroid at this time.

Irradiation with 500 R of X-rays was followed after 24 h by a statistically significant rise in ^{131}I uptake by the thyroid of male and female guinea pigs in comparison with control guinea pigs, but maxima and minima occurred at the same times of the day, although at a higher level.

The rise in ^{131}I uptake by female and male guinea pig thyroid at various times during 24 h after termination of exposure to X-rays is consistent with the view that in certain doses X-rays first cause a rise in thyroid activity, which is followed by a gradual drop [7, 14, 17, 19].

Increased accumulation of ^{131}I in the thyroid of X-irradiated animals at various times of the day is related to increased synthesis and release of TSH in the hypophysis [4, 5]. In turn, changes in thyroid activity after irradiation are accompanied by morphological changes in the hypophysis and by changes in TSH content [2, 24].

The changes in kinetics of iodine, particularly in uptake of ^{131}I by the thyroid of X-irradiated guinea pigs, should be attributed to changes in activity of the hypothalamus and hypophysis. Whole-body irradiation of animals causes changes in activity of the hypothalamus [1, 13, 20, 21].

Changes in the hypothalamic activity are reflected by changes in hypothalamic levels of TRF which, in turn, influence TSH synthesis and release [22, 23]. Simultaneously, levels of thyroxine change in X-irradiated animals [9].

Lastly, measurements of thyroid radioactivity have revealed that the highest difference between the iodine uptake of controls and irradiated animals occurs at night. The reason for this is the varying sensitivity of animals to X-irradiation.

This conclusion is supported by the studies of PIZZARELLO and coworkers [15, 16] and HELLWIG and ROSENKRANZ [12] who demonstrated that sensitivity of strains of mice and rats to ionizing radiation is subject to a circadian rhythm. On the whole, animals were more sensitive to X-rays in the nighttime.

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THE EFFECT OF PERPHENAZINE TREATMENT DURING THE ORGANOGENESIS IN RATS

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Abstract

The teratogenic effect of perphenazine was studied in closed-bred Wistar rats. Rats were given the substance orally continuously on days 7 to 14 of pregnancy, or as a single dose on one of days 9—15. The doses were considerably higher than the specific neuroleptic doses of perphenazine (20—150 mg/kg). The fetal mortality and malformation rates were significantly higher in the perphenazine-treated groups than in the controls. The results were compared with the earlier results of methophenazine treatment. Perphenazine was found to be more toxic for rat embryos than methophenazine.

Introduction

The effect of neuroleptic phenothiazine derivatives on the embryonic development in various species has been examined by several authors. The opinions are inconsistent on their embryotoxic and teratogenic effect.

Single i. p. doses of chlorpromazine given on different days of pregnancy increased fetal mortality and caused hydronephrosis and skeletal malformations in rats, depending on the day of treatment [5]. Chlorpromazine alone was not teratogenic on chick embryos, but promoted the teratogenicity of insulin [8].

Besides the above alterations, hydrops fetalis and hydrocephalus internus developed in higher percentage after s. c. injection of trifluoperazine in rats [6].

Fetal resorption was frequently found if the rats were treated with prochlorperazine before the 7th gestational day. Treatment after the 7th day was often followed by malformations, for instance, cleft palate occurred in fetuses treated on the 13th day of pregnancy [13]. In another series of experiments [16] only two rat embryos showed cleft palate of those treated in a similar way [1].

High occurrence of retrognathia, cleft palate, and micromelia was reported on Wistar rat fetuses after single doses of methophenazine given on the 14th day of pregnancy [7]. The applied doses were much above the specific neuroleptic doses.

Phenothiazine derivatives containing the piperazine-ring in N side-chain are more teratogenic than those containing here an alkyl group [4, 7]. Methophenazine was first synthesized by TOLDY and co-workers [15] from perphenazine by the esterification of its hydroxyl group with trimethoxybenzoic acid. This substitution decreased the toxic effects of perphenazine (hepatotoxicity, leucopenia, tachycardia, hypotension, etc.) [2]. The teratogenic effect of the former compound was studied earlier [7], while the embryotoxic and teratogenic effects of perphenazine [N-hydroxyethyl-(N')-2-chloro-10-phenothiazinyl-(propyl)-piperazine] in comparison with similar effect of methophenazine have been the subject of the present studies. In addition, the teratogenic effect of methophenazine given on the 15th gestational day was studied, since the effect exerted on this particular day had not been investigated. We intended to show whether the mentioned substitution of hydroxyethyl group of perphenazine decreases the teratogenic effect of this substance.

Material and method

Closed-bred virgin female Wistar rats, weighing 150 to 200 g, were bred with males of the same strain, weighing 250 to 300 g. The first day of pregnancy was determined on the basis of the presence of spermatozoa in the vaginal smear. Preparations were stained with 1% aqueous methylene-blue solution. The pregnant rats were separated, food and water were given ad libitum.

The value of acute LD_{50} (single dose, 8-day's observation) was established on non-pregnant female Wistar rats of the same body weight and calculated by Litchfield–Wilcoxon's method [9]. The acute oral LD_{50} value of perphenazine was 350 ± 35 mg/kg. Since the perphenazine base is poorly soluble in water, it was suspended in 1.25% methylcellulose solution and given in a volume of 5 ml/kg. Freshly-prepared suspension was administered always in the morning by means of a stomach tube. After the treatment the pregnants were placed in separated cages and were continuously observed. Both treated and control animals were weighed every second day. Grouping is shown in Table 1.

Caesarean section was made on the 21st day of pregnancy. The fetuses were taken out with the uterus-horns; the live and died fetuses or early resorptions were counted in each horn. The offsprings were weighed and external examination was performed. A half of the fetuses were fixed in Bouin's solution and dissected with modified Wilson's technique [19]. The others were fixed in 96% ethanol and skeletons were examined after KOH alizarin red S staining [10, 14]. If it was necessary, histological seral sections were prepared. These were stained with hematoxylin and eosin.

Fetal mortality rate was calculated on the basis of total implantation count while the rates of retardation and malformations were related to the live fetuses. The statistical significance of both mortality and malformations was calculated by the $\varkappa^2$ test. The "p" values are presented in Table 2. Fetuses in which one or more sternal ossification centres were missing or double-shaped, or one or more ventral ossification centres of vertebrae were underdeveloped were regarded as retarded [10]. The percentage of malformations was calculated for live fetuses.

Results

The results are summarized in Tables 2, 3, and 4, and in Fig. 1. Both fetal mortality and the incidence of malformations were significantly higher in the groups treated with perphenazine than in the control groups (Table 2).

Table 1

Grouping and dosage in teratological studies with perphenazine

Group	Substance	Daily dose	Total dose	Days of treatment	Number of pregnant rats/died females during study
C	Control	ø	ø	ø	15/ø
M.C.	Solvent	5 ml/kg	40 ml/kg	7—14	10/ø
P/20	Perphenazine	20 mg/kg	160 mg/kg	7—14	10/ø
P/90/ 9	Perphenazine	90 mg/kg	90 mg/kg	9	9/ø
10				10	9/ø
11				11	10/ø
12				12	10/ø
13				13	9/ø
14				14	11/1
P/120/ 9	Perphenazine	120 mg/kg	120 mg/kg	9	9/ø
10				10	11/ø
11				11	10/ø
12				12	11/ø
13				13	14/ø
14				14	10/ø
P/150/ 9	Perphenazine	150 mg/kg	150 mg/kg	9	11/1
10				10	9/2
11				11	13/1
12				12	16/ø
13				13	13/ø
14				14	18/1
15				15	9/ø

The following three types of malformations were typical: cleft palate, retrognathia, and micromelia. Their incidence depended on the dose and on the day of treatment. Cleft palate appeared in fetuses of mothers treated on the 11th day or later. Its incidence and degree reached the maximum when 150 mg/kg of perphenazine was given on the 15th day of pregnancy (15th day — 150 mg/kg — 85.2% cleft palate — Table 4, Fig. 1). Retrognathia was observed in the group treated on the 13th gestational day or later. All the fetuses had retrognathia in the group of mothers having received 150 mg/kg of perphenazine on the 15th day; the incidence of retrognathia showed a sudden decrease with reducing the dose (Table 4, Fig. 1). Micromelia in high percentage

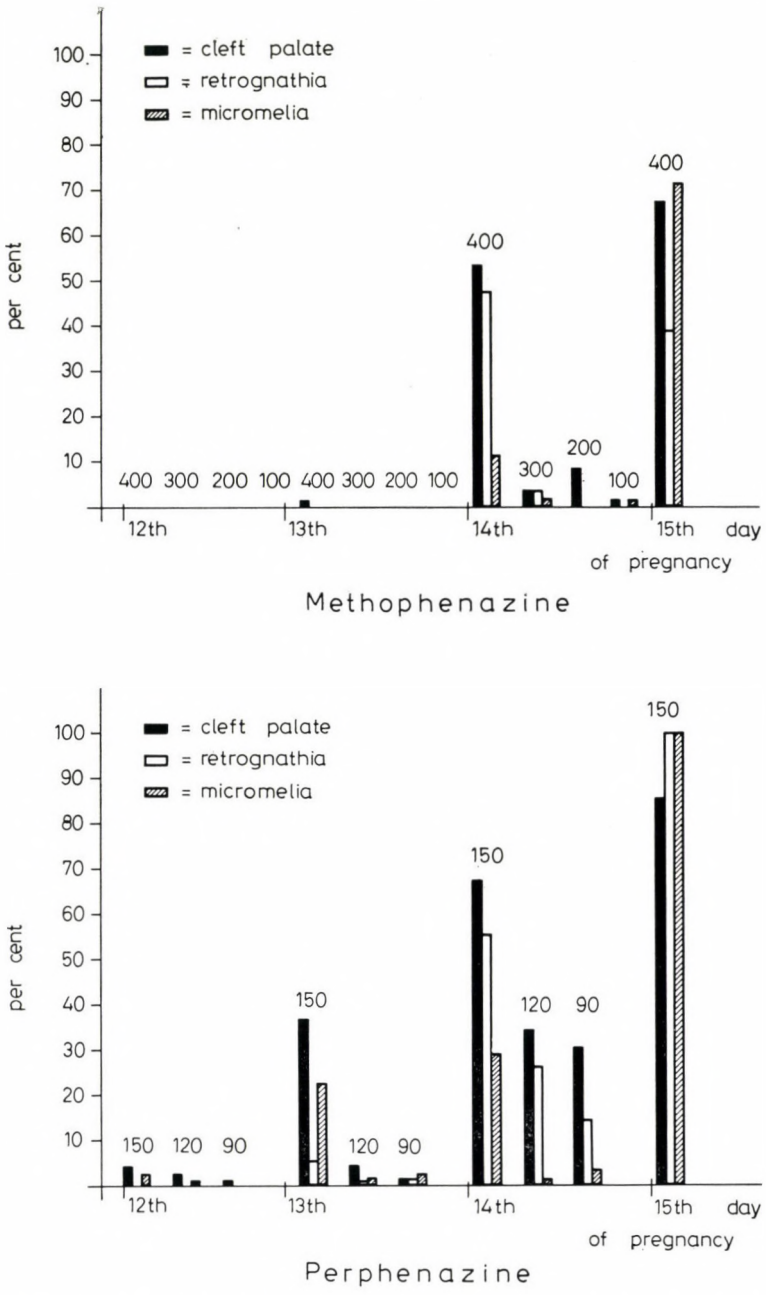

Fig. 1. Comparison of perphenazine and methophenazine with regard to the induction of cleft palate, retrognathia, and micromelia. The given doses of each substance are presented over the columns

Table 2

The effect of perphenazine treatment on embryos of pregnant rats

Group	Implantations	Resorptions		Living fetuses	Normal fetuses	Retardations		Malformations	
		No.	per cent			No.	per cent	No.	per cent
C[1]	159	10	6.3	149	118	26	17.4	5	3.3
M.C.[2]	108	7	6.5	101	66	33	32.6	2	2.0
P /20/ 7—14[3]	118	40	33.9*	78	30	30	38.4	18	23.1*
P /90/ 9	101	63	62.4*	38	17	16	42.1	5	14.8**
10	97	60	61.9*	37	25	8	21.6	5	13.5***
11	105	36	34.3*	69	26	26	37.7	17	24.6*
12	112	25	22.3*	87	54	20	23.0	13	14.9**
13	87	10	11.7	77	42	13	16.9	22	28.6*
14	104	15	14.4***	89	25	25	21.1	39	43.8*
P /120/ 9	80	66	82.5*	14	7	6	42.8	1	7.1
10	123	80	65.0*	43	27	10	23.3	6	14.0**
11	102	47	46.0*	55	23	23	41.8	9	16.3*
12	124	38	30.6*	86	39	29	33.7	18	20.9*
13	151	41	27.2*	110	47	36	32.7	26	23.6*
14	99	30	30.3*	69	9	21	30.4	40	56.5*
P /150/ 9	90	79	87.8*	11	4	3	27.3	4	36.3*
10	72	59	81.9*	13	3	6	46.1	4	32.5*
11	133	77	57.9*	56	25	16	28.6	15	26.8*
12	164	46	28.1*	118	55	29	24.6	34	28.8*
13	144	87	60.4*	57	15	14	24.6	28	49.1*
14	199	35	18.0**	164	26	10	6.1	119	72.6*
15	104	16	15.4***	88	0	0	0.0	88	100.0*

[1] untreated control, [2] medium control, [3] perphenazine (dose in mg/kg/ day of treatment)
* $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$

required 150 mg/kg perphenazine. If this was administered on the 15th day, all fetuses developed micromelia (Table 4, Fig. 1). It was remarkable on the skeletons stained with alizarin red S that longitudinal growing of long bones was disturbed, while the appositional ossification not. The 21-day-old embryos of mothers treated on the 15th gestational day with 150 mg/kg perphenazine had long bones similar in length to those of the 20-day-old untreated embryos (damaged enchondral ossification), while the thickness of long bones was equal with that of the 21-day-old control embryos (intact periosteal ossification).

Table 3

Visceral malformations in the embryos of perphenazine treated pregnant rats
(The frequencies of malformations are expressed in per cent of living fetuses)

Group	Hydrops fetalis	Anophthalmia, microphthalmia	Cerebral malformations	Hernia umbilicalis	Thymus accessorius superior	Hydroureter + hydronephrosis	Other urogenital malformations
C[1]	—	—	—	—	0.7	2.0	—
M.C.[2]	—	—	—	—	—	1.0	—
P /20/ 7—14[3]	—	1.3	—	—	—	16.7	—
P /90/ 9	—	5.3	2.6	—	—	—	—
10	—	—	2.6	—	—	2.6	—
11	—	1.4	4.3	1.4	2.9	5.8	1.4
12	1.1	—	—	—	—	3.4	—
13	5.2	—	3.9	—	5.2	14.3	5.2
14	—	—	1.1	1.1	7.9	11.2	—
P /120/ 9	—	7.1	—	—	—	—	—
10	—	—	—	—	2.3	9.3	2.3
11	—	—	—	—	1.8	5.5	—
12	1.2	—	1.2	—	3.5	10.5	—
13	—	—	0.9	8.9	6.4	4.5	—
14	—	1.4	—	—	7.2	17.4	—
P /150/ 9	—	—	—	—	—	9.1	—
10	—	15.4	—	7.7	7.7	15.4	—
11	—	1.8	—	3.6	3.6	5.4	—
12	—	—	0.8	4.2	7.6	4.2	—
13	—	—	5.3	3.5	8.8	7.0	—
14	0.6	—	1.2	1.2	7.3	7.9	2.4
15	—	—	—	—	1.1	8.0	2.3

[1] untreated control, [2] medium control, [3] perphenazine (dose in mg/kg/ day of treatment)

Hydroureter and hydronephrosis occurred in all experimental groups independent of the day of treatment. In the control and solvent control groups these anomalies were generally unilateral and the kidneys remained practically intact. The ureters in the fetuses of the perphenazine-treated mothers became bilaterally very thick, with very thin wall. This was combined with hydronephrosis, and normal nephrons were hardly visible histologically. The incidence of serious hydronephrosis was the highest in the groups of

Table 4

Skeletal malformations in the embryos of perphenazine treated pregnant rats
(The frequencies of malformations are expressed in per cent of living fetuses)

Group	Missed vertebrae	Rib anomalies	Calcification anomalies	Cleft palate	Retrognathia	Micromelia	Club foot	Other skeletal anomalies
C[1]	0.7	—	—	—	—	—	—	—
M.C.[2]	1.0	—	—	—	—	—	—	—
P /20/ 7—14[3]	—	—	1.3	—	—	3.8	—	—
P /90/ 9	7.9	7.9	—	—	—	—	—	—
10	2.7	5.4	—	—	—	—	2.7	—
11	4.3	—	—	—	—	2.9	—	—
12	6.9	—	1.1	—	—	—	—	—
13	—	—	1.3	1.3	1.3	2.6	—	—
14	—	1.1	—	30.4	14.6	3.4	—	—
P /120/ 9	—	—	—	—	—	—	—	—
10	2.3	—	—	—	—	—	—	—
11	7.3	—	—	1.8	—	—	—	—
12	7.0	—	—	2.3	—	1.2	—	—
13	—	—	—	4.5	0.9	1.8	1.8	—
14	2.8	—	—	34.8	26.1	1.4	—	—
P /150/ 9	9.1	—	—	—	—	—	18.2	—
10	—	—	—	—	—	—	—	—
11	7.1	—	—	—	—	—	7.1	—
12	5.1	—	—	4.2	—	2.5	4.2	0.8
13	3.5	—	—	36.8	5.3	22.8	—	—
14	0.6	2.4	1.2	67.1	55.9	29.3	1.8	—
15	4.5	6.8	1.1	85.2	100.0	100.0	—	—

[1] untreated control, [2] medium control, [3] Perphenazine (dose in mg/kg/ day of treatment)

fetuses treated with 20 mg/kg/day perphenazine on days 7—14, viz., 16.6%, vs. 2.0% (untreated control) and 1.0% (methylcellulose control).

Hydrocephalus internus, hydrops fetalis, missed vertebrae, and, generally bilateral, anophthalmia or microphthalmia developed in all perphenazine-treated groups. In two instances fused ribs and in one case each of other rib anomalies together with missed vertebrae, encephalocele and hernia umbilicalis developed. A single fetus with very serious hydrops fetalis had in addition micromelia and skin absence on the lumbosacral region. In this region histo-

logical serial sections revealed myelodysplasia. Neurons were absent in the left side of the spinal cord, the medullar substance was diminished and remarkable round-cell infiltration was present, especially in the grey matter.

The applied doses of perphenazine were toxic for the pregnant rats as shown by their weight loss of mothers and prolonged cataleptic state. Several animals even died (Table 1).

Discussion

From the present data the conclusion can be drawn that the esterification of the —OH group of perphenazine with trimethoxybenzoic acid decreased, besides the toxic effects in an adult animal, the teratogenicity of the drug (Fig. 1).

BEALL [1] did not find any malformations in animals treated with lower doses of perphenazine though toxic effects appeared in animals treated with 7 mg/kg/day (out of 25 animals 10 died).

Cleft palate was induced by methophenazine only when the animals were treated on the 14th and 15th gestational days and its frequency decreased with reduction of the dose very quickly (15th day: 400 mg/kg — 66.7%; 14th day: 400 mg/kg — 53.5%, 300 mg/kg — 3.6%, 200 mg/kg — 8.5%, 100 mg/kg — 1%; 13th day: 400 mg/kg — 1.4%). On the 13th day cleft palate could not be induced by any of the applied doses, while after perphenazine treatment cleft palate was observed even if the mother was treated on day 11 (Table 4, Fig. 1). Except for a single case, microphthalmia or anophthalmia did not occur in methophenazine experiments. As a failure characteristic of both substances the incidence of hydronephrosis in the fetuses was very high, which is in good agreement with earlier teratological investigations on phenothiazine derivatives [4, 5, 6, 7].

For the teratogenic effect of perphenazine riboflavine deficiency and disturbed FAD synthesis and carbohydrate metabolism seem to be responsible [4, 11, 17].

NIELSEN and co-workers [12] have found significantly more chromosome aberrations in psychiatric patients treated with perphenazine, therefore, a chromosome-aberration-inducing effect of this drug in rat embryos cannot be excluded, either.

Finally, on the base of KOH alizarin red S staining, calcification disturbances and altered lengthening of bones might play an important role in the development of micromelia. This resembles the skeletal anomalies of human fetuses with congenitally very low alkaline phosphatase values [18].

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HISTOPATHOLOGY OF THE ENDOCRINE PANCREAS OF A FRESH-WATER FISH, *CLARIAS BATRACHUS* L.

VII. EFFECTS OF HYDROCORTISONE AND THYROXINE ADMINISTRATION

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Abstract

Hydrocortisone treatment caused a hyperglycaemic state in *Clarias batrachus* within an hour. The blood glucose values remained significantly increased for 6 days. An initial increase in liver glycogen was followed by a decrease below the normal value. The muscle and brain glycogen levels remained unaffected. A mild degranulation of B cells was seen in the early hours and a conspicuous vacuolation and necrosis of both A and B cells appeared 72—96 h after treatment. Administration of thyroxine resulted in hyperglycaemia within 15 h but a distinct hypoglycaemic condition was seen 4 days after the treatment. Considerable drain in the liver glycogen deposits was noticed between 24 and 72 h while the muscle glycogen showed an increase. The brain glycogen did not change noticeably. Islets of the fishes autopsied between 72 and 96 h contained severely damaged B cells. The A cells remained unaffected. It is suggested that, in addition to several other hormones, hydrocortisone and thyroxine play an important role in carbohydrate metabolism and blood sugar homoeostasis in *C. batrachus*.

Introduction

Besides glucagon and growth hormones, the hyperglycaemic agents, the secretory products of certain other well-known endocrine glands, such as the adrenal and the thyroid, are considered to be important tools for exploring the regulatory mechanism of insulin secretion from B cells of the pancreatic islets. Pertinent literature delineating the role of certain adrenocorticosteroids and thyroxine shows that these agents participate in the development of diabetes mellitus, concomitant with selective degenerative changes in the Langerhans islets [2, 4, 9, 13]. Information about modifications in tissue glycogen concentrations is, however, scanty [11, 12]. In the present studies the effects of hydrocortisone and thyroxine on the carbohydrate metabolism of the freshwater fish *Clarias batrachus* were investigated, viz., the glucose level of the blood and the glycogen levels of liver, muscle and brain were followed up. Furthermore the islet parenchyma of the treated animals was examined.

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Material and method

A 1% solution of hydrocortisone (Lot no., E648; CIBA Research Centre, Basel) in 0.9% saline was prepared just before use. The solution was injected i. m. to a group of 45 fish at a dose of 100 mg/kg body weight. Animals were killed 1, 5, 15 and 24 h after the treatment and, subsequently, at one-day intervals up to the 7th day. A freshly-prepared 1% solution of thyroxine (Eltroxin, B.D.H., India) was administered i. m. to 35 catfishes at a dose of 5 mg/kg body weight. The animals were killed at the predetermined intervals up to 5 days (Fig. 9). Another batch of 35 fish were injected with 10 g/kg body weight of 0.9% saline to serve as controls for both experiments. Blood glucose and the glycogen contents of various tissues were estimated according to the methods described earlier [10]. Statistical analysis was made after SINEDECOR [17]. Paraldehyde-fuchsin was used as routine staining procedure for the material fixed in Hellys' fluid for 12—18 h. Fishes were starving during the experiment at a water temperature of 24 ± 1 °C.

Results

Effect of hydrocortisone

Blood glucose. The blood glucose value in 120 untreated catfishes averaged 61.2 mg/100 ml [10]. Administration of hydrocortisone resulted in hyperglycaemia within an hour (91 ± 4.97 mg/100 ml). The values remained about the same level for 72 h. A further increase in the glycaemia was observed 96 h after the injection (109.5 ± 16.36 mg/100 ml) and all the treated fish were hyperglycaemic till the end of the experiment (Fig. 1).

Tissue glycogen. Hydrocortisone caused an increased deposition of glycogen in the liver within an hour, and the values increased from 58.06 mg/g (the normal mean value in 120 animals [10]) to 71.3mg/g 15 h after the injection. There was, however, a loss of glycogen from liver 24 h after the administration, reaching the lowest mean value, 40.9 ± 2.23 mg/g, at 96 h. Recuperation to normal values was observed by the end of the experiment (Fig. 1). The muscle and the brain glycogen concentrations were not modified to a significant extent and the values were within the normal range throughout the experimental period (Fig. 2).

Islet histology. The pancreatic islets of untreated control fish show well-recognizable A and B cells (Fig. 3). In the islets of hydrocortisone-treated fish a mild degranulation of B cells was observed within 5 h and, also, 15 h after the treatment; some islets showed a distinct loss of granulation in these cells (Fig. 4). An islet examined 48 h post-injection appeared to have considerably damaged cellular components (Fig. 5). Later, the necrobiotic changes in the islets were more severe and persistent: all B cells and some A cells contributed to the formation of large necrotic areas (Fig. 6). Atrophy and degranulation of B cells was prolonged for 4 days after the injection of hydrocortisone, but the A cells appeared approximately normal in configuration (Fig. 7).

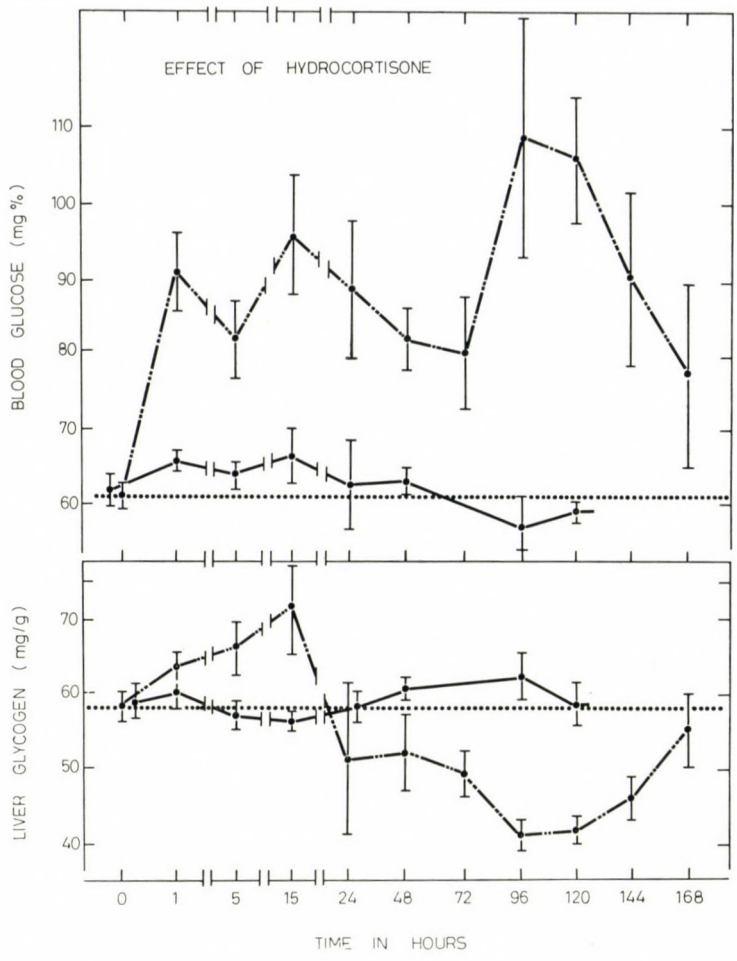


Fig. 1. Modifications in the levels of blood glucose (mg/100 ml) and liver glycogen (mg/g) of *Clarias batrachus* following treatment with hydocortisone. ——— control fish; —··—··— treated fish; — — — mean of 120 normal untreated fish. Each point represents the mean of 4 determinations, and carries an indication of the standard error of the mean

Effect of thyroxine

Blood glucose. Within an hour after treatment a hyperglycaemic condition was produced (92.5 ± 6.02 mg/100 ml), and the blood glucose reached its maximal level (116 ± 8.81 mg/100 ml) at 15 h. A pronounced decrease in the plasma glucose, which led to the appearance of a distinct hypoglycaemic state (46 ± 8.5 mg/100 ml), was observed 96 h post-injection. The blood glucose continued to drain, and a low level (44.3 ± 4.6 mg/100) ml was recorded at the end of the experiment (Fig. 8).

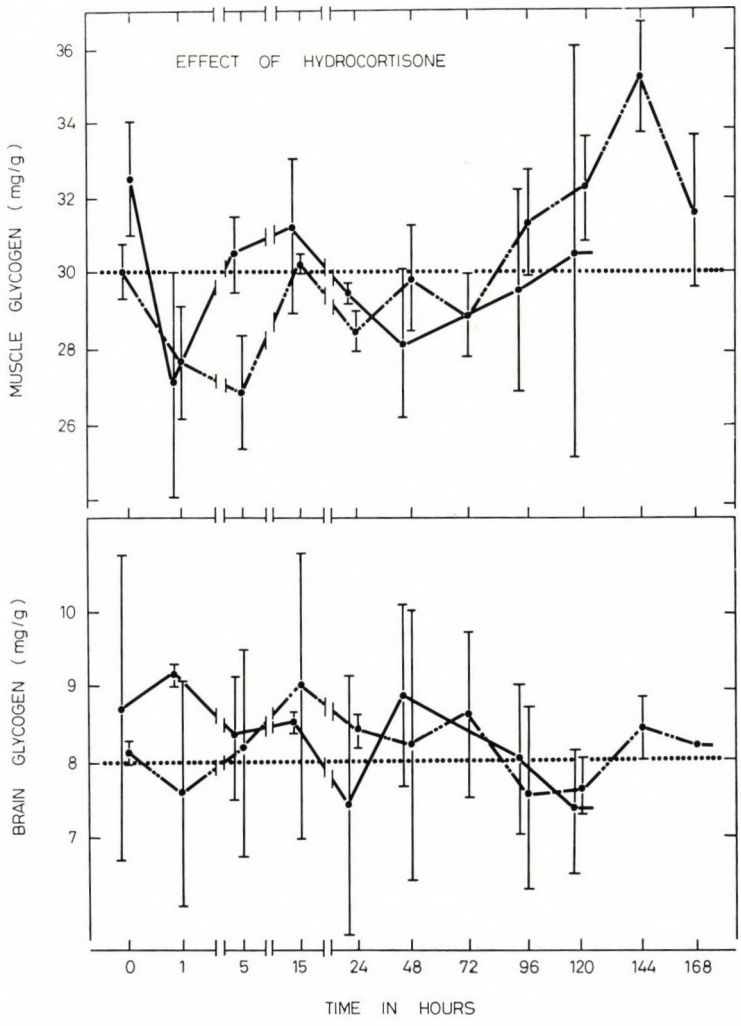


Fig. 2. Modifications in the glycogen levels of muscle and brain (mg/g) of *Clarias batrachus* following treatment with hydrocortisone. Designations same as in Fig. 1

Tissue glycogen. A decrease was registered in liver glycogen content (47.9 $\pm$ 4.35 mg/g) within 5 h after thyroxine administration. A further reduction in its amount (34.2 $\pm$ 3.39 mg/g) was recorded in the next few hours. The values, however, increased steeply by the 4th day and reached the base level when the experiment ended (Fig. 8). In the muscle glycogen, the hormone evoked a gradual increase up to 24 h (39.6 $\pm$ 2.59 mg/g). Restoration of the normal muscle glycogen concentration was evident within 48 h (Fig. 9). The brain glycogen stores were not noticeably altered, except for a slight increase between 5 and 24 h (Fig. 9).

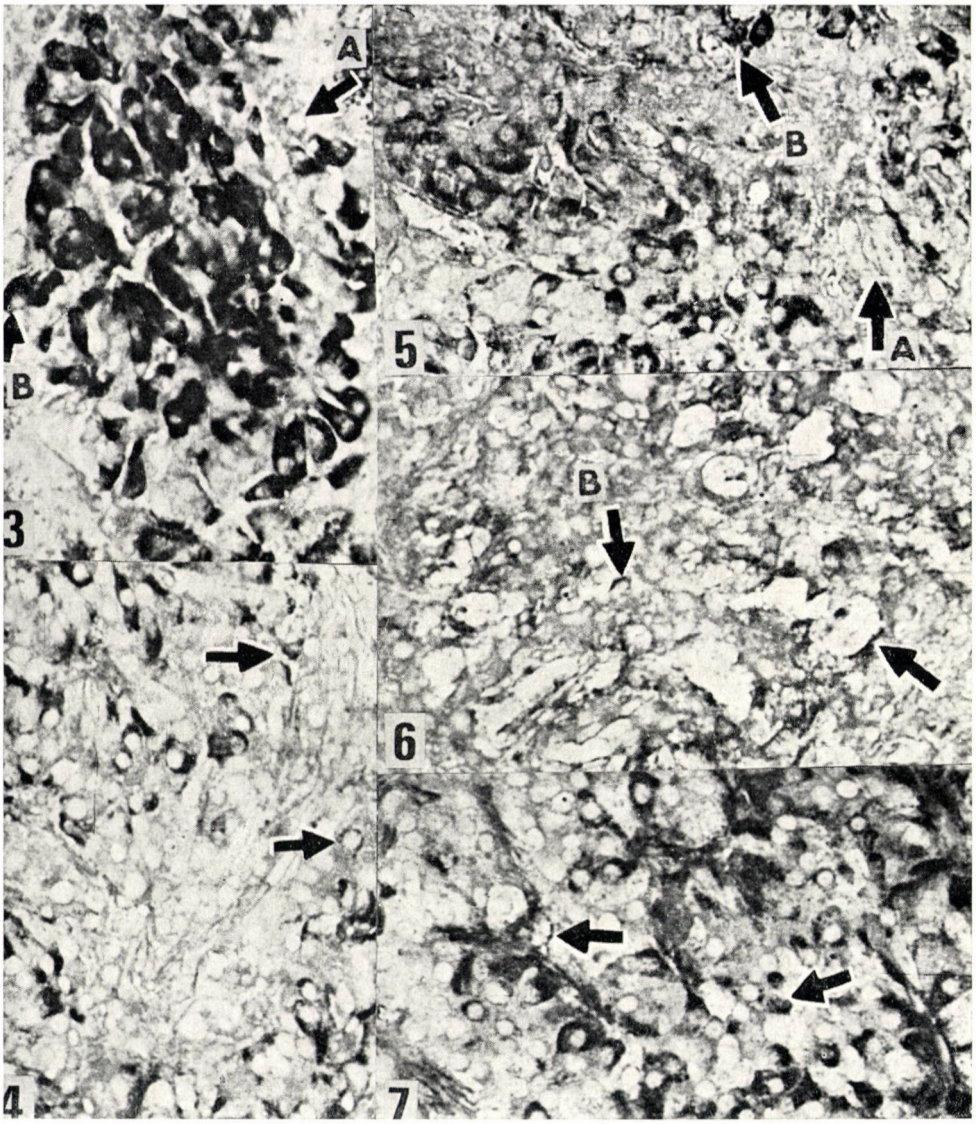


Fig. 3. Photomicrograph of the pancreatic islet of untreated *Clarias batrachus* showing normal A and B cells. AF stain, ×400

Figs 4—7. Photomicrographs of the pancreatic islets of *Clarias batrachus* showing changes in B cells (arrows) after treatment with hydrocortisone. AF stain, ×400. Fig. 4 = After 15 h; Fig. 5 = after 48 h; Fig. 6 = after 72 h; Fig. 7 = after 96 h

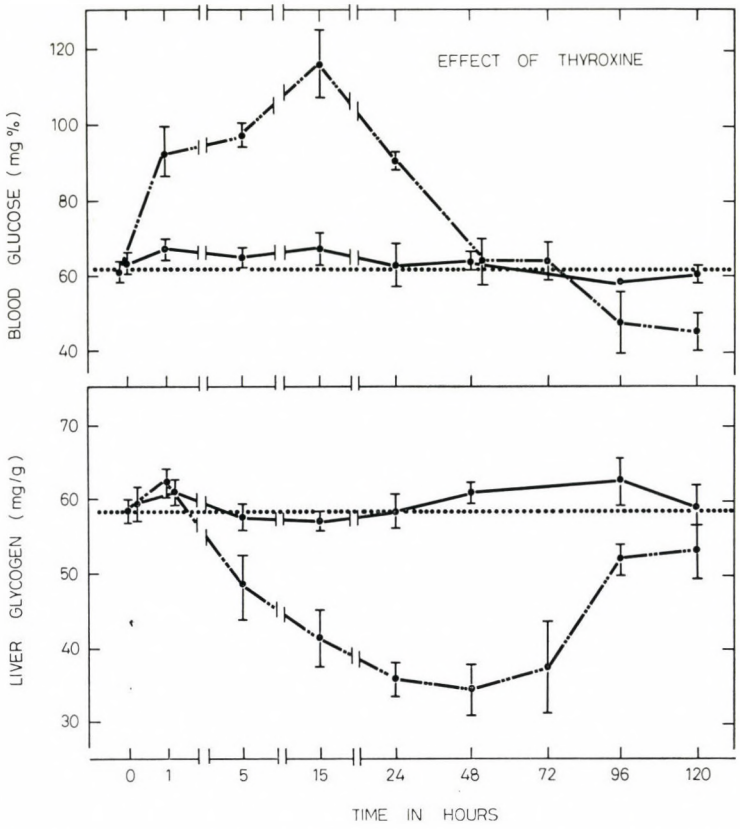


Fig. 8. Modifications in the levels of blood glucose (mg/100 ml) and liver glycogen (mg/g of *Clarias batrachus* treated with thyroxine. Designations same as in Fig. 1

Islet histology. No appreciable change in the islet cell elements was observed during the first 5 h after thyroxine administration. A few necrotic and degranulated B cells were observed 5 h after the injection (Fig. 10), and this condition persisted up to the 48th hour (Fig. 11). Almost all B cells appeared to be in a state of degranulation in the islets examined at 72 h (Fig. 12). The necrotic condition of B cells was more pronounced in the islets of the fishes killed at 96 h (Fig. 13). A normal granulation of the islet B cells was not recuperated even by the 120th hour and a number of them had reduced granular cytoplasm (Fig. 14). The A cells, except a few, remained unaffected (Fig. 14).

Although the saline-injected control animals showed a slight increase in the blood glucose level up to 15 h (Fig. 1), no marked changes could be observed in the glycogen contents of their organs (Figs 1 and 2). Neither of the hormones caused any change in the usual behaviour of the fish.

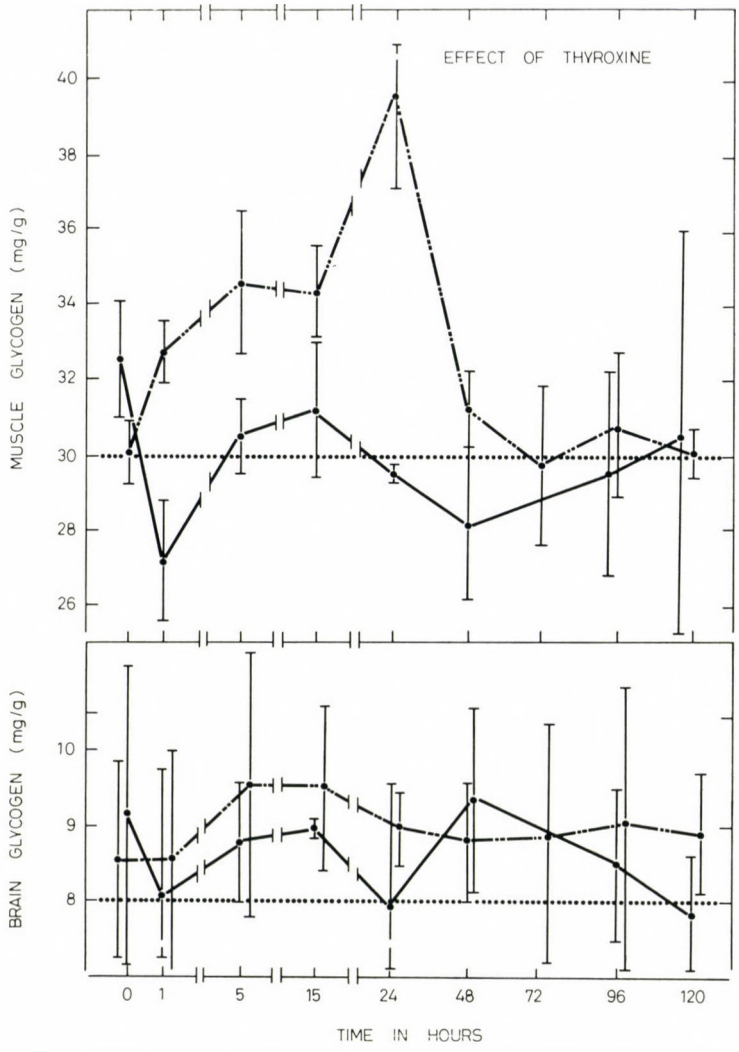
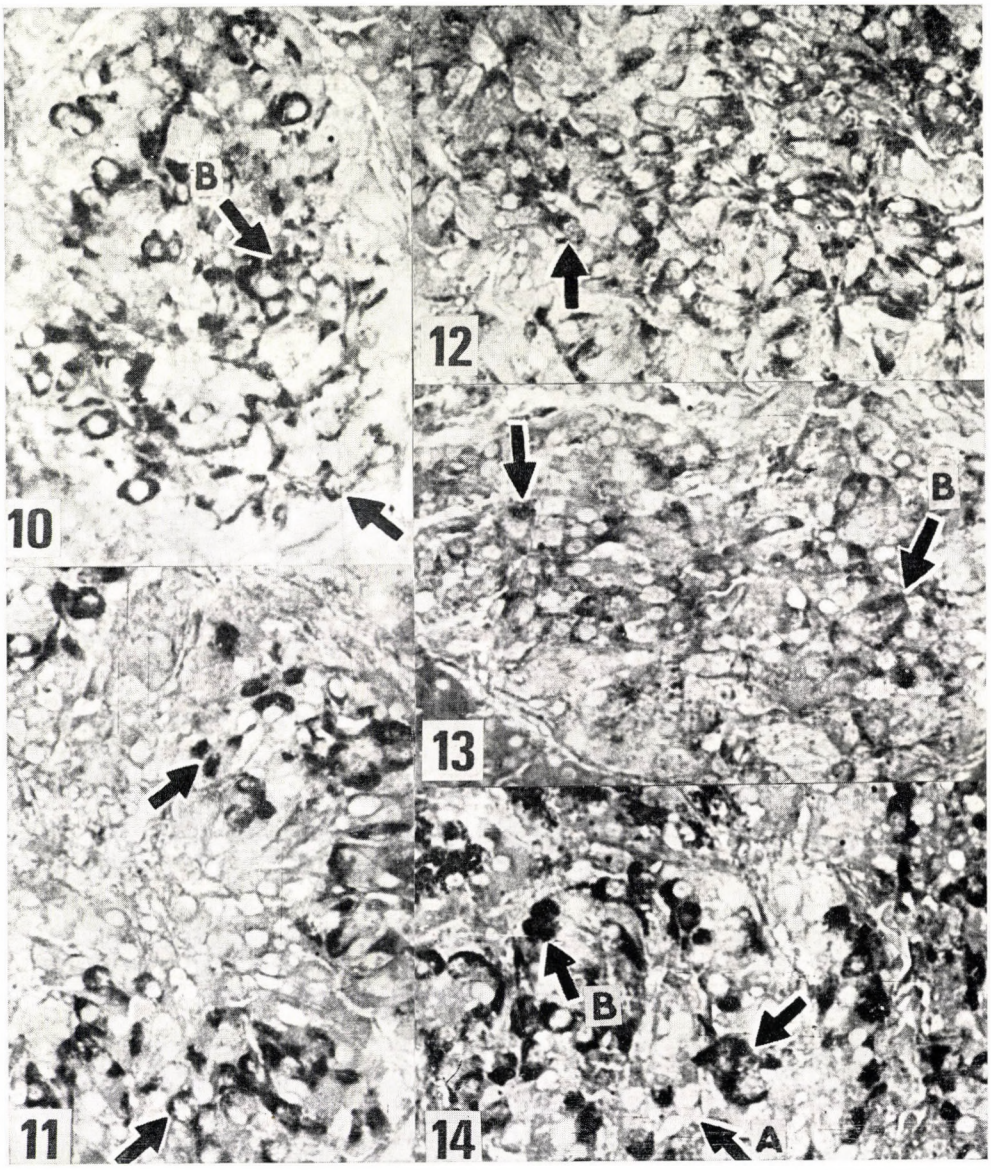


Fig. 9. Modifications in the levels of muscle and brain glycogen (mg/g) of *Clarias batrachus* after treatment with thyroxine. Designations same as in Fig. 1



Figs 10—14. Photomicrographs of the pancreatic islets of *Clarias batrachus* after treatment with thyroxine. The arrow indicates changes in the B cell configuration. AF stain, ×400. Fig. 10 = after 5 h; Fig. 11 = after 48 h; Fig. 12 = after 72 h; Fig. 13 = after 96 h; Fig. 14 = after 120 h

Discussion

Administration of adrenocorticoids to fasting normal or adrenalectomized animals caused a rise in the blood glucose level and a striking increase in the liver glycogen [8]. The data presented here on *C. batrachus* are comparable to those obtained in some other teleosts [4, 13]. NACE [13] observed the highest blood sugar level, 210 mg/100 ml, in *Opsanus tau*, the toad fish, 4 days after treatment with hydrocortisone. FALKMER [4] observed that only 6 of 28 sculpins (*Cottus scorpius*) were hyperglycaemic at the end of the experiment. In *C. batrachus*, a marked hyperglycaemic response was observed within an hour. Although all the injected fishes showed a parallel increase in the blood glucose, there were great individual differences in the degree of hyperglycaemia. A slow decrease in the blood glucose was noted 6 days after the treatment, but normal values were not recuperated by the end of the experiment. Like in *Notopterus notopterus* [11] and *Salmo gairdnerii* [14], administration of hydrocortisone increased the liver glycogen deposits of *C. batrachus* also. The lack of any change in the muscle and brain glycogen contents shows that the carbohydrate metabolism of these tissues is not influenced by the hormone.

FALKMER [4] observed small patches of necrotic B cells in the principal islets of hydrocortisone-treated *Cottus scorpius*. The present investigation shows that *C. batrachus* is much more sensitive to hydrocortisone than *Cottus scorpius*. Concomitant with a rise in the blood glucose level, the B cells underwent marked alterations in their cellular composition, which included degranulation followed by vacuolation, atrophy and necrosis. The A cells remained normal, except when the entire islet tissue was severely damaged.

It is difficult to assess the cause of the hyperglycaemia produced after hydrocortisone treatment in *C. batrachus*. It has been demonstrated that adrenocortical hormones increase body carbohydrate stores by promoting gluconeogenesis [1]. As liver and muscle did not show a fall in their glycogen reserves, it seems justified to conclude that hydrocortisone administration results in the synthesis of glucose from certain non-carbohydrate precursors, such as proteins, a part of which is also deposited as glycogen in the liver. Later, as a result of fostered glycogenolysis, the glycogen stores of the liver are decreased with the resultant increase in blood glucose. An increase in the blood glucose stimulates the secretion of insulin from the B cells, which consequently become damaged. The A cell damage may be explained to be due to exhaustion in them, so as to maintain a normal homeostatic balance.

Thyroxine, at a pharmacologically high dose (5 mg/kg) to *Myxine glutinosa*, produced some hyperglycaemia 24 h after the first injection but after 8 days of treatment, a hypoglycaemic condition appeared [5]. A marked hyperglycaemia was observed in *Rana temporaria* also [16]. However, SINGH

and co-workers [15] failed to show any effect of thyroxine on the blood sugar level in normal rats. Thyroxine administration in the fish under report, *C. batrachus*, evoked hyperglycaemia reaching the maximal value 15 h after the treatment. Thereafter, a gradual fall in the blood sugar resulted in a hypoglycaemic phase, 4 days after the injection. As in other teleosts [6], the increase in the blood glucose of *C. batrachus* appears to be due to hepatic glycogenolysis. However, McNABB [12] observed a marked decrease in the glycogen stores of muscles and an increase in the glycogen content of liver in thyroxine-treated leopard frog, *Rana pipiens*. In *C. batrachus*, while the brain glycogen remained unaffected, the muscle glycogen increased up to 24 h, but reached the base level by the next day. The cause of such diversities in the results remains to be investigated.

Dogs made 'thyroid-diabetic' revealed lesions of the B cells of the Langerhans islets; disappearance of granulation, increase in size, vacuolation, pycnosis, disintegration and, finally, disappearance of the B cells from the atrophied islets [3, 9]. Similarly, thyroxine treatment elicited changes in the B cell configuration of *C. batrachus*, the effect being more significant during the later part of the experiment when the fishes were hypoglycaemic.

It is known from the studies of ELGEE and WILLIAMS [3] that in rats the rate of degradation of insulin is increased by administration of thyroxine and reduced by the removal of the thyroid. Since the level of blood sugar controls insulin release and *vice versa*, it may be contended whether the action of thyroxine on carbohydrate metabolism is to bring about an increased demand of insulin so as to suppress the hyperglycaemia evoked by hepatic glycogenolysis. The excess secretion of insulin from the degenerating B cells most probably caused a hypoglycaemic state in a late phase of the experiment.

The available information does not indicate a direct influence of hydrocortisone and thyroxine on the pancreatic islets. As suggested by GORBMAN and BERN [7], the effects of these hormones on carbohydrate metabolism might not be 'regulatory' and they are not directly sensitive to the changes in the blood glucose. Through their effects on gluconeogenesis, amino-acid metabolism, lipogenesis, phosphorylation, etc., the hormones may create an imbalance in homeostasis which has to be tackled by the pancreatic hormones. The data cited here strongly suggest that the two hormones play a significant role in carbohydrate metabolism of *C. batrachus*.

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CROSS-RESISTANCE OF TRANSFORMED MOUSE CELLS TO SOME DRUGS

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Abstract

The Colcemid-resistant L—53 cell strain was examined for cross-resistance to metaphase inhibitors (Vincristine, Vinblastine, estradiol-17β), an antitumor antibiotic (Rubomycin C) and an alkylating agent (Lycurim), compared with the Colcemid-sensitive L cells. The L-53 cells proved to be resistant besides colchicine to Vincristine, Vinblastine and estradiol-17β concerning their antimitotic effect. The comparison of the viability of L and L-53 cells in the presence of Rubomycin C and Lycurim showed a resistance of the L-53 cells to Rubomycin C, while the effect of Lycurim was the same on both cell lines. The chromosome-mutagenic action of Lycurim was also equal on both cell lines.

Introduction

Under the influence of a drug, tumours may acquire resistance besides the same drug to several others. For example, Chinese hamster cells resistant to actinomycin D were found to be cross-resistant to Mithramycin, Vinblastine, Vincristine, Puromycin, Daunomycin, Colcemid and mitomycin C [1]. The mechanism of the cross-resistance is still unknown.

In this study we investigated cross-resistance in cultured mouse cells L and L-53. L-53 is a mutant cell line derived from L cells; it was selected for resistance to Colcemid [2]. According to Rosenblat and co-workers [3] its resistance is due to a decreased plasma membrane permeability to Colchicine and Colcemid. We compared the sensitivity of L and L-53 cells to some drugs from different chemical classes viz., alkaloids (Colchicine, Vincristine [Vcr], Vinblastine [Vbl]), a hormone (estradiol-17β), an antibiotic (Rubomycin C or Daunomycin) and an alkylating derivative of a sugar (Lycurim: dimethyl-sulphonyloxyethylamino-didesoxymesoerythrit-dimesylate).

These drugs have different modes of action on molecular level. Colchicine and its derivative Colcemid are metaphase inhibitors (antimitotics). These substances bind to molecules of tubulin — a microtubular protein —

and thus disrupt mitotic spindle [4]. Vcr and Vbl are also spindle poisons and also bind to molecules of tubulin, but the binding sites on the protein for Vinca-alkaloids and Colchicine are different [5].

The steroid hormone estradiol-17β is also a metaphase inhibitor. The precise mechanism of its action on the mitotic spindle is unknown. To disturb mitosis high concentrations ($\sim 10^{-4}$ mol/l) are needed, thus its action may be non-specific.

The antitumour antibiotic Rubomycin C (Daunomycin) is known to bind to DNA of chromosomes [6]. Lycurim is an alkylating drug [7]. Thus, these two substances are not spindle poisons.

In this report the results of our studies on the sensitivity of L and L-53 to four metaphase inhibitors and two cytotoxic drugs which do not disrupt mitotic spindle are presented. To reveal the degree of sensitivity of these cells to antimitotics, we counted the cells in different stages of mitosis. The cellular sensitivity to cytotoxic drugs was estimated by counting the number of cells in cultures after propagation in the presence of the drug.

In the present study we investigated the resistance of L and L-53 cells to different drugs, supposing that the differences in their resistance spectra are related to the decreased permeability of the cytoplasmic membrane of the L-53 cells to Colchicine.

Material and method

Culture conditions. L and L-53 cells were maintained in monolayer cultures. The culture medium for L cells consisted of a mixture of equal volumes of Eagle's essential medium and 5% solution of lactalbumin hydrolysate (1 : 1) supplemented with Monomycine (100 units per ml) and bovine serum (10%). The medium in which the L-53 cells were cultured contained, in addition, 0.2 μg/ml Colcemid; 5 to 7 days prior to the experiments Colcemid was omitted from the medium.

Drugs and chemicals. The following chemicals were used: Colcemid (CIBA), Colchicine (Merck, GFR), Vincristine (Elly Lilly L Co., USA), Vinblastine (Richter, Hungary), estradiol-17β (Koch Light, England), Rubomycin C (Institute of Antibiotics, USSR) and Lycurim (Richter, Hungary).

Evaluation of the antimitotic effect of metaphase inhibitors (Colchicine, Vcr, Vbl, estradiol-17β). Cell suspensions containing 2×10^5 cells/ml were placed in small flasks (2 ml per flask) with glass coverslips on the bottom. Drugs were added 24 h after seeding and the cultures were incubated in the presence of the drug for 2 h. The antimitotic effect of several drug concentrations was measured by the reduction of the percentage of postmetaphase mitotic cells (ana- and telophases); 100 mitoses per coverslip were counted. Late prophases, metaphases (both normal and C-metaphases), anaphases and telophases (or reconstructed nuclei) were taken into consideration.

Evaluation of the viability of cells propagated in the medium supplemented with the drugs (Rubomycin C and Lycurim). To study the effect of drugs on cell growth, we planted L and L-53 cells in small flasks with bottom area approximately 3 cm^2, 0.6×10^5 cells per cm^2. After 24 h the drugs were added to the cultures and after 48 h most cells were removed from the glass by trypsin treatment, and counted.

Assay of mutagenic action of Lycurim. Cells, 1×10^6 per flask, were seeded in flasks with bottom area 20 cm^2. Twenty-four hours later Lycurim was added to the cultures for another 24 h, and flame-dried chromosome preparations were made.

Results

The sensitivity of L and L-53 cells to spindle poisons

In the Tables 1, 2 and 3 the results of the comparison of sensitivity of Colcemid-resistant and sensitive cells to metaphase inhibitors (or spindle poisons) are presented. These drugs arrest dividing cells in C-metaphase, thus by counting cells in various phases of mitosis one can evaluate the effect of the drug on the spindle of the studied cells. In the Tables the percentages of postmetaphase cells are given.

L-53 cells proved to be resistant to Colchicine (Table 1). 0.5 μg/ml of the drug arrested all L cells in C-metaphase (postmetaphase cells were absent).

Table 1

Antimitotic effect of Colchicine on L and L-53 cells
(percentages of postmetaphase stages of mitosis)*

Strain	Control	Concentration of Colchicine (μg/ml)		
		0.05	0.1	0.5
L	49.0 $\pm$ 8.0	39.7 $\pm$ 6.1	31.7 $\pm$ 10.2	6.7 $\pm$ 6.7
L-53	52.0 $\pm$ 2.9	47.0 $\pm$ 7.0	55.5 $\pm$ 3.5	48.5 $\pm$ 3.5

Strain	Control	Concentration of Colchicine (μg/ml)		
		1.0	5.0	10.0
L	49.0 $\pm$ 8.0	—	—	—
L-53	52.0 $\pm$ 2.9	51.3 $\pm$ 2.9	57.5 $\pm$ 0.5	41.0 $\pm$ 5.0

* Results were obtained in 2 experiments. 200 mitoses per experimental group per experiment were counted

Even 10 μg/ml Colchicine did not inhibit cell division in L-53 cultures. Thus, these cells are 20-fold as resistant to the antimitotic action of Colchicine as wild-type cells are.

L-53 cells also possess some degree of resistance to estradiol-17β (Table 2). The difference in sensitivity of the studied cells was estimated in the presence of the hormone in concentrations 0.25 — 5.0 $\times$ 10^{-4} mol/l. Only some L cells remained in ana- and telophases while 13—20% of the L-53 cells were in postmetaphase stages of mitosis (Table 2).

The sensitivity of L and L-53 cells to Rubomycin C and Lycurim

The viability of L and L-53 cells cultured in the presence of two drugs which do not affect mitotic spindle was compared (Tables 4 and 5). The sensitivity of cells to these agents was determined by counting the cells per

Table 2

Antimitotic effect of estradiol-17β on L and L-53 cells
(percentages of postmetaphase stages of mitosis)*

Strain	Control	0.5% ethanol (solvent of estradiol)	Concentration of estradiol-17 β ($\times 10^{-4}$mol/l)		
			0.25	1.25	5.0
L	50.4 ± 3.9	49.0 ± 2.8	24.0 ± 9.4	27.0 ± 0.9	2.7 ± 2.6
L-53	53.0 ± 7.6	43.5 ± 3.5	33.5 ± 3.5	20.5 ± 3.5	13.5 ± 8.2

* Results were obtained in 3 experiments. 200 mitoses per experimental group per experiment were counted

L-53 cells were found to be highly resistant to two other metaphase inhibitors, viz., Vcr and Vbl (Table 3). Vcr in a concentration of 0.1 μg/ml arrested all dividing L cells in C-metaphase. The same effect was obtained in L-53 cultures with the 100-fold concentration of the drug. L-53 cells proved to be also approximately 100-fold more resistant to Vbl. Postmetaphase stages of mitosis were almost absent in L cultures treated with 0.01 μg/ml and disappeared in L-53 population in the presence of 1.0 μg/ml of Vbl.

Table 3

Antimitotic effect of Vincristine and Vinblastine on L and L-53 cells
(percentages of postmetaphase stages of mitosis)*

Strain	Control	Vincristine (μg/ml)			
		0.01	0.1	1.0	10.0
L	50.4 ± 3.9	16.5 ± 7.3	0.25 ± 0.5	0	—
L-53	53.0 ± 7.6	49.5 ± 3.5	49.2 ± 11.2	12.5 ± 2.1	0.5 ± 0.7

Strain	Control	Vinblastine, (μg/ml)			
		0.001	0.01	0.1	1.0
L	50.4 ± 3.9	47.2 ± 1.3	8.5 ± 11.4	0	—
L-53	53.0 ± 7.6	—	54.0 ± 5.1	52.0 ± 1.5	1.0 ± 0.0

* Results were obtained in 3 experiments. 200 mitoses per experimental group per experiment were counted

flask after propagation of the cultures in the presence of drugs. These experiments have shown that the L-53 cells are resistant to the cytotoxic action of Rubomycin C.

In Table 4 the results of two experiments are shown. In the first experiment Rubomycin C was added to the culture 24 h, and in the second one 2 h.

Table 4

*Effect of Rubomycin C on the number of L and L-53 cells after 48 h of cultivation**

Concentration of Rubomycin C (μg/ml)	L				L-53			
	Experiment I		Experiment II		Experiment I		Experiment II	
	Number of cells**	Per cent	Number of cells	Per cent	Number of cells	Per cent	Number of cells	Per cent
0	1.64	100	1.70	100	0.87	100	0.96	100
0.01	1.79	109.4	0.50	29.6	1.12	128.7	—	—
0.1	0.63	38.7	0.45	26.7	1.34	154.2	0.74	77.5
0.5	0.41	25.2	0.22	13.3	0.92	106.4	0.48	50.7
10.0	0.20	12.7	0.10	6.0	0.54	61.9	0.26	27.6
50.0	0.02	1.4	0.03	2.1	0.56	63.9	0.11	12.1

* Results were obtained in 2 experiments. Cells from 2 flasks per experimental group per experiment were counted
** Number of cells $\times 10^5/cm^2$

Table 5

*Effect of Lycurim on the number of L and L-53 cells after 48 h of cultivation**

Concentration of Lycurim (μg/ml)	L		L-53	
	Number of cells**	Per cent	Number of cells	Per cent
0	2.43 ± 0.25	100	1.42 ± 0.31	100
0.1	2.44 ± 0.18	100	1.31 ± 0.24	92.2
0.5	2.22 ± 0.14	91.3	1.09 ± 0.12	76.7
1.0	2.18 ± 0.15	89.7	1.11 ± 0.11	78.1
10.0	1.02 ± 0.04	41.9	0.71 ± 0.13	50.0
50.0	0.49 ± 0.12	20.1	0.35 ± 0.01	24.6

* Results were obtained in 3 experiments. Cells from 2 flasks per experimental group were counted
** Number of cells $\times 10^5/cm^2$

after seeding. In both experiments a high degree of drug resistance was revealed (approximately 100-fold).

In the first experiment 0.1 μg/ml Rubomycin C substantially diminished the number of L cells harvested from the flask while even 50 μg/ml had a lesser effect on L-53 cells. In the second experiment lower concentrations of Rubomycin C were effective. Apparently, this is connected with a greater drug sensitivity of the cells immediately after planting. Obvious difference in the viability of L and L-53 cells propagated in Rubomycin C supplemented medium was seen also in this experiment.

The growth of L cells was inhibited up to 26.7% by 0.1 μg/ml of the drug, the antibiotic had almost the same effect on the L-53 cultures in a concentration of 10.0 μg/ml.

Thus, these experiments show that L-53 cells are cross-resistant both to metaphase inhibitors and to antitumour antibiotic, i.e., to drugs with different modes of action on the cell.

Cell lines L and L-53 proved to be similarly sensitive to Lycurim, which inhibited the growth of both lines in a concentration range from 0.5 to 50 μg/ml (Table 5).

Mutagenic action of Lycurim on L and L-53 cells

The action of Lycurim on the chromosomes of L and L-53 cells was comparatively studied (Table 6). With increasing concentration of the drug the cells with chromosome aberrations rose in number. The same dose of Lycurim had approximately the same mutagenic effect on the cells of both cell lines, indicating that the L-53 line had not acquired cross-resistance to this alkylating drug.

Table 6

*Mutagenic action of Lycurim on L and L-53 cells**

Concentration of Lycurim (μg/ml)	L			L-53		
	Number of cells analyzed	Aberrant cells		Number of cells analyzed	Aberrant cells	
		number	per cent		number	per cent
0	50	1	2	50	5	10
0.1	50	5	10	50	5	10
0.5	50	10	20	50	10	20
1.0	50	13	26	43	19	44.1

* The results were obtained in one experiment

Discussion

Our data show that a cell line that had been selected for resistance to Colcemid is cross-resistant to a number of drugs of different chemical classes and different modes of action.

The range of the drugs to which cellular cross-resistance may occur has been discussed in the literature. On the basis of cross-resistance it is possible to distinguish several groups of drugs [1, 8, 9]. Our data and those of some other authors have shown that to one of these groups belong to Colchicine and

its derivatives, Vinca-alkaloids, Daunomycin (Rubomycin C), Actinomycin D and, probably, some other substances. We have shown previously that L-53 cells have also some degree of cross-resistance to the non-ionic detergent Tween 80, which does not penetrate the cell [10]. Thus, the range of the drugs of this group is evidently very wide. Lycurim and other alkylating agents form another group of drugs.

Cross-resistance to very different agents is connected with alterations of the plasma membrane of the cells. Colchicine-resistant Chinese hamster cells proved to be cross-resistant to the drugs of the same group [1, 9]. It seems that the cell membrane alterations which lead to cross-resistance to different chemicals are not highly specific, though, the fact that the L-53 cells resistant to Colchicine and some other drugs have retained their sensitivity to Lycurim points to some degree of specificity. The subline of Chinese hamster cells resistant to actinomycin D was cross-resistant to six other drugs and sensitive to nine substances. Among these substances there were antitumour antibiotics, antimetabolic and alkylating agents and hormones [1].

This specificity of membrane alterations conferring cross-resistance may be connected with similar alterations of the plasma membrane of different drug-resistant cell lines. The nature of membrane alterations is still unknown. It has been reported [11, 12] that the membrane glycoproteins and glycolipids of resistant cells are somewhat modified. Possibly, the changes in membrane lipids are the cause of the relative resistance of L-53 cells to Tween 80. Further studies of the phenomenon of cross-resistance may elucidate the cellular mechanisms of drug resistance. It is reasonable to study both the range of substances to which cross-resistance occurs and the molecular basis of membrane alterations conferring cellular drug resistance.

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THE HAEMATOPOIETIC SUPRANEURAL ORGAN OF ADULT, SEXUALLY IMMATURE RIVER LAMPREYS (*LAMPETRA FLUVIATILIS* [L.] GRAY) WITH PARTICULAR REFERENCE TO AZUROPHIL LEUCOCYTES

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Abstract

The haematopoietic tissue in the supraneural organ of the freshwater river lamprey (*Lampetra fluviatilis* L. GRAY) was studied in sexually immature animals. Besides erythro- and granulopoietic elements, macrophages, reticular cells, fibroblasts and glycogen-rich fat cells were seen. Developing granulocytes of the lamprey contain one type of azurophil granules originating from small cytoplasmic (Golgi) vesicles. The lamprey's azurophil granulocytes seem to be homologous with those of fishes. However, the granulocytes of fishes, studied thus far, show granules with only one type of inclusion, whereas in lamprey the granulocyte inclusions are variable in size and shape. Thus, lamprey granulocytes are, in this respect, reminiscent of similar cells of higher vertebrates. The PAS and alkaline phosphatase reactions, common markers of vertebrate neutrophil leucocytes, are very weak in the haematopoietic tissue granulocytes of the lamprey, and intense in the blood cells of the same animal. Lamprey granulocytes, similarly to the granulocytes of *Chondrostei* and *Elasmobranchiata*, do not stain with peroxidase, naphthol-AS-D-chloroacetate esterase and sudan black B. The haematopoietic tissue contains a relatively high number of degenerated granulocytes.

Introduction

Lampreys are often used in comparative studies because in many structural features they resemble fossil *Cephalaspidomorphi* from the Orovician, Silurian, and Devonian periods. Furthermore, their life cycle contains several remarkable traits. The larvae (ammocoetes) of river lampreys spawn in fresh water, undergo after about four years metamorphosis and migrate to the sea; after a period of growth and fattening in the sea they return to fresh water in autumn, where they stop feeding. The following spring they reach sexual maturity, spawn, and die a few weeks later. Before natural death profound changes occur in their blood and hematopoietic tissue. The erythrocytes disintegrate, the hematocrit values decrease [27], and the hematopoietic supraneural organ undergoes atrophy, especially in females (LARSEN, unpublished).

Among the about 30 recognized species of lampreys the peripheral blood cells have been studied by light microscopy in *Lampetra wilderi* (= *lamottenii*) [18], *Petromyzon* (= *Lampetra*) *planeri* [37], and *Petromyzon marinus* [35]. Hematopoiesis in lampreys has been recorded to occur in a number of organs viz., the typhlosole or spiral valve of the intestine, the kidney, the branchial region, the region posterior to the pineal gland [13, 36].

The purpose of the present study was to extend previous comparative studies of azurophil granulocytes [20] and, further, to investigate the changes at sexual maturation in a haematopoietic organ known in *Petromyzon marinus* to produce granulocytes, the supreneural myeloid organ ("fat body" or "protovertebral arch tissue") [12, 13, 14, 36]. The present report includes a description of the supraneural organ and its cells in adult river lampreys about three months before development of secondary sex characters starts. A later publication will describe the conditions in sexually mature lampreys.

Material and methods

Five adult lampreys (3 females and 2 males) used in this study were caught in Varde å, Jutland, Denmark; they arrived in the laboratory on November 14 and were kept in tapwater at 11 °C. They had small wounds in the skin, but were otherwise healthy. They were killed between November 19 and 25, 1972, i. e., when they were sexually immature. The animals were anaesthetized by placing them in a 0.1% solution of MS 222 (methane sulphonate of m-amino benzoic acid ethyl ester, Sandoz) for a few minutes.

Light- and electron microscopy. From small fragments of the supraneural organ, taken from the middle part of the body, smears and touch preparations (imprints) were made and air-dried. Whole profiles of the organ, 2—3 mm thick, were fixed in 10% solution of phosphate-buffered formol (0.1 mol/l, pH 7.2) and embedded in paraffin. Small blocks 1 to 2 mm in diameter were fixed in 1% solution of phosphate-buffered osmium tetroxide (0.1 mol/l, pH 7.2), at 4 °C for 90 min, dehydrated in acetone, and embedded in Durcupan (Fluka). Sections, 1 μm thick and ultrathin, were cut with glass knives (Knife Maker, LKB) in an Ultrotome III (LKB) ultramicrotome.

Smears and touch preparations were stained with May-Grünwald-Giemsa solution and tested for alkaline [19] and acid [29] phosphatase, α-naphthylacetate-, naphthol-AS-acetate- [30], naphthol-AS-D-chloroacetate-esterase [28] and diaminobenzidine-peroxidase [15]. The periodic acid-Schiff reaction, Sudan black B and diaminobenzidine [16] stainings were also used. Paraffin and 1 μm thick plastic-embedded sections were stained with either haematoxylin-eosin [21] or toluidine blue [42], ultrathin sections contrasted with uranyl acetate-lead citrate [43]. Ultrathin section were studied in a Tesla BS 242E or 613 electron microscope at 60 and 80 kV.

Results

Anatomy

In cross sections the haemopoietic supraneural organ appeared as a round body, red, brownish, yellowish-red or yellow in colour and 3 to 5 mm in diameter, situated dorsally to the spinal cord and extending from the gill region to 0—2 cm anterior to the cloaca.

Light microscopy

The supraneural organ of the November lamprey is composed of haematopoietic, fat and reticular cells, macrophages and fibroblasts. It is well circumscribed, but not encapsulated. The number of fat cells dispersed among other elements varies from one animal to the other, capillaries are small in number. Collagen and reticulin fibres are few. Granulo- and erythropoietic elements are not separated; granulopoietic elements usually predominate in number (Fig. 1). A few large macrophages with engulfed material (cell débris) and few granules were also seen (Fig. 9). One μm thick sections show granulocytes with a large number of granules; erythropoietic cells can be recognized by the empty appearance of their cytoplasm. Besides these, a few strongly stained acidophil structures with processes reaching into the spaces between the neighbouring cells were encountered (Fig. 1). At the light microscopic level it was not possible to identify the nature of these structures. The periodic acid-Schiff reaction was negative, except for the narrow cytoplasm of fat cells (Fig. 2a).

In touch preparations the neutrophil granulocytes, owing to the high number of azurophil granules, were conspicuous (Fig. 3). In the immature forms (myelocytes) the nuclei, lying eccentrically, were round or oval with more or less deep indentations. The more mature granulocytes showed a bilobated nucleus; only very few cells with three nuclear lobes were seen. Cells with eosinophil or basophil granulation were not found.

Histochemistry

The periodic acid-Schiff (PAS) and the alkaline phosphatase reactions of the neutrophil granulocytes were very weak, although blood neutrophils of the same animals gave intense reactions (Figs 4a, b). In touch preparations a suprisingly large number of PAS-positive granules were seen among the cells and assumed to represent glycogen originating from disrupted fat cells (Fig. 2b). The diaminobenzidine-peroxidase and naphthol-AS-D-chloroacetate-esterase reactions, the Sudan black B and diaminobenzidine stainings were invariably negative, and the naphthylacetate-, naphthol-AS-acetate-esterase and acid phosphatase reactions weak.

Electron microscopy

The granulocytes appeared as round or oval cells about 16—21 μm in diameter (Figs 5, 6). The nuclear structure of myelocytes (mononuclear) and of the granulocytes (bilobated) were similar, the usually small amount of heterochromatin being somewhat larger in the latter cells; the high degree

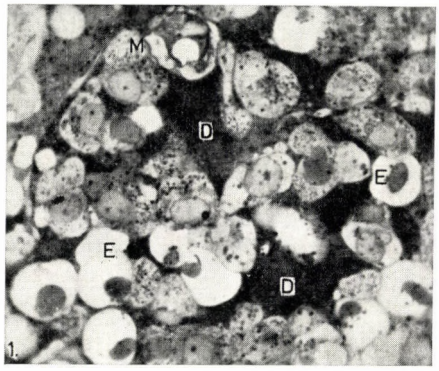

Fig. 1. Immature granulocytes with distinct granules, a macrophage (M), erythroblasts (E) with seemingly empty cytoplasm and irregular black structures (D) corresponding to degenerated granulocytes. Supraneural organ, 1 μm thick section, toluidine blue, pH 10; $\times 860$

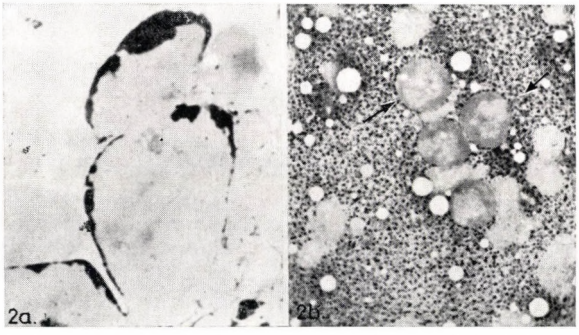

Fig. 2a—b. (*a*) Positive PAS reaction in thin cytoplasm of supraneural organ tissue fat cells. One μm thick section, $\times$ 480; (*b*) PAS-positive granules originating from disrupted fat cells scattered over the field. The PAS reaction of granulocytes is very weak (arrows). Touch preparation of the supraneural organ. $\times$ 800

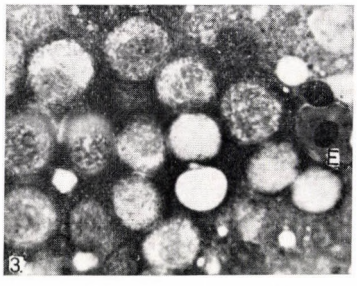

Fig. 3. Granulocytes with azurophil granules, erythroblasts and an erythrocyte (E). Touch preparation of the supraneural organ, May-Grünwald-Giemsa, $\times$ 840

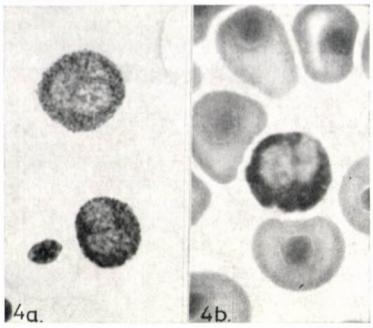

Fig. 4a—b. (*a*) Fine PAS-positive granules and (*b*) strongly positive alkaline phosphatase reaction in blood granulocytes. Blood smear, × 980

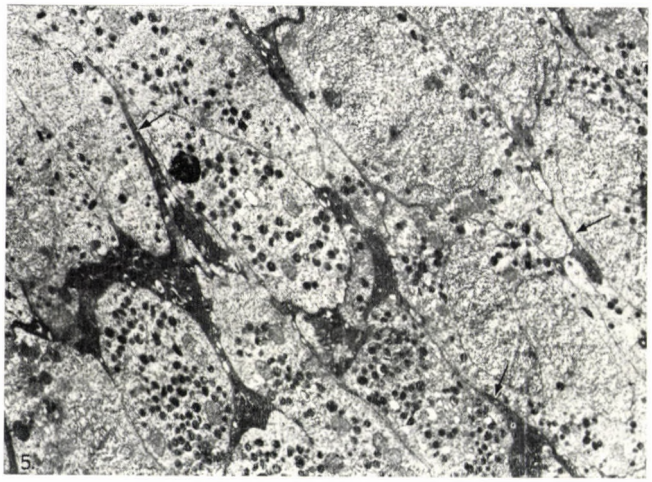

Fig. 5. Supraneural organ. Granulocytes with electron-dense granules. Between the cells parts of degenerated granulocytes and their thin projections (arrows). × 3 700

of nuclear pycnosis seen in mammalian granulocytes was not observed. Mitotic figures were frequent. Moreover, a few mitochondria, lipid droplets, glycogen granules in small groups, Golgi regions and cisternae of ergastoplasm were encountered. The predominant cytoplasmic organelles of the granulocytes were the granules 0.2 to 0.7 μm in diameter and variable in shape and structure. They were surrounded by a trilaminar membrane and seemed to originate in the immature cells from small vesicles, possibly Golgi vesicles, evenly distributed over the whole cell profiles. The development of granules seems to start with the appearance in the vesicles of a slightly electron dense material, which, parallel to an increase in the granule size, increases gradually in amount

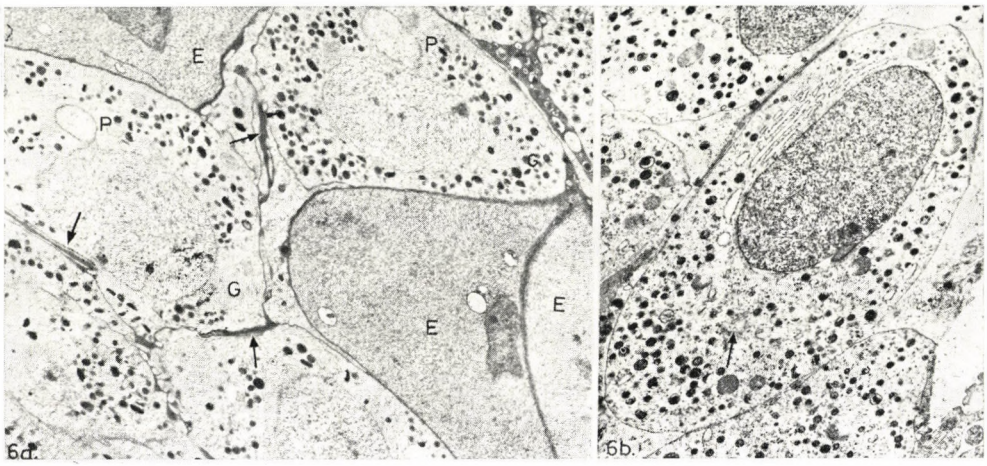

Fig. 6a—b. (*a*) Supraneural organ. Erythrocytes (E), granulocytes (G) with nuclear pockets (P) and granules varying in size. At arrows thin projections of degenerated granulocytes. × 4900. (*b*) Granulocyte with a small group of parallel ergastoplasmatic cisternae (Döhle body), few mitochondria, Golgi region (arrow). × 6 300

and electron density. The electron dense cores of the granules were round, oval or polygonal, and in the more mature forms inclusions were seen. Fig. 7 shows ring-, rod-, needle-shaped and irregular inclusions; in some of the more elongated inclusions a fine fibrillary structure was present. Our findings on granules indicate that not more than one type can be distinguished with certainty; however, the appearance of the granules varies greatly.

Two to four profiles or narrow ergastoplasmatic cisternae arranged in parallel order were frequently seen at the periphery of granulocytes with many azurophil granules, reminiscent of the so-called Döhle bodies (Fig. 6b). These structures, seen in mammalian blood neutrophil granulocytes, appear in conditions when neutrophil production by, and release from, the bone marrow are increased [31].

The deeply acidophil structures seen in the light microscope were identified as granulocytes in different stages of breakdown with decaying nuclei and granules in the very electron dense cytoplasm. These cells, appearing "compressed", invaded with very thin processes the interstitial spaces between the neighbouring cells (Figs 5, 6a and 8). Some of the decaying cells showed intense, cystic dilatation of their few ergastoplasmic cisternae and clumped glycogen granules.

The fat cells, demarcated from the haematopoietic elements by a basement membrane, contained varying amounts of fat. The narrow cytoplasm of these cells was tightly packed with glycogen granules, forming rosette-like

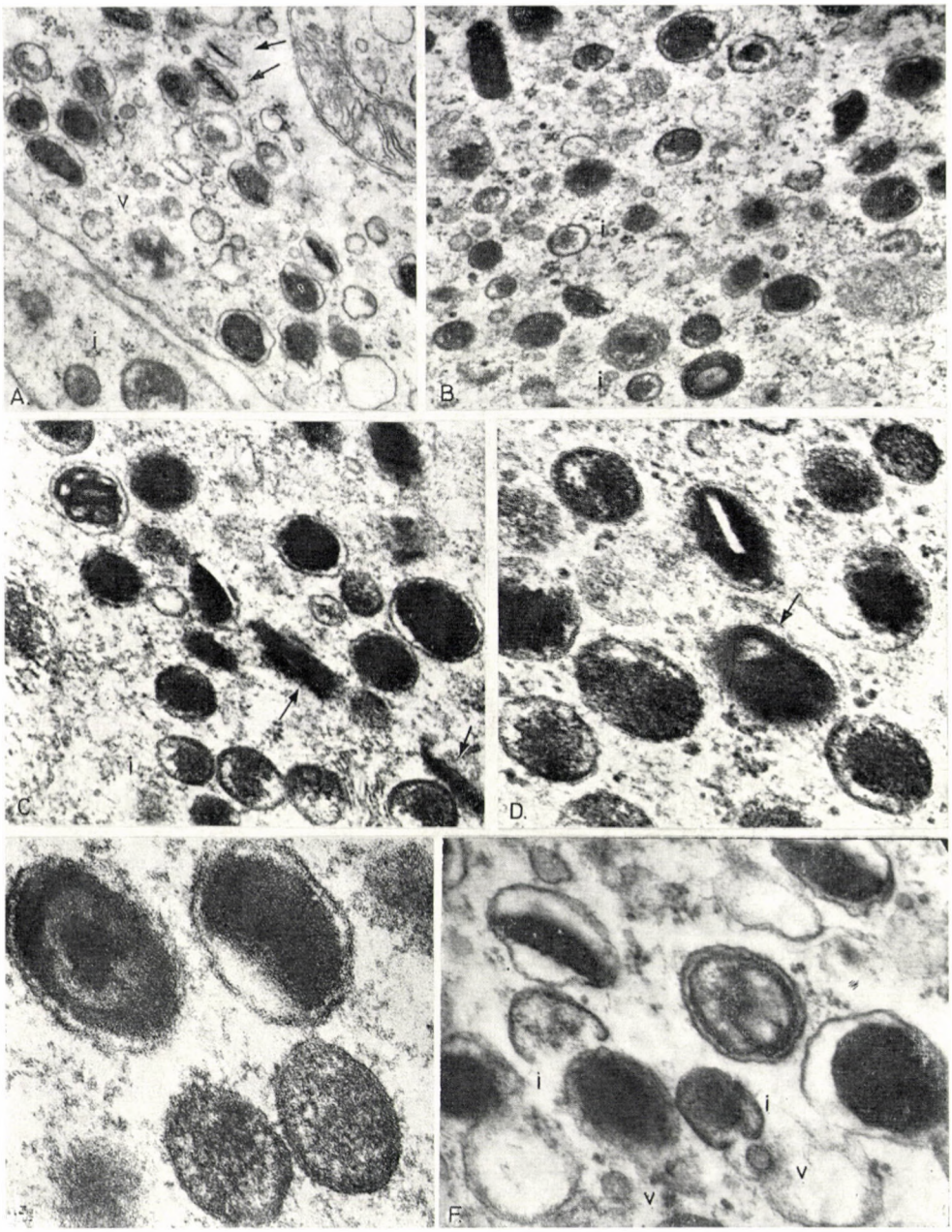

Fig. 7a—f. Granules of supraneural organ granulocytes. (*a*) Needle-like (arrows) and irregular inclusions, Golgi vesicles (V) and immature granules (I). × 21 000 (*b*) Immature (I) and mature granules with cores of varying electron density, rod-shaped and ring-like inclusions. × 24 000 (*c*) Besides round granules with electron-dense cores there is one with multiple ring-like structures and two others with rod-like inclusions (arrows). At the bottom of the field four immature granules (I). × 38 000 (*d*) Granules with cores of varying electron density, in one of them part of a ring-like inclusion (arrow), in another a thin empty rod. × 46 500 (*e*) A ring-like and a round inclusion in granules surrounded with a trilaminar membrane. At the bottom two immature granules with very fine vesicles of low electron density. × 65 000. (*f*) The granules in this field show various stages of granule formation. Small vesicles (V), immature granule (I) and mature granules with ring-like and round inclusion. × 54 500

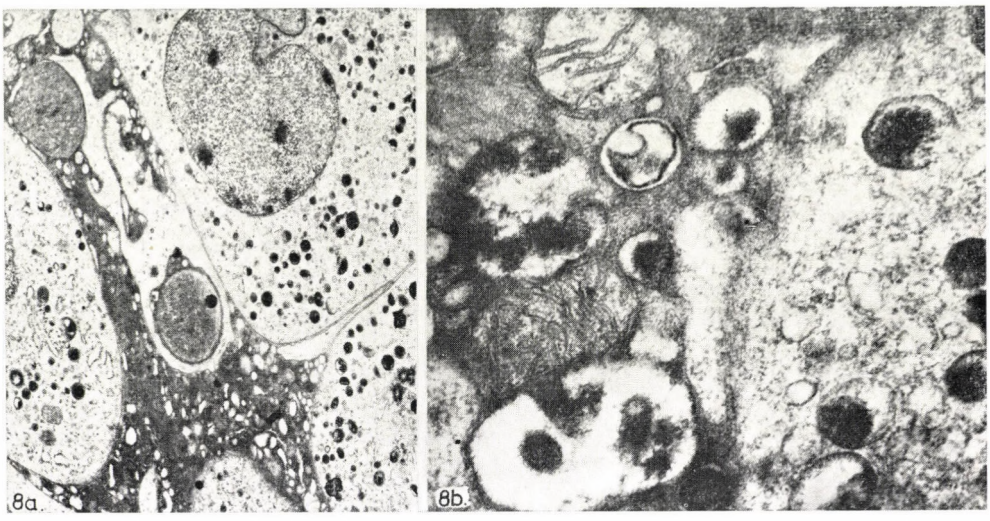

Fig. 8a—b. (*a*) Supraneural organ, parts of intact granulocytes and a degenerated cell with two nuclear lobes, one seemingly surrounded by another cell. The granules appear as empty holes, the cytoplasm is very electron dense. × 6100. (*b*) On the right, an intact cell with a few granules, on the left the cytoplasm of a decaying cell is very dense. Inside the granules remnants of the original structures, a mitochondrion with cristae. × 41 000

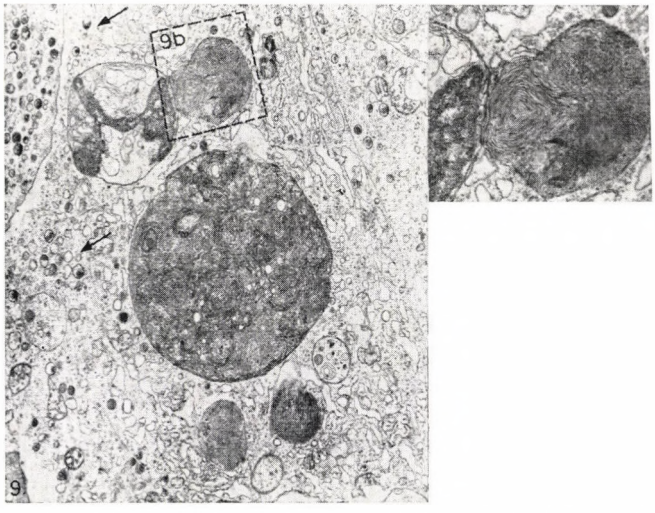

Fig. 9a—b. Supraneural organ, (*a*) a macrophage with various inclusions (phagolysosomes), a few granules (arrows) similar to granulocyte granules. × 6100. (*b*) Phagolysosome with whorl-like structure (myelin body). × 14 100

structures. In the region of the basement membrane, inside the cytoplasm, many pinocytic vesicles were present. The cytoplasm of some glycogen-free fat cells contained degenerating mitochondria.

Discussion

The term "supraneural organ" for the haematopoietic tissue located dorsally to the spinal cord was preferred to the term "fat body" or "protovertebral arch tissue" [14, 36]. Although the supraneural organ contains fat cells it should not be confused with the real fat body found in connection with the gonad in ammocoete larvae [44]. Protovertebral arches (*arcus neuralis*) are the rudimentary vertebrae which have nothing to do with the supraneural organ. The supraneural organ with differentiating precursor and more mature haematopoietic elements and fat cells is comparable to the bone marrow in higher vertebrates.

At the light-microscopic level developing granulocytic elements of the supraneural organ are easily recognized and their similarity to developing granulocytes of higher vertebrates is conspicuous. This may be due, among others, to the characteristic granulation seemingly common to all vertebrates, even to those in which the mature granulocytes have large eosinophilic granules (reptiles, birds, rabbit, guinea pig, pseudoeosinophil or *heterophil* granulocytes) [17]. This morphological constancy of the granulocytes is, however, incomplete, i.e., there are some clear-cut differences between the developing granulocytes of various classes of higher vertebrates.

In mammals the *neutrophil* granulocytes have at least two distinct populations of granules. The larger primary or azurophil granules, binding metachromatic dyes when immature [38], are peroxidase-positive, they possibly represent lysosomes and appear earlier during cell differentiation than do the secondary, specific or neutrophil granulation. The formation of the latter begins at the myelocytic stage [3, 4, 10]. It was generally agreed that this later granule carries the enzyme alkaline phosphatase. Recent observation, however, would suggest characterizing secondary granules as peroxidase-less and lactoferrin-lysozyme-containing structures, since alkaline phosphatase appears as a fraction clearly separated from secondary granules [41]. Substantial ultrastructural, cyto- and biochemical evidence supports the existence of a third type of granule [1, 2, 5, 9, 38, 40].

The developing heterophil granulocytes of birds and reptiles contain two granule populations [7, 34]. Concerning the presence of secondary granules in amphibian cells, conflicting opinions are known [8, 39]. In fishes developing granulocytes contain only one population of granules, which is azurophilic (*azurophilic granulocytes*, [35]).

In the freshwater river lamprey one population of granules, azurophilic, in both blood and haematopoietic tissue cells was found, probably homologous to the azurophilic granules of the granulocytes of fishes.

In spite of this similarity, fish and lamprey granulocytes differ in a number of cytochemical and ultrastructural features. Cytochemically lamprey

azurophil granulocytes of both the supraneural organ and of the blood are peroxidase-, naphthol-AS-D-chloroacetate-esterase- and Sudan black B-negative. The species of *Teleostei* thus far studied have been found peroxidase-positive, others (*Chondrostei, Ganoidei: Acipenser ruthenus, Elasmobranchiata, Selachidea: Scyliorhinus canicula*) negative [11, 20]. Thus, in this respect, the granulocytes of the November lamprey are similar to those of fishes of lower order. The finding of FEY [11] of a weak positive peroxidase reaction in the granulocytes of adult *Lampetra planeri* (and a negative reaction in larvae) might reflect differences in various species of *Cyclostomes*.

At the electron-microscopic level the developing granulocytes of *Teleostei* (kidney), *Chondrostei* (spleen) and *Elasmobranchiata* (spleen) contain one population of granules, the more mature forms of which carry only one type of inclusion [20]. Azurophil granules of the lamprey, on the other hand, are similar to azurophil granules of mammals, which contain rod-, needle-, ring-like and irregularly shaped inclusions. The presence of a fine fibrillar structure in some of the elongated inclusions of the lamprey azurophil granulocyte granules — as seen in other vertebrates — indicates that the structures of azurophil granules of all vertebrates have some features in common.

The positivity of the alkaline phosphatase and of the PAS reactions is, with a few exceptions, a common feature of blood neutrophils of vertebrates. The strong reactions in blood granulocytes, and the very weak seen in the supraneural organ haematopoietic tissue cells of the lamprey, suggest that in cells entering the blood synthesis of the enzyme and glycogen proceeds with maturation. Since some of the granulocytes of the haematopoietic renal tissue of *Teleostei* give intense alkaline phosphatase and PAS reactions, our findings indicate that in the lamprey granulocytes enter the blood at an earlier stage of cell differentiation than in *Teleostei*. This occurrence of undifferentiated blood cells in peripheral blood has also been found in *Lampetra lamottenii* [18] and in *Petromyzon marinus* [36].

In view of the absence of peroxidase activity from the azurophil granules of lamprey leucocytes the question of their bactericidal capacity may be raised. It is known that this enzyme is necessary for killing of bacteria [32]. However, besides the peroxidase-halide-hydrogen peroxide-generating system, other bactericidal mechanisms were shown to be active in rabbit and human granulocytes [33]. The peroxidaseless chicken granulocytes might also kill bacteria by cationic proteins of their primary granules [6]. The presence of these peroxidase-independent bactericidal factors in lamprey granulocytes is still to be proven.

The finding of degenerated leucocytes in the supraneural organ, though unusual concerning their high number, may occur also in haematopoietic tissues of higher vertebrates. To understand their significance, further studies of haematopoietic tissues are needed, especially in other life stages of the river

lamprey. The degenerated leucocytes are seemingly in contact, through their projections, with large surface areas of other cells, suggestive of an interaction between normal and decaying cells. The possibility that these projections are artefacts resulting from mechanical injury in the course of processing, would speak for a high degree of mechanical vulnerability of these cells.

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ARE MAST CELLS INVOLVED IN ADJUVANT ACTION?

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Abstract

A variety of adjuvant substances have been found to reduce the absolute and relative number of peritoneal mast cells in mice. This observation is discussed in the light of literary data suggesting a potential role of mast cells in the regulation (modification) of the immune response.

Introduction

In our previous work we have found mast cell damage and a considerable decrease in the absolute and relative number of peritoneal mast cells (MC) of mice following endotoxin (ET) administration [29]. As ET is a potent adjuvant [28], the question arose whether MC or their damage might be involved in the adjuvant effect. Therefore, we have tested a variety of substances with adjuvant activity for their ability to decrease similarly to ET the peritoneal MC count.

Material and method

The materials to be tested were administered i.p. to randomly bred 14—17 weeks old BALB/c male mice. In each series of experiments control mice of the same age receiving saline were used. Usually at day 4, peritoneal cell suspension and peritoneal smears were obtained as described previously [29].

For counting MC in the peritoneal fluid, a rapid test was developed. This is based on the observation that the number of MC stained with toluidine blue in formalin-treated buffered peritoneal fluid proved to be approximately twice as great and was more reproducible than that in the formalin-untreated samples. The former technique showed a good correlation with the alcian blue–safranine method. To 0.8 ml peritoneal cell suspension 0.2 ml of 36% neutralized (pH 7.0) formalin was added and the mixture was incubated in a water bath (25 °C), usually for 150 min. Then 0.2 ml of the formalin-treated cell-suspension was added to 0.025 ml of 0.1% aqueous solution of toluidine blue and the number of metachromatic cells was counted in a Burker chamber. (After 4 h of incubation with formalin the enumerable MC count began to decrease, due to cell clumping.) The following substances were tested.

Endotoxin prepared from *E. coli* O86 according to Westphal's method [43] kindly supplied by Dr. L. BERTÓK, Budapest); Dextran, MW: 5×10^5; dextran sulphate MW: 5×10^5; Bayol-F and Arlacel-A for Freund's incomplete adjuvant (IFA) were obtained from Serva; dextran, MW higher than 5×10^6 (Nutritional Biochem. Corp.); Retinol (vitamin A alcohol), calf thymus histone and bovine serum albumin cryst. (Sigma); polyinosinic acid–polycytidylic acid (Poly I : C) A-grade (Calbiochem); Polymyxin-B (Pfizer); protamine sulphate (Roche); saponin albus (Reanal); Human serum albumin (HSA) and *Bordetella pertussis* vaccine (Insti-

tute "Human"; kindly supplied by Dr. Z. CSIZÉR); Arquad-2HT (di-octadecyl dimethyl ammonium chloride) a gift of Dr. GALL and Dr. RÉTHY, resp.; Tilorone-HCl (Merrell-National Labs.); Silica, particle size of 5—7 μ were used.

The materials were administered i.p. in 0.2 ml of neutralized saline. Retinol was injected in 0.02 ml dimethylsulphoxide (DMSO); IFA was emulsified in saline.

Results

Table 1 shows that beside the applied MC-disruptors (polymyxin-B, protamine sulphate), most of the adjuvant materials tested decreased the absolute and relative number of peritoneal MC. These substances include

Table 1

*The effect of various substances on the peritoneal mast cell count**

Treatment		No. of animals	Adjuvant activity	WBC, $\times 10^3 \pm$ SE	MC, $\times 10^3 \pm$ SE	Decrease in MC-count
Saline		(6)	—	21.9 ± 3.2	214 ± 53	—
BSA	3 mg	(6)	—	25.5 ± 2.3	220 ± 15	—
HSA	3 mg	(6)	—	21.8 ± 2.0	228 ± 34	—
Polymyxin-B	0.5 mg	(6)	n.k.**	30.0 ± 4.0	11.9 ± 5	+
Protamine-SO_4	1 mg	(5)	n.k.**	25.6 ± 2.1	8.3 ± 2	+
Histone	1 mg	(5)	+ (20)	39.8 ± 6.5	21.5 ± 7	+
Saline		(9)	—	17.2 ± 0.6	215 ± 19	—
Endotoxin	25 μg	(5)	+ (28)	16.5 ± 2.7	36 ± 16	+
IFA***	0.1 ml	(6)	+ (18)	21.5 ± 2.1	31 ± 7	+
B. pertussis	3×10^9	(5)	+ (16)	37.8 ± 4.8	92 ± 28	+
Poly I : C	0.5 mg	(5)	+ (38)	16.9 ± 2.3	151 ± 7	+
Silica	10 mg	(6)	+ (1)	15.3 ± 1.8	11 ± 3	+
Saline		(9)	—	17.1 ± 1.5	191 ± 8	—
Saponin	0.1 mg	(6)	+ (20)	20.4 ± 6.3	18 ± 4	+
Arquad 2HT	0.2 mg	(6)	+ (19)	15.3 ± 2.0	61 ± 12	+
Tilorone	3 mg	(6)	+ (10)	15.1 ± 1.6	10 ± 4	+
DMSO	0.02 ml	(6)	—	16.3 ± 0.9	105 ± 19	+
Retinol	3.2 mg	(6)	+ (14)	27.8 ± 1.9	25 ± 8	+
Saline		(6)	—	18.9 ± 3.5	192 ± 27	—
Dextran MW 5×10^6	1 mg	(6)	± (7)	20.4 ± 4.1	99 ± 26	+
Dextran MW 5×10^5	3 mg	(6)	± (7)	21.0 ± 3.5	175 ± 35	—
Dextran-SO_4 MW. 5×10^5	3 mg	(6)	+ (11)	21.8 ± 2.7	177 ± 20	—

* 4 days after treatment
** n.k. = not known
*** 5 weeks after treatment

endotoxin, IFA, silica, arquad-2HT, saponin, *B. pertussis* vaccine, Poly I : C, tilorone and vitamin A. Dextran of high molecular weight caused a marked decrease, but similar doses of dextran and dextran sulphate with a mol. weight of 5×10^5 did not influence the MC count. Weak soluble antigens lacking adjuvanticity (BSA and HSA) had no effect on the MC counts.

Discussion

The results show that most of the adjuvant substances tested decreased the peritoneal MC count in mice, presumably due to MC damage (degranulation and sequestration of MC). It may be noted that many adjuvant substances studied by GALL [19] are also known as potential MC disruptor agents.

Though only rough correlations appear between the MC-damaging capacity and the adjuvant activity of substances, this may be due to the complex and different mechanism by which the various adjuvants exert their action. It seems to be conceivable that the involvement of MC in the action of certain adjuvants may have some causal relationship with the adjuvant activity. The following data and considerations seem to support this view.

MC are widely distributed in the organism and assumed to be of lymphatic, presumably of thymic, origin [8, 23]; therefore they might have some role in the defensive mechanisms.

A good correlation was found between the adjuvant effect and the lysosome-labilizing property of various adjuvants [40]. It is likely that membrane labilization is not restricted to the lysosomes but involves also the MC, which represent one of the most sensitive cell types. MUNDER and co-workers observed an elevated rate of lysolecithin formation in the course of adjuvant action [31] and considered this substance an endogenous adjuvant [32]. It is worthy to recall that phospholipase A and lysolecithin are potential MC disruptor agents [26].

It was shown that MC accumulate in the regional lymph node shortly after the local application of antigens [37, 44]. Thus, the possibility is given that MC may exert some direct influence on the immune response.

MC are stores of various biologically active substances which may have some regulatory influence on the immune response. Of MC constituents heparin is thought to be one of the candidates which are able to influence immune response. Among other polyanions, heparin has been shown to have adjuvant activity [42]. Though heparin has slighter activity in this respect than other polyanions, its effect is apparently more specific, as it lacks the ability to cause blockade in the mononuclear phagocytic system [5]. Heparin as well as other polyanionic substances are capable of increasing the rate of thymidine incorporation into the spleen cells [42].

In contrast to glucocorticoids, heparin causes lymphocytosis [4, 34, 41] and is able to antagonize the lymphopenic effect of glucocorticoids [34]. The potentiation of the adjuvant action of endotoxin and that of IFA by heparin on the development of 7S immunological memory has also been demonstrated (unpublished observation, J.J. and E. K.). Heparin is known to influence the activity of complement [13, 21] and the latter was reported to be necessary for tolerance induction [2] or antibody formation to T-dependent antigens [35, 36]. Heparin might be liberated from MC in the course of the degranulation process locally without getting into the circulation.

The possibility has not been ruled out that not only free heparin but also MC granules by themselves may modify the immune response.

Biogenic amines liberated from MC also might have some immunoregularoty influence. Histamine is considered to be the physiological activator of the reticuloendothelial system [27] and the function of the latter may alter the immune processes.

As it has recently been shown histamine receptor-bearing lymphoid cells exert a suppressive effect on antibody response [3] and so do some biogenic amines [30]. It can be assumed that MC surrounding the regional lymph node secrete histamine (or other amines) [39] continuously, thus rendering the suppressor T-cells effective. Adjuvants would act by depleting the histamine stores of MC.

Thus, there are several routes by which MC might influence immune response:

(a) by releasing heparin or heparin-containing granules;
(b) by secreting or releasing amines into the surrounding tissues;
(c) by direct contact with the lymphoid cells (peripolesis) [9];
(d) by other means.

The involvement of MC in the action of many adjuvants is compatible with the earlier findings that adjuvants generally induce inflammatory phenomena, cell damage [6] with the liberation of some tissue products [33], and some of them induce granuloma formation. The relationship between MC and inflammation is well known.

It appears, however, that MC disruption *per se* does not explain as a whole the adjuvant effect of some potent adjuvants, as their mode of action may be complex and different. It seems more likely that the involvement of MC (or their constituents) might only contribute to other effects of some adjuvants (e.g. to the direct effect of adjuvants on T or B lymphocytes).

The way in which some adjuvants affect MC may be different. Beside agents acting directly on MC (surface-active agents, cationic compounds) other agents may act through the complement system (e.g., C3a and C5a anaphylatoxins), as anaphylatoxins can degranulate MC [12]. It has been

found that ET [22, 24], polyanions and polycations [21], and a wide variety of different T cell independent immunogens and mitogens [15] are capable of activating the alternate pathway of complement. Thus, C3 activation by a nonspecific route would also reasonably explain the complement derived anaphylatoxin formation and the involvement of MC in this event.

The failure of dextran sulphate to affect MC despite the fact that it activates complement *in vitro* [25] can be explained by assuming that it neutralizes the cationic anaphylatoxins generated and thus protects MC, like the anionic polycarboxylic acid reported by FERNÖ and co-workers [17].

Even if MC do not appear to be necessary for antibody production in a completely *in vitro* system, this does not exclude the possibility that MC (and their constituents) may exert some modifying (regulatory) influence on the immune response *in vivo*. Our results and other data in the literature suggest this possibility.

The question whether MC (or their constituents) do actually play a part in immunostimulating (or suppressing) effect of various adjuvants — and if so, by what mechanisms — remains to be elucidated.

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Acta biol. Acad. Sci. hung., **27**(1), 65—70 (1976)

FERTILIZATION AND FUSION OF FREE ENDOSPERM NUCLEI IN *PIMPINELLA ANISUM* L.

SHORT COMMUNICATION

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(Received 1975—03—18)

The mosaic endosperm [4], xenia and metaxenia [2], the high and different degrees of polyploidy of secondary endosperm cells [6], have been well-known phenomena in one or more species for many years.

During investigation of *Pimpinella anisum* from an embryological point of view, I saw some primary and secondary phenomena on and in the carpels, from which four direct or indirect routes were connected with the endosperm cell population. The phenomena have been unknown so far.

The anise brings about, after double fertilization [3, 5], 256 free triploid endosperm nuclei (Fig. 3) in 8—12 days. During this time, the two styli grow (Figs 1, 2), the postflorale nectar secretion continues [7], the micropyle remains open and the zygote is still at rest.

Simultaneously with the formation of free endosperm nuclei, many

Fig. 1. Flower after double fertilization in the embryo sac

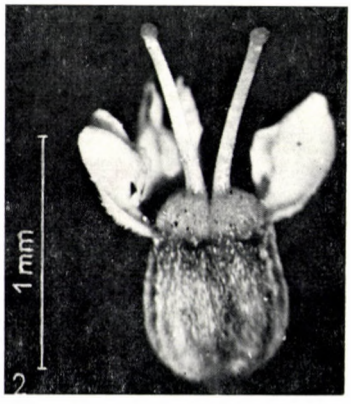


Fig. 2. Bicarpel with the well developed styli, the shape when the secondary fertilization and fusion inside the carpels evolves

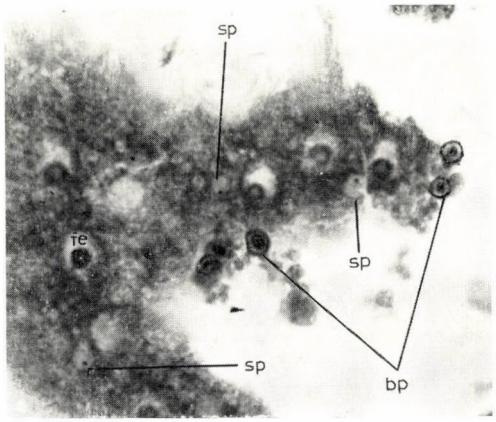
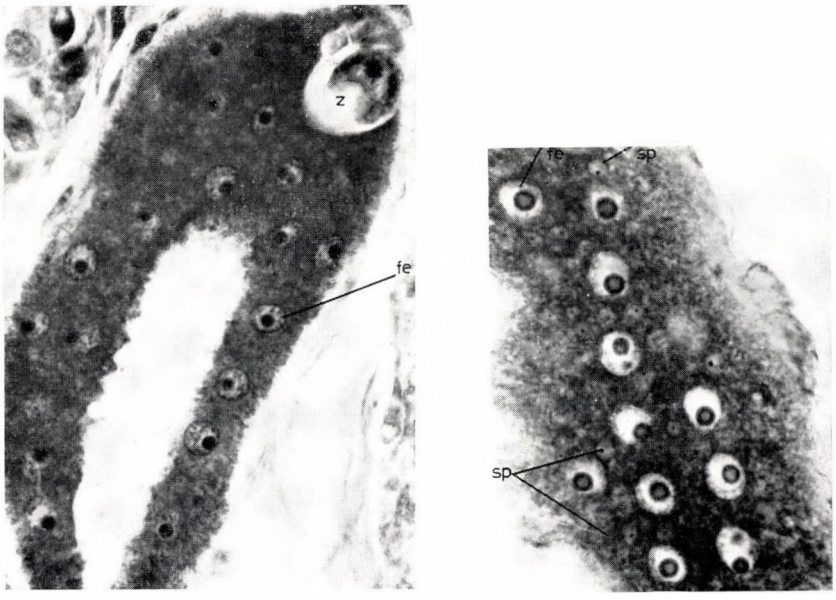


Fig. 3. Free endosperm nuclei, among them many small pollen tubes, × 850
Abbreviations: bp = big pollen tubes, cy = cytoplasme, fe = free endosperm nucleus, fer = fertilized endosperm nucleus, fu = fusiform free endosperm nucleus, mg = microgamete, nu = nucleoli, sp = small pollen tubes, z = zygote



a) b)

Fig. 4. Different sizes of pollen tubes among the free endosperm nuclei, × 800

pollen grains appear on the surface of stigma, the pollen tubes subsequently penetrate at first the style and later the cytoplasm, among the free endosperm nuclei (Figs 3, 4, 5, 10, 11). The pollen tubes are variable in size (Fig. 4). Near the large pollen tube (which is dark lilac-coloured among the free blue endosperm nuclei staining with toluidine blue), the endosperm nucleus is

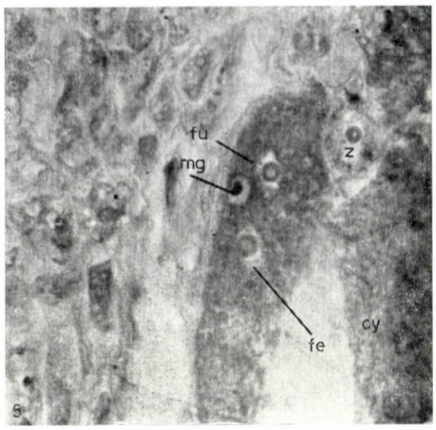


Fig. 5. Fusiform free endosperm nucleus near the generative cell in the mass of cytoplasm, × 750

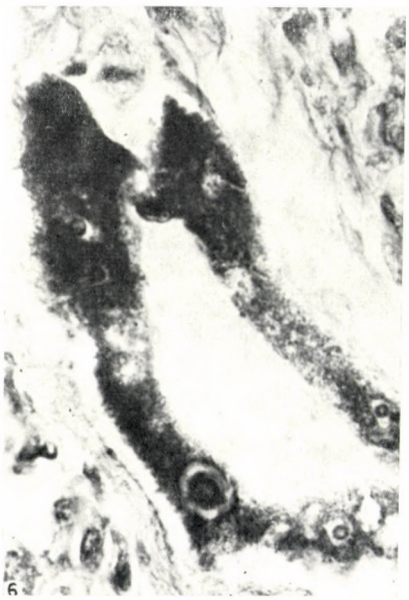


Fig. 6. A big, round fertilized and many unfertilized free endosperm nuclei, × 750

fusiform (Fig. 5). As the next step the fertilization of some free endosperm nuclei takes place (Fig. 6). These nuclei become tetraploid. In addition, two or more free endosperm nuclei fuse (Figs 7, 8, 9, 10), and later the same ensues nucleoli (Fig. 11). The free endosperm nuclei need the presence of numerous small pollen tubes to fuse (Figs 3, 10).

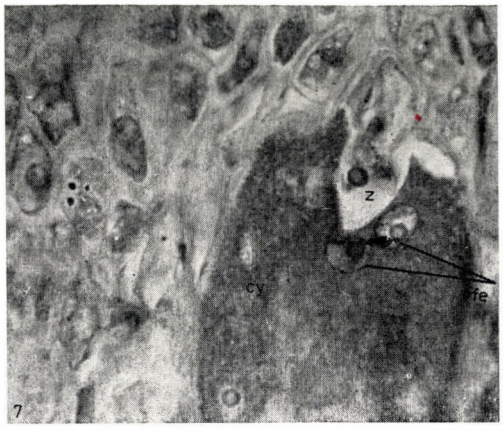


Fig. 7. Two free endosperm nuclei of equal size before the fusion, under the zygote, × 750

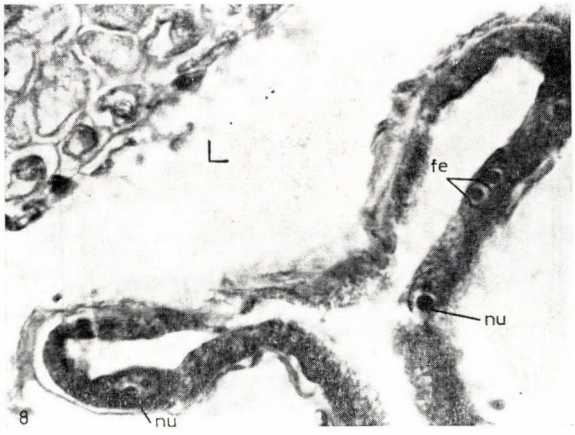


Fig. 8. Stages of fusion between two endosperm nuclei, × 750

These latter may represent later the different degrees of polyploidy of endosperm cells — depending on the number of fused free endosperm nuclei.

After the fertilization of certain endosperm nuclei and the fusion of others, these gradually form the membranous cell walls around the cytoplasm containing nucleus.

I designate these phenomena as the secondary fertilization and the fusion of the free endosperm nuclei in the anise.

Considering that the secondary endosperm cells take form, and the cell differentiation into "normal" cell begins, after these simultaneous processes, I suppose that free endosperm nuclei cannot develop into complete cells without secondary fertilization and fusion [1].

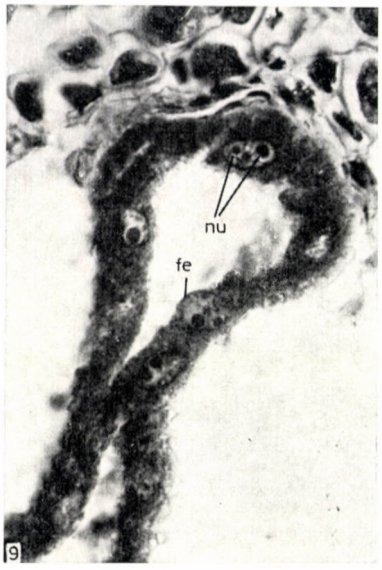
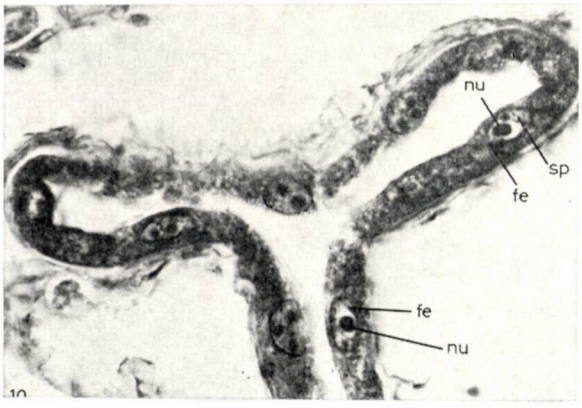


Fig. 9. Fused endosperm nuclei with two or three nucleoli, × 750



10. Different phases of the fusion of free endosperm nuclei, in the presence of small pollen tubes, × 750

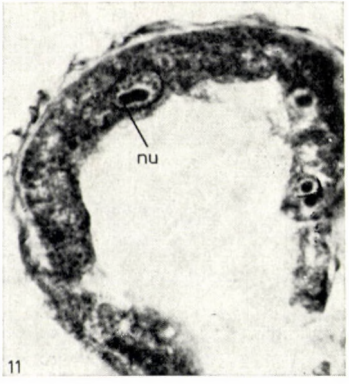


Fig. 11. Fusion inside the nucleus between the nucleoli, × 750

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IDENTIFICATION OF MOUSE CHROMOSOMES IN RODENT SOMATIC CELL HYBRIDS

SHORT COMMUNICATION

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(Received 1975—08—01)

It has been reported by HILWIG and GROPP [4] that "33258 Hoechst" compound [2,2(4-hydroxyphenyl)-6-benzimidazolyl-6-(1-methyl-4-piperazyl)--benzimidazoldihydrochloride] causes elongation at the pericentric regions of mouse chromosomes. This phenomenon is species-specific in a sense that it was not found either in human or in other rodent chromosomes. The species specificity of elongation seemed to be a valuable tool to distinguish mouse chromosomes in interspecific somatic cell hybrids. KIM and GRZESCHIK [5] reported that they were able to select the mouse chromosomal set in human–mouse hybrid cells, by treating the cells with "33258 Hoechst". We have attempted to use this technique in the case of somatic cell hybrids of rodent origin. We have studied mouse–Chinese hamster hybrid cells for this purpose.

A9HT (HGPRT$^-$) mouse and A23 (TK$^-$) Chinese hamster lung cells were fused according to the standard procedure [3]. The hybrids were selected and cultivated in F12 HAT medium [1], supplemented with 5% fetal calf serum (Flow Lab.). The application of a 16-h treatment with 50 μg/ml "33258 Hoechst" (kindly provided by Dr. H. LOEWE, AF Hoechst) exerted different effects on the fusion partners. We have observed pericentric elongation virtually on the whole mouse chromosomal set (Fig. 1) but none in Chinese hamster cells.

It is well known that from the hybrid cells usually one parental chromosomal set is preferentially lost during cultivation. From mouse–Chinese hamster hybrids chromosomes of both parents can be lost [2]. We have treated the hybrid cells with 50 μg/ml "33258 Hoechst" for 16 h (previously the hybrids were grown in HAT medium for three weeks after the fusion has been made). Chromosomes were prepared according to the standard procedure, after a 2-h mitotic arrest with 0.25 μg/ml colcemide (CIBA), and stained with 5% Giemsa or banded with the trypsin digestion method as described by RAY

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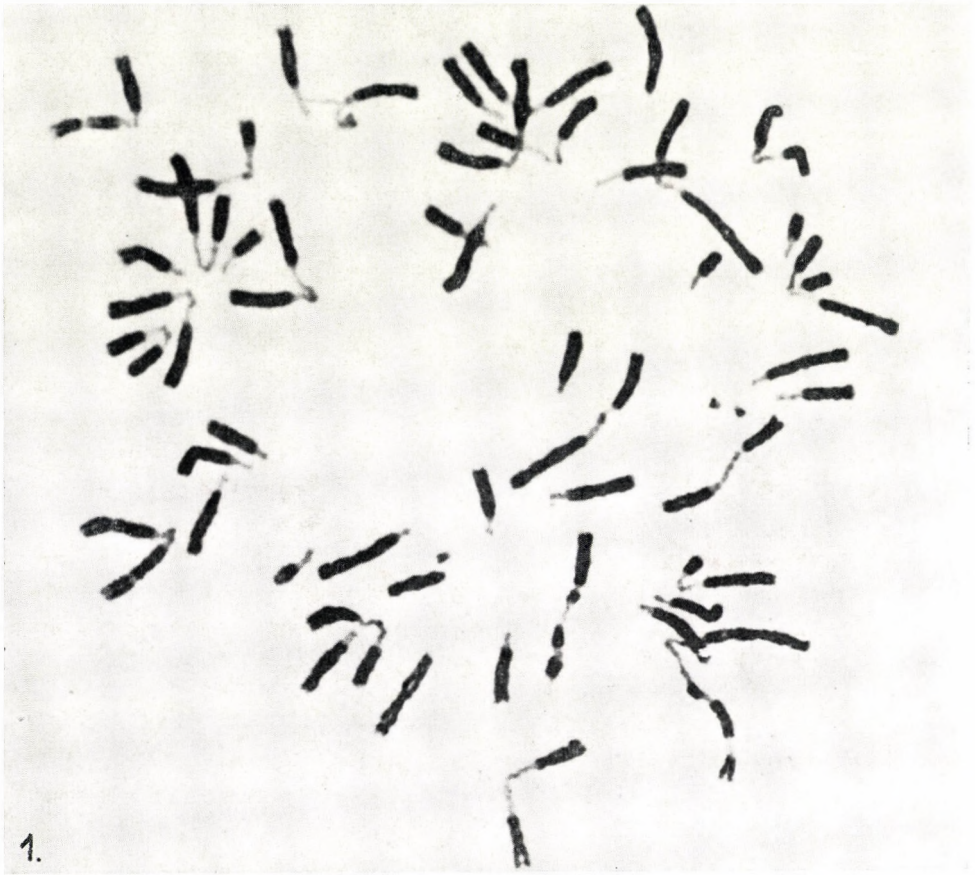

Fig. 1. Metaphase plate of A9HT mouse cell treated with "33258 Hoechst". (The cell line has several metacentric chromosomes beside the submetacentric and aerocentric ones. Note the elongated pericentric regions.)

and HAMERTON [6]. On the Giemsa-stained spreads there are chromosomes with elongated pericentric regions (Fig. 2). According to their C-banding pattern obtained by trypsin digestion they proved to be mouse chromosomes (Fig. 3).

In the mouse-Chinese hamster hybrids it is not necessary to use G- or

Fig. 2. Metaphase plate of A9HT-A23 hybrid cell treated with "33258 Hoechst". Arrows indicate the hamster chromosomes

C-banding in the first step to discriminate the chromosomes; for a quick test the "33258 Hoechst" treatment seems to be suitable. However, it would be of interest to know what is the difference in the pericentric regions between the mouse and Chinese hamster chromosomes, responsible for the specific effect. This question is under investigation.

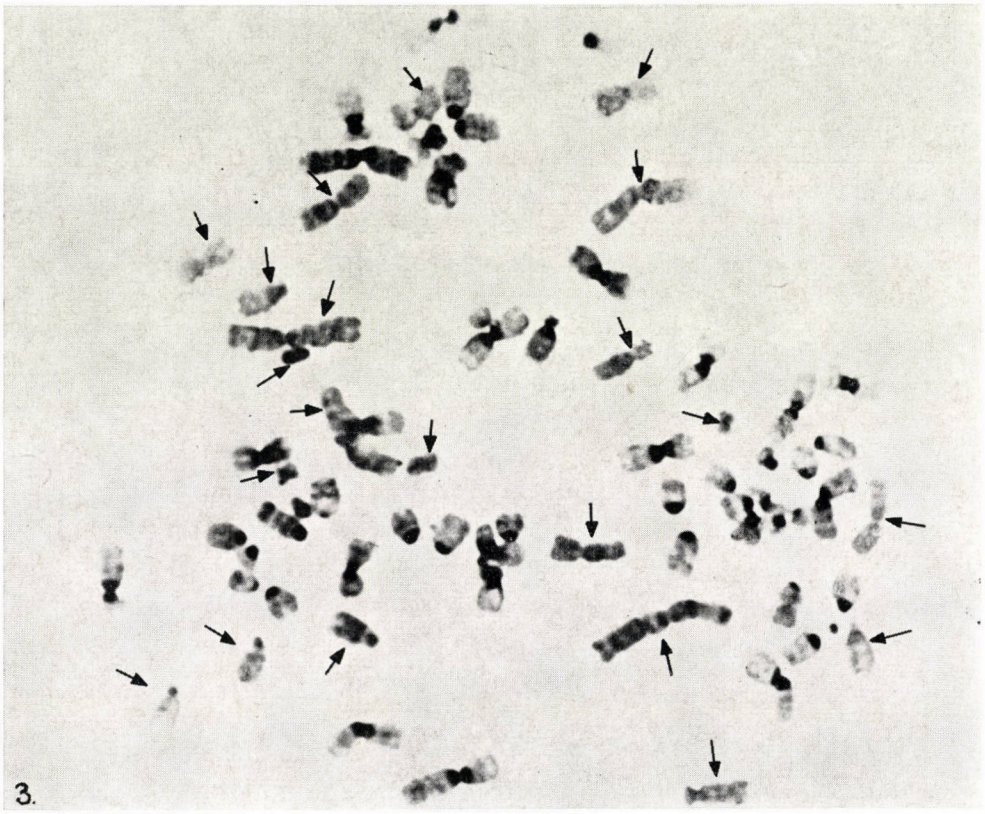

Fig. 3. Methaphase plate of A9HT-A23 hybrid cell stained with the C-banding technique. Arrows indicate the hamster chromosomes

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CHANGING IMMUNOREACTIVITY OF THE LHRH-CONTAINING NERVE TERMINALS IN THE ORGANON VASCULOSUM OF THE LAMINA TERMINALIS*

SHORT COMMUNICATION

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(Received 1975—09—13)

KAWAKAMI [3] and BROWNSTEIN and co-workers [2] reported that luteinizing hormone-releasing hormone (LHRH) was detected in the extracts made from the medial prechiasmatic area including the *organon vasculosum laminae terminalis* (OVLT). This finding is consistent with those of BARRY and co-workers [1], ZIMMERMAN and co-workers [12] and NAIK [6], who found LHRH-containing neural elements in the medial prechiasmatic area. WEINDL [9] noticed the similarity between the OVLT and the median eminence. Both contain nerve endings terminating on, or near, the basement membrane of the external surface of the organ, and synaptic and granulated vesicles are evident in the terminals [10]. Recently WENGER [10] reported that four and fourteen days after removal of the ovaries the larger granulated vesicles, possibly carrying peptide hormones, almost disappeared from the nerve terminals of the OVLT. We decided, therefore, to investigate whether synchronous changes in the oestrous cycle could be detected in the immunoreactivity of the OVLT.

The brains of female rats killed at various stages of the oestrous cycle were fixed in ZAMBONI's [11] fixative, dehydrated in graded alcohols, and embedded in polywachs 1000 (Chemische Werke Hüls AG, 4370 Marl). LHRH was detected on 5 μm thick sections according to the method of MAZURKIEWICZ and NAKANE [5]. The antiserum to LHRH was generated in rabbits. This antiserum appeared to be specific, because of lack of cross-reaction with other hypothalamic and pituitary hormones in the radioimmunoassay system for LHRH [7]. Sheep anti-rabbit γ-globulin was conjugated with horseradish-peroxidase (Sigma, type VI) according to the method of KAWAOI and NAKANE

* This work was supported in part by the Hungarian Academy of Sciences and in part by Population Council Grant No. M74. 86, USPHS Grants AM 06555 and AM 07467, and the Veterans Administration.

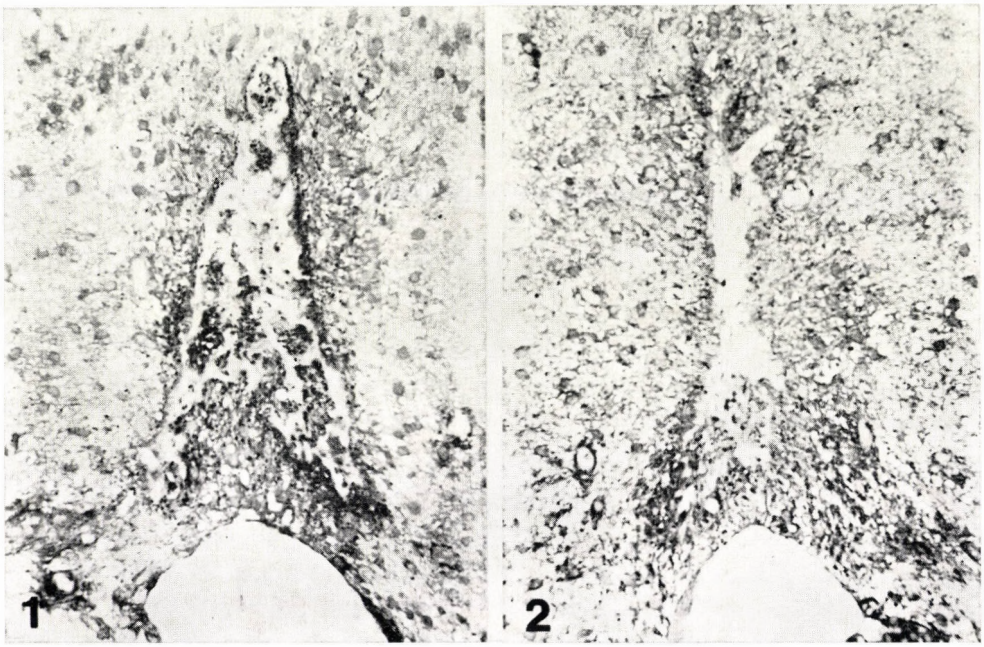

Fig. 1. OVLT of an intact adult rat killed in proestrus. Numerous reaction products of DAB due to the binding of anti-LHRH are visible on, or in the immediate vicinity of, the capillaries of the OVLT

Fig. 2. OVLT of an intact adult rat killed in metaestrus. Note the reduced amount of reaction products of DAB in the OVLT, as compared to Fig. 1

[4]. The reacted sections were developed for peroxidase using diaminobenzidine (DAB) and hydrogen peroxide as substrate. Specificity of the staining was tested by using normal rabbit serum or anti-LHRH absorbed with LHRH instead of the specific antiserum.

Figure 1 shows the OVLT of an intact adult rat killed at the proestrous stage of the cycle. Numerous dot-like DAB reaction products, due to the binding of specific antibodies and corresponding obviously to cross-sectioned nerve fibres or terminals, are visible on, or in the immediate vicinity of, the capillaries of the OVLT. The LHRH-containing nerve fibres terminating in the OVLT originate from LHRH-containing nerve cells situated in the suprachiasmatic area, between the anterior commissure and the optic chiasm, as well as in the neighbourhood of the OVLT [8]. These immunopositive nerve cells as well as the majority of the fibres were observed only at the end of diestrus and proestrus. A comparison of Fig. 1 and Fig. 2 indicates clearly that the immunoreactivity of the neural elements in the OVLT is much lower at metaestrus than at proestrus. This finding together with that of WENGER [10] suggests that the OVLT might play some role in the control of the cyclic

release of gonadotrophic hormones. This suggestion is in agreement with that of KAWAKAMI [3] who found that the bioelectrical activity of the medial prechiasmatic area containing the OVLT and that of the arcuate nucleus–median eminence region fluctuated in the same manner in accordance with the estrous cycle.

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RECENSIONES

BLOOM, W., FAWCETT, D. W.: **A textbook of histology**
W. B. Saunders Company, Philadelphia—London—Toronto (1975), pp. 1033, figs. 901.

This is the 10th edition of "A textbook of histology". Previously it had been edited by the late W. BLOOM and his co-authors. This last edition was prepared by D. W. FAWCETT.

This edition is based mostly on the previous ones, but some chapters have been thoroughly revised. That was necessary because of the great amount of the new data as well as the changed aspect of some questions.

The book is not only a synopsis of histology, needed for university examinations, but the newest histophysiological and biochemical observations are summarized in it, besides the particular, clear and instructive, light and electronmicroscopic description of various organs.

The textbook consists of 36 chapters. The first part deals with methodical questions, the second part with cytology. In the following chapters the different tissues and organs are described.

An accurate reference list belongs to each chapter and an alphabetic subject index is at the end of the book.

Illustrations are crucial part of histological textbooks. In this case the authors add an extraordinary good photo documentation to the text. There are about 900 light and electron micrographs and partly coloured schematic drawings in the book. The interesting and very instructive scanning electron micrographs help the good understanding of the text too.

The excellent photographs and the making-up of the book demonstrate again the high standard of Saunders Company's work.

L. KOVÁCS (Budapest)

PEAKER, M., LINZELL, J. L.: **Salt glands in birds and reptiles**
Monographs of the Physiological Society No. 32. Cambridge University Press, Cambridge (1975), pp. 307, £ 9.0.

This book is a comprehensive survey of data concerning the anatomy and physiology of the avian and reptilian salt glands, which play an important role in the regulation of salt and water content of the organism.

The first part of the monograph is devoted to the avian salt glands. It contains the following chapters: structure of salt glands, methods employed in its study, nervous control of secretion, blood flow, the secretory mechanism, factors affecting the concentration of nasal fluid, hormones and salt-gland secretion, adaptation of the gland, integration between the gland and other organs and comparative physiology of the nasal glands.

The second part of the book includes chapters on the salt glands of marine and terrestrial reptiles and a brief survey of the evolution of salt glands.

The book is written at a high level, the authors succeeded in bringing together in an accessible form the most important data of the literature. The volume is well illustrated and the detailed list of references makes it easy to survey the subject. It may be recommended for all biologists interested in comparative physiology and especially for those working in the field of regulation of ion and water content of the organisms.

J. KOVÁCS (Budapest)

WALKER, W. F., Jr.: **Vertebrate dissection**
W. B. Saunders Company, Philadelphia (1975), pp. 397.

The purpose of this book was to give a laboratory manual and detailed dissection guide on vertebrate anatomy, together with some information about the theoretical aspects of the anatomical transformations that have occurred during vertebrate evolution. On this basis some topics have of necessity been omitted. The manual contains extensive treatise on the macroscopic anatomy of fishes (aquatic environment), primitive tetrapodes (transition from the aquatic to the terrestrial environment), and mammals (terrestrial animals). The stress put on the mentioned groups gives a very clear line of evolution and adaptation of vertebrates for students and specialists as well. On the other hand, this selection results in a nearly total lack of data concerning avian and reptilian anatomy.

In the first chapters the reader will find descriptions of primitive chordates. The next parts deal with the comparative anatomy of different organ systems of fishes, amphibians and mammals. The last parts of the volume are devoted to the technical problems of dissection, e.g., to the killing and preservation of animals, injection into the vascular system, etc. A selected list of references and a subject index are also included.

The book is well-printed and excellently illustrated (part of the figures are made in colours). It represents a highly valuable source of information on practical vertebrate anatomy both for students and instructors.

J. KOVÁCS (Budapest)

International Union of Physiological Sciences

XXVIIth INTERNATIONAL CONGRESS OF PHYSIOLOGICAL SCIENCES

PARIS – 1977

The Congress will take place from Monday July 18th to Saturday July 23rd 1977. Reception desk will open on Sunday July 17th at the University Paris VI, quai Saint Bernard, Paris.

A new formula has been chosen for the general organizing scheme of the Congress. Its first part will be devoted to lectures covering the major fields of the Physiological Sciences. A few lectures will emphasize the relationship of Physiology with other biological disciplines and/or with socio-economical problems.

The second part the Congress begins on Wednesday the 20th. It aims to promote contacts between students working in the same area. Therefore the Scientific program will be divided into sections representing the different fields of the Physiological Sciences. For each section half-day symposia and free presentations (communications or posters) will take place, without any overlap between them in order to increase the attendance at the various events. Each section will have its proper conference rooms, amphitheaters and poster surfaces. Sections will meet in the same geographical area so that a close relationship between the different specialists might be preserved. Congress participants will be able to choose their section(s) of interest.

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Any further information concerning the 1977 Congress can be obtained through each National Physiological Society or by writing directly to Congress Secretary:

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I. Dési

DAS GEHEIMNISVOLLE GEHIRN

Dieser Band ist ein Nachdruck des im Jahre 1973 erschienenen Werkes. In populärwissenschaftlichem Stil gehalten, vermittelt es der breiten Öffentlichkeit Kenntnisse über Struktur und Funktion des Gehirns. Der Leser erfährt von den Vorgängen, die in seinen Gehirnzellen beim Lesen eines Buches, bei der Aneignung und beim Wiedervergessen des darin behandelten Stoffes vor sich gehen. Auch werden die »Geheimnisse« des Traumes, der Hypnose und der Suggestion enthüllt. Der Lesestoff enthält außerdem Wissenswertes über Gehirnmodelle und »Kunsttiere«. Der Autor gibt einen recht großen Überblick über alle bisherigen Erkenntnisse, die die Funktion des Gehirns betreffen. Vorerst schildert er kurz das peripherische und vegetative Nervensystem, die grundlegende biologische Tätigkeit der einzelnen Gehirnteile und kommt dann ausführlicher auf die Gehirnfunktionen höherer Ordnung zu sprechen. Er beschreibt sodann die wichtigsten Forschungsmethoden der Neurobiologie und schildert interessante Experimente mit Ratten, Hunden, Affen, ja sogar mit Stieren.
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FORMATION OF ROOT HAIRS BY THE WHEAT TRITICUM VULGARE HOST. TRICHOBLASTS

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(Received 1974—10—24)

Abstract

The process of root hair formation has been studied by light-, transmission- and scanning electron microscopy. In the course of root hair development a break of the outer cell wall is observed by electron microscopy. It apparently occurs after the break of the fibrillar layer. The break of the outer layer of the cell wall is assumed to represent the break of the outer mucilage, the cuticle and the adjoining amorphous matrix with irregularly oriented cellulose microfibrils. The scheme of successive ultrastructural changes in the outer cell wall pattern during root hair formation is presented.

Introduction

The interest in the problems of root epidermis cell differentiation and formation of root hairs is increasing. These processes seem to be conditioned by a special state of epidermis cells in the process of supplying the plant with minerals and water. The root epidermis cells (the rhizodermis cells) of some plant species are determined to develop into trichoblasts or atrichoblasts, i.e., root hair or hairless initials, at an early stage of development.

The determination of epidermis cells of the cereal group, so-called festucoid type, into trichoblasts and atrichoblasts is conditioned by the last asymmetrical division of the meristematic cell [7, 27, 28, 29]. Wheat is a typical representative of this group. The considerable distinctions in activities of trichoblast and atrichoblast enzymes and the changes in the amounts of DNA, RNA and protein during differentiation were reported previously [4—6, 8—11, 17, 18]. These metabolic distinctions between trichoblasts and atrichoblasts are reflected in their ultrastructure. Some data about early appearance of the ultrastructural distinctions of the trichoblasts and atrichoblasts have been obtained by electron microscopy [1, 2].

These distinctions were retained up to the complete development of the hairless cells and the completion of the trichoblasts' differentiation, whose morphological expression is displayed in the root hair development.

There is plenty of information about the influence of different factors on the growth and development of root hairs [16[. Nevertheless, the mechanism of root hair development is not quite clear.

In the present work the process of root hair formation was investigated by light- and electron microscopy.

Material and method

The roots of 2-day-old seedlings of wheat *Triticum vulgare* HOST. var. REDGRAIN were used. Seeds were germinated in the dark at 25—27°C on moist filter paper in Petri dishes. Roots of seedlings of 10—15 mm length, having root hairs, were used for appropriate treatments.

Light microscope studies. Roots of wheat seedlings were fixed in Carnoy mixture [3] for 2 h at room temperature, hydrolysed in 1 N HCl for 8—10 min at 60°C and stained by Feulgen's technique [3]. Using fine dissecting needles, strips of epidermis were separated from the roots on glass slides and placed in a drop of 40% acetic acid. The 2—3 layers of epidermis strips thus obtained were covered with a cover slip. After slight crushing one-layer strips were obtained. The cover slip edges were sealed with a vaseline–paraffin mixture (1 : 1).

For the detection of RNA the wheat roots were fixed in Brodsky mixture for 1 h and stained with chrome–gallocyanin by Einarson's technique [3]. After staining the roots were immersed in 1% KOH for 5—10 min to dissolve the lamellar substance binding cell files together. Using the technique as described above the strips of epidermis were obtained.

Scanning electron microscope studies. The plant materials were prepared by a modified freeze-drying technique [14, 22]. The unfixed wheat roots were cut off from the seedlings and freezed for 30—45 min. The freeze-drying was carried out with a special technique for 2.5—3 h. The dried samples were covered with a thin gold film in a vacuum evaporator Hitachi HUS-4. These samples were examined in a Hitachi HSM-2A scanning electron microscope, operating at 25 kV.

Transmission electron microscope studies. The wheat root segments from root hair zone were fixed in 4% glutaraldehyde dissolved in 0.1 mol/l phosphate buffer of pH 7.2—7.4 for 1 h, then washed in the same buffer and post-fixed in 2% OsO_4 for 2 h. The fixation was carried out at room temperature (23°C). The plant material was dehydrated in an alcohol–acetone series, saturated with acetone–epoxy mixture and embedded in epoxy resin according to the modified Luft's schedule [24]. The sections were obtained with LKB-8801 ultramicrotome, stained with lead citrate by Reynolds' technique [26]. The sections were viewed in a Hitachi HU—11K electron microscope operating at 75 kV.

Results

The spring-crop wheat *Triticum vulgare* HOST. is a typical representative of festucoid cereals. The preliminary determination of root epidermis cells into trichoblasts and atrichoblasts of festucoid cereals is conditioned by the last asymmetrical division of the initial merismatic cell [7, 27, 28, 29].

As a consequence of asymmetrical mitosis two epidermal cell types are produced. The shorter, denser apical cell is determined to develop the root hair. The longer basal cell remains hairless. The strict alternation of long hairless cells and short trichoblasts in one cell row is a typical feature for a given type of rhizodermis. The ratio of trichoblasts to atrichoblasts is constant for a particular grass species and can serve as an important taxonomic feature [27].

Light microscopy

In the epidermis strips of wheat roots stained by gallocyanin short trichoblasts with denser cytoplasm and long atrichoblasts alternated in the same longitudinal cell row.

The ratio of the two epidermis cell types to each other was constantly 1 : 1 (Fig. 1a).

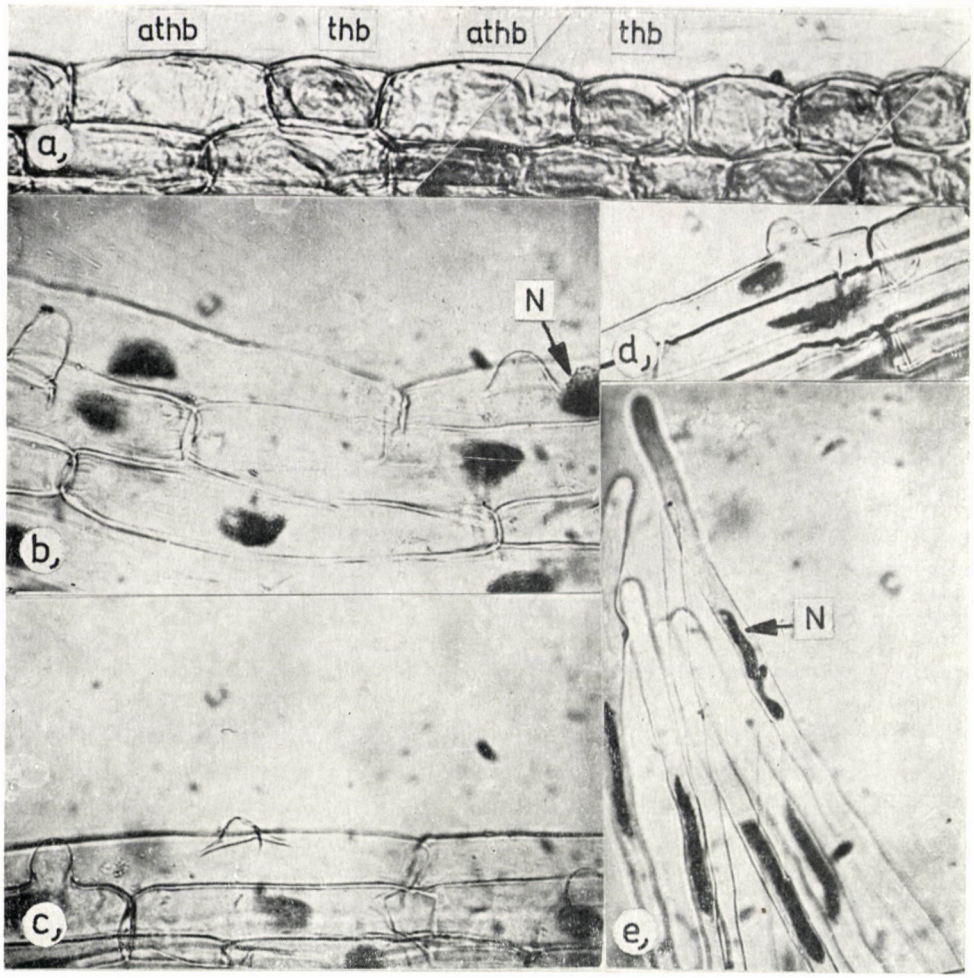

Fig. 1. Differentiation of wheat rhizodermis. Formation of the root hairs. *a* — Cell types of rhizodermis: trichoblasts (thb) and atrichoblasts (athb); chrome—gallocyanin, × 480; *b*, *c*, *d*, *e* — location of the nucleus during the development of root hair (N=nucleus); × 480

In the course of root hair development the swelling of cell wall occurs opposite to the nucleus or adjacent to it (Figs 1b—c). Then, during the growth of the root hair, the nucleus moves into the growing root hair tube and usually occupies a central location. The nucleus is frequently strongly elongated (Fig. 1d). The wheat root hair developed in the apical department of the trichoblast cell, under acute angle to the cell wall surface (Fig. 1e). This is a typical feature of root hair development of festucoid cereal grasses [27, 29].

Scanning and transmission electron microscopy

The ultrastructural changes in the pattern of the outer cell wall during root hair development are of essential significance. The presence of clearly delimited layers, viz., the inner fibrillar and outer amorphous layers, in the structure of the outer rhizodermis cell walls and root hair walls at their base have been distinguished by many investigators [12, 20, 30]. The amorphous matrix of the outer layer contains pectin, protein substances, hemicellulose and irregularly orientated microfibrils of cellulose. The inner fibrillar layer contains cellulose microfibrils laid down in parallel longitudinal arrays, impregnated with lignin pectic and nonpectic substances. These data are consistent with the multiple growth pattern of the cell wall proposed by HOWINK and ROELOFSEN [21]. The outer amorphous layer continues along the root hair wall, whereas the inner fibrillar layer originates only further back from the hemispherical root hair tip [12]. The presence of mucilage and a thin cuticle film covering the outer amorphous layer of the outer cell walls of root epidermis cells and root hairs has been discovered in many plants [20, 30]. The presence of root cuticle is defined more clearly by histochemical staining of the outer cell walls with dimethylaminoazobenzene [20]. Data about the presence of cuticle on the outer surface of rhizodermis cell walls have so far remained without general acceptance.

The process of the root formation was investigated also by scanning electron microscopy. For this purpose the chemical fixation of plant materials and the preparation of ultra-thin sections are not necessary and this allows to avoid considerable changes originating in the course of the procedures. The changes that occurred after freeze-drying were negligible. The possibility to observe the outer surface of objects is an essential advantage of scanning electron microscopy.

The sequential stages of the root hair formation by wheat trichoblasts are presented in Figs 2a—c. The local swelling of the cell wall on the root surface, observed by scanning electron microscopy, is the first stage of root hair formation. In the next stage of hair growth the outer layer of the trichoblast cell wall breaks. Then this layer of the outer trichoblast wall is raised by the growing root hair tube and is turned back mainly to one site from the formed hollow (Figs 2a—b). Subsequently, the ruptured and raised part of the outer layer of the cell wall appears to die and slough off from the root surface as the latter cannot be detected near the base of the developed hair (Fig. 2c). The break apparently represents the break of mucilage, root cuticle and an adjoined amorphous matrix with the irregularly oriented cellulose microfibrils.

Sometimes in the course of the root hair formation the break of the outer cell wall may not be observable. But the absence of the distinct picture of

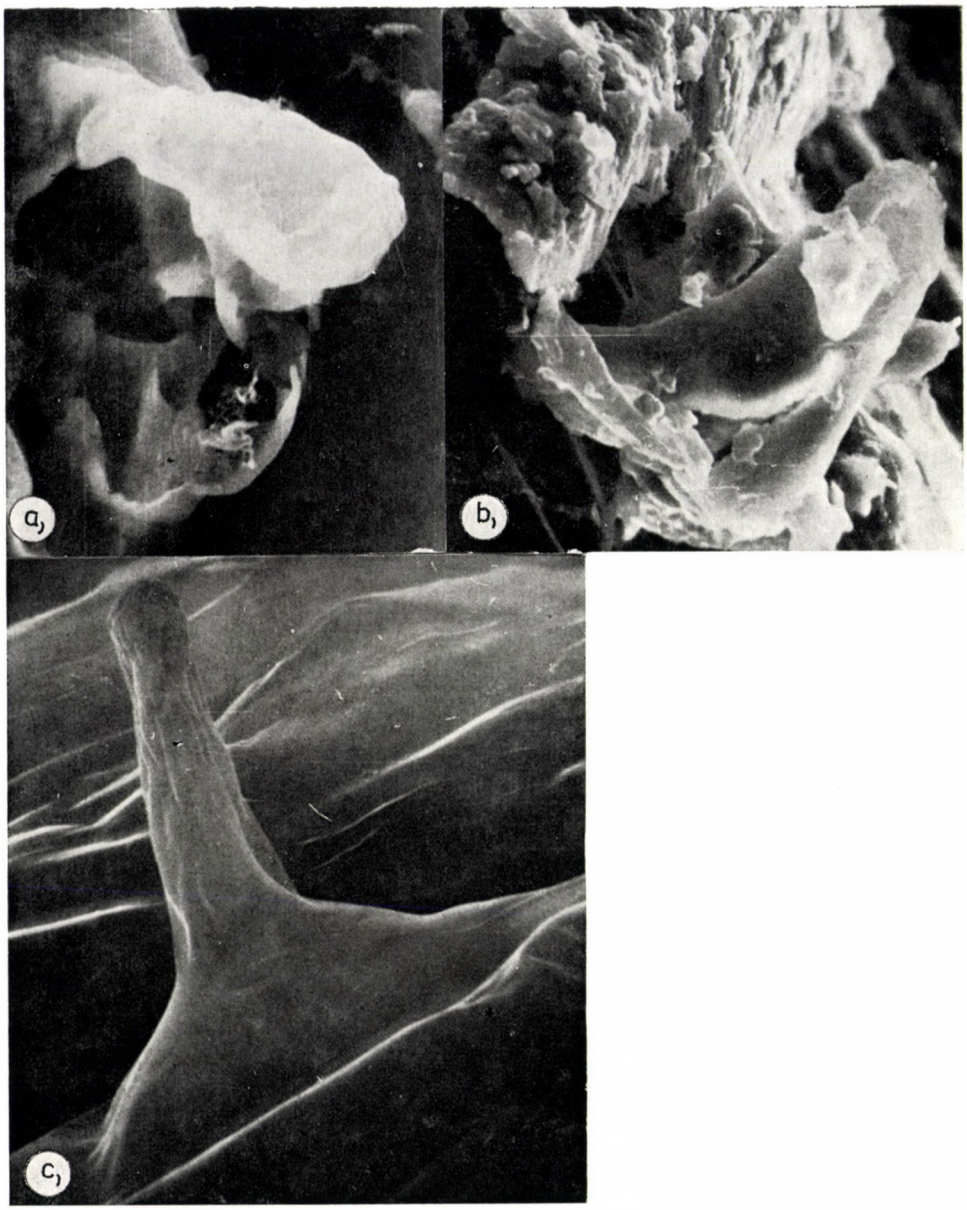

Fig. 2. Developmental stages of root hair formation. *a*—the ruptured wall is raised and is turned back mainly to one site from the formed hollow. Scanning electron micrograph, ×1 400; *b* — the outer layer of the cell wall is sloughing off from the root surface. It cannot be detected near the base of the developing hair. Scanning electron micrograph, ×1 400

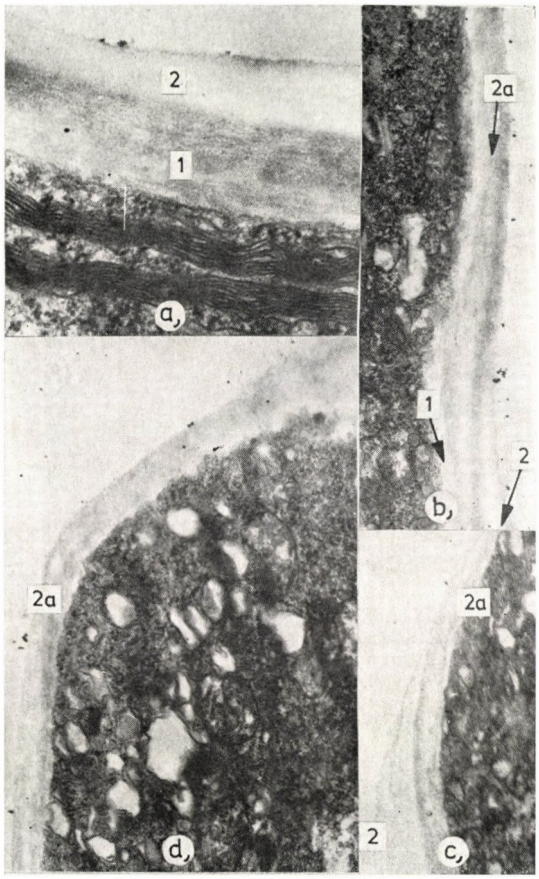

Fig. 3. Structure of the outer cell wall and of the root hair. *a* — Structure of the outer trichoblast cell wall. Gl — OsO_4, ×48 000; *b* — structure of the cell wall of trichoblast near the base of the root hair (1 = inner fibrillar layer, 2 = outer amorphous layer of the trichoblast cell wall, 2a = outer amorphous layer of the root hair). Gl — OsO_4, × 30 000; *c* — structure of the outer layer of trichoblast cell wall further back from the base of the root hair. Gl — OsO_4, ×15 000; *d* — structure of the outer layer of the root hair apex. Gl — OsO_4, ×26 000

break does not mean that such a phenomenon does not occur. This opinion has been supported by transmission electron microscopy (Figs 3a—d). The presence of three distinct layers of the outer trichoblast cell wall occurs near the base of the root hair. The more electron dense inner layer contains cellulose microfibrils oriented parallel to the longitudinal axis of the cell; the middle and the outer layers are amorphous. The root cuticle and mucilage could not be clearly detected with the given fixative schedule. In the course of the root hair extension visible thinning of the outer layer adjoined closely to the middle amorphous layer was observed (Figs 3b—c). Then the outer layer of the trichoblast cell wall disappeared further back from the base of the hair, whereas

the middle amorphous layer continued into the root hair wall. The microfibrils of the inner layer become more irregular in distribution, and friable, towards the hair tip. The presence of the inner fibrillar layer could not be detected at the root hair apex (Fig. 3d).

It appears that the absence of the distinct picture of the break of the outer cell wall may be explained in such a way that the ruptured outer amorphous layer of the trichoblast cell wall in the given cases is closely adjointed to the cell wall of the developing root hair.

Discussion

The physiological differentiation of the rhizodermis cells into trichoblasts and atrichoblasts takes place earlier than the morphological changes appear. This is supported by the appearance of differences in the activities of the particular enzymes in the immature trichoblasts and atrichoblasts [4—11, 17, 18]. Thus, it appears possible that the root hair development of festucoid cereal grasses is connected with a special direction of their metabolism conditioned by the last asymmetrical cell division [7, 17, 18]. The latter in turn is conditioned by the cell polarity [29]. The polarity of the root cells as a whole root is conditioned by a gravitation and a distribution of auxin [15].

The presence of certain environmental conditions is necessary for the root hair development of a great number of plants, though the festucoid cereal grasses develop the root hairs in a wide range of environmental changes [16].

The trichoblasts as compared with the daughter hairless cells possess higher phosphatase, cytochrome oxidase, succine dehydrogenase and peroxidase enzyme activities; the large nucleus and the nucleoli contain large amounts of RNA, ribonucleophosphate and proteins. The trichoblasts have all features of the meristemic cells, viz., high activities of the particular enzymes, the absence of large vacuoles, a weak development of the plastids, a great number of the ribosomes in the cytoplasm, large amounts of proteins and RNA, and the ability to synthesize DNA. However, they are unable to divide. Based on the peculiarities cited above, CUTTER and FELDMAN [17] considered the trichoblasts as supermeristematic cells whose developmental process was delayed, though the typical meristematic features have been retained.

The literary data concerning the amount of DNA in the trichoblasts are inconsistent. Investigating rhizodermis differentiation in *Hydrocharis*, CUTTER and FELDMAN [18] found that at the moment of the completion of the earliest developmental stages (before root hair formation) the trichoblast nuclei contained 8-times as much DNA as the nuclei of the neighbouring hair-

less cells, and the latter contained the double DNA amount as compared with the protodermis cells. Therefore, the development of trichoblasts in *Hydrocharis* is associated with endomitosis, resulting in a higher degree of polyploidy. Furthermore, there are some data in literature about root hair development by the diploid trichoblasts for 19 species of angiospermic plants [31].

Preliminary results of quantitative photometric determination of DNA in the rhizodermic cell nuclei of wheat and maize, stained by Feulgen's technique (unpublished data), have shown that root hairs are developed by the trichoblasts with the nuclei of all classes of the ploidy represented in the zone of differentiating cells. The DNA increases in amount in a large part of the rhizodermis cells during differentiation. Many investigators consider polyploidy as a necessary factor of differentiation [17, 18, 19, 25].

Literary data and our preliminary results suggest that polyploidy, being a necessary factor of the differentiation of the rhizodermis cells for a large number of higher plants, is not necessary for root hair development.

The high degree of trichoblast polyploidy in *Hydrocharis*, associated with endomitosis, appears to represent a specific feature of the *Helobiae*. The delay in the embryonic developmental stage is a general feature of the trichoblasts' origin and development [2, 18]. Its appearance might be attributed to prolonged divisions of complex type, to endomitosis or to asymmetric mitosis.

The root hair formation is a morphological expression of the rhizodermis cell differentiation. Although at the present time little is known of a rule of the nucleus in the root hair growth, based on literary data and our own observations it may be concluded that the formation and the development of the root hair is in close connection with the topography and the activity of the nucleus. The literary data concerned with the behaviour of the nucleus are contradictory. One investigator [13] reported that the nucleus occurred constantly adjacent to the root hair tip, others [23] observed a continuous shift of the nucleus during the hair tube extension.

The pattern of the outer cell wall undergoes considerable changes in the course of root hair formation. According to our observations obtained by scanning and transmission electron microscopy and on the basis of literature data [1, 12, 16, 20, 30], we offer the following scheme of the successive ultrastructural changes.

In the presented scheme (Figs 4a—d), the structure of the outer cell wall displays an outer amorphous and an inner fibrillar layer. The former contains the irregularly oriented cellulose microfibrils in a matrix of hemicellulose, pectic and protein substances. The inner fibrillar layer is formed by dense cellulose microfibrils oriented parallel to the longitudinal axis of the cell and impregnated with the lignin and various substances of pectic and nonpectic nature.

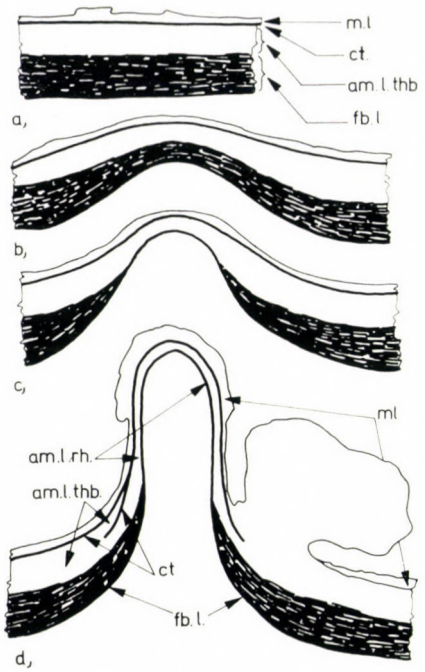

Fig. 4. Changes in structure of the outer cell wall in the course of the root hair development. (fb. l. = inner fibrillar layer; am. l. thb. = outer amorphous layer of trichoblast; am. l. rh. = outer amorphous layer of root hair; ct = cuticle; ml. = mucilage); *a* — structure of the outer cell wall; *b* — structure of the cell wall of trichoblast in the first stage of hair development; *c* — break of the inner fibrillar layer in consequence of the root hair growth; *d* — break of the outer layer of the trichoblast cell wall

The cell wall near the base of the root hair consists of an outer and a middle amorphous layer and an inner fibrillar layer. The middle amorphous layer, being a newly formed root hair wall, continues along the whole hair. The outer amorphous layer, representing the outer layer of the trichoblast cell wall, and the inner fibrillar layer are displayed only along short length of the cell wall near the base of the root hair. The external surface of the amorphous layers of the outer cell walls and the root hair walls is covered with thin cuticle and the layer of mucilage.

The structural organization of the cell wall is a result of the cytoplasmic metabolic activity and represents an integral structure. The presented scheme (Figs 4a—d) gives the whole picture of changes in the structure of the outer cell walls during the process of root hair development. The local swelling of the outer wall is the first stage of root hair formation. The swelling of the cell wall appears opposite to the nucleus or nearby.

The elongation of the root hair tube seems to ensue not only as a result of the local extension of the outer cell wall, but also as a result of the synthesis of

new structures of the amorphous layer. The synthesis of new cell wall structures is undoubtedly the main factor of root hair growth. The inner fibrillar layer, being less elastic than the amorphous layer, breaks during the elongation of the root hair. The presence of a break is obvious from data of transmission electron microscopy as well. In accordance with these data, the fibrillar layer is absent along the whole root hair and it is displayed only further back from the root hair tip. Then the break of the outer layer of the cell wall, observed by scanning electron microscopy, ensues. The break of the outer layer involves the mucilage, the cuticle, and the adjoined amorphous matrix with the irregularly oriented cellulose microfibrils (Fig. 4d). The presence of two amorphous layers (outer and middle layers) and the inner fibrillar layer in the basal part of the root hair wall is displayed. The outer amorphous layer itself is the old outer layer of the trichoblast cell wall, the middle amorphous layer is a newly formed layer of the root hair wall. The absence in some cases of the clear picture of the break of the outer layer of the trichoblast wall may be due to the close contact of the ruptured outer amorphous layer with the root hair wall. Thus the break of the outer cell wall seems to be essential in the process of root hair formation in wheat. Different mechanisms of root hair formation are not expected in other plants either. The elucidation of the mechanism of root hair formation in other plants and the solution of a number of related problems need further investigations.

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EFFECT OF THYROID HORMONE ON INITIATION OF PERSISTENT OESTRUS IN THE RAT

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Abstract

When rats are exposed to continuous illumination persistent oestrus and polyfollicular ovaries develop. Thyroidectomy at 24 days of age (juvenile rats) prevents the onset of persistent oestrus and polyfollicular ovaries under exposure to constant light, and irregular ovulation continues to occur. Replacement with 1.75 μg L-thyroxine in these rats produces a prolonged oestrus. However, in adult persistent-oestrous rats (90 days after exposure to continuous illumination) thyroidectomy does not interrupt persistent vaginal cornification. In rats receiving 100 μg of testosterone propionate subcutaneously at 5 days of age, persistent oestrus and polyfollicular ovaries develop. Thyroidectomy at 24 days of age (juvenile) prevents the onset of persistent oestrus and the development of polyfollicular ovaries, however, ovulation is not observed. Replacement treatment with 1.75 μg L-thyroxine in these rats produces a prolonged oestrus and polyfollicular ovaries. However, in adult persistent oestrus rats, thyroidectomy at 130 days of age does not interrupt persistent vaginal cornification. From these facts, it may be inferred that circulation of a physiological level of thyroid hormone in juvenile rats is necessary for the development of oestrogen binding receptors in the hypothalamus. Therefore, a hypothyroid state during the juvenile stage interferes with the development and maturation of hypothalamic controlled pituitary ovarian function.

Introduction

When rats are exposed to continuous illumination, the oestradiol binding capacity of the medial preoptic area (mPOA) is reduced [10] and persistent oestrus and polyfollicular ovaries develop [3]. In rats receiving androgen treatment neonatally, the oestradiol binding capacity of the medial preoptic area (mPOA) is reduced [6], the cyclic release of gonadotropin is blocked, and persistent oestrus and polyfollicular ovaries develop [1].

The hypothyroid state changes hypothalamic responsiveness to oestrogen [9] and causes an erratic critical period of gonadotropin secretion [8].

An alteration of hypothalamic responsiveness to oestrogen by thyroidectomy may make it possible to interfere with the onset of persistent oestrus and polyfollicular ovaries in rats exposed to continuous illumination or treated with androgen. This consideration prompted the present study into the effect of hypothyroid state on the onset of persistent oestrus and ovulation in rat exposed to continuous illumination or treated neonatally with androgen.

Material and method

Sprague Dawley female rats from our laboratory breeding colony were maintained under controlled temperature (24°C±2) and lighting schedule (light on at 5 h and off at 19 h) and provided laboratory food and tap water *ad lib.* In the following experiments rats were placed under continuous illumination at 40 days of age.

Experiment 1. Removal of thyroid gland, including parathyroid gland, was performed at 24 days of age. Vaginal opening and degree of vaginal cornification were observed and daily vaginal smears were recorded. Laparotomy for confirmation of ovulation (examination of tubal ova or corpus luteum) was performed between 8 and 10 a.m. on the day of oestrus smear. Necropsy was performed on all animals at 130 days of age and the ovaries were examined histologically.

Experiment 2. Removal of thyroid gland, including parathyroid gland, was performed at 24 days of age. Daily injections of 1.75 μg L-thyroxine in 0.1 ml saline (Sigma Chem. Co., St. Louis, Mo., purified by recrystallization) were given subcutaneously at 8 h and 16 h for 21 days beginning 90 days after exposure to continuous illumination (130 days of age). Daily vaginal smears were recorded and ovulation was observed. As a control purpose, 0.1 ml saline was injected daily for 21 days. The ovaries were removed after cessation of L-thyroxine treatment and examined histologically.

Experiment 3. Removal of thyroid gland, including parathyroid gland, was performed in rats after exposure to continuous illumination for 90 days. Daily vaginal smears were recorded. Laparotomy was performed once a week for confirmation of ovulation. The ovaries were examined histologically 8 weeks after thyroidectomy.

For the following experiments testosterone propionate (100 μg) was injected into female rats from our laboratory breeding colony at 5 days of age.

Experiment 4. Removal of thyroid gland, including parathyroid gland, was performed at 24 days of age. Vaginal opening and degree of vaginal cornification were observed and daily vaginal smears were recorded. Laparotomy for confirmation of ovulation (examination of tubal ova and/or corpus luteum formation) was performed between 8 and 10 h on day of oestrus smear. All animals were sacrificed at 130 days of age, and the ovaries were examined histologically

Experiment 5. Removal of thyroid gland, including parathyroid gland, was performed at 24 days of age. Daily injections of 1.75 μg L-thyroxine in 0.1 ml saline (Sigma Chem. Co., St. Louis, Mo., purified by recrystallization) were given subcutaneously at 8 h and 16 h for 24 days beginning at 130 days of age. Daily vaginal smears were recorded. The ovaries were removed after cessation of L-thyroxine treatment and examined histologically. As a control purpose, 0.1 ml saline was injected daily for 24 days beginning at 130 days of age in thyroidectomized rats.

Experiment 6. Removal of thyroid gland, including parathyroid gland, was performed at 130 days of age. Daily vaginal smears were recorded; laparotomy was performed once a week for determination of ovulation. The ovaries were examined histologically 8 weeks after thyroidectomy.

Results

Experiment 1. When intact rats were exposed to continuous illumination at 40 days of age, 38 of 39 rats became anovulatory and showed polyfollicular ovaries with persistent oestrus (Table 1). Thyroidectomy caused a delay of onset of puberty [8].

When thyroidectomized rats were exposed to continuous illumination at 40 days of age, only 2 of 26 rats became anovulatory and showed polyfollicular ovaries with persistent oestrus. Results in the other 24 rats were irregular oestrus cycles and confirmed ovulation; polyfollicular ovaries were not observed. Ovarian histology revealed primarily secondary follicles with corpora lutea.

Experiment 2. In these thyroidectomized rats under continuous illumination, injections of 1.75 μg L-thyroxine produced a prolonged oestrus (Table 2)

Table 1

Influence of hypothyroid state in juvenile rats on ovulation and vaginal cycles under exposure to continuous illumination

Treatment regimens	No. of rats	No. of rats showing persistent oestrus	No. of rats showing tubal ova or corpora lutea
Group 1			
Intact control rats	28	27	1
Thyroidectomized rats	13	2	11
Group 2			
Intact control rats	11	11	0
Thyroidectomized rats	13	0	13

Thyroidectomy was performed at 24 days of age, and these rats and intact control rats were exposed to continuous illumination at 40 days of age. Daily vaginal smears were taken and corpora lutea were examined histologically at 130 days of age (90 days after exposure to continuous illumination).

which ceased, on average, at 12.3 days after cessation of L-thyroxine treatment. Development of polyfollicular ovary and ovulation were not observed (Fig. 1).

Table 2

Effect of L-thyroxine on ovulatory cycles in thyroidectomized rats under exposure to continuous illumination

Treatment regimens				Onset of prolonged oestrus		No. of rats showing corpora lutea
Agent	Doses (μg)	Duration (days)	No. of rats	No. of rats	Initial days** (days $\pm$ S.E.)	
Saline	0.1 ml	21	13	0	—	13
L-thyroxine*	1.75×2	21	11	11	7.2 ± 2.5	0

* L-thyroxine (1.75 μg) was given subcutaneously at 8 h and 16 h
** Initial days of the appearance of prolonged oestrus

Experiment 3. In rats which were exposed to continuous illumination for 90 days persistent oestrus and polyfollicular ovaries developed. Removal of thyroid gland did not interfere with persistent vaginal cornification. Histological examination of ovary at 30 days after thyroidectomy determined polyfollicular ovary with no corpus luteum (Table 3).

Experiment 4. When intact rats received 100 μg of testosterone propionate at 5 days of age, 20 of 22 rats became anovulatory with persistent oestrus. The ovaries were polyfollicular with no corpus luteum. When thyroidectomy

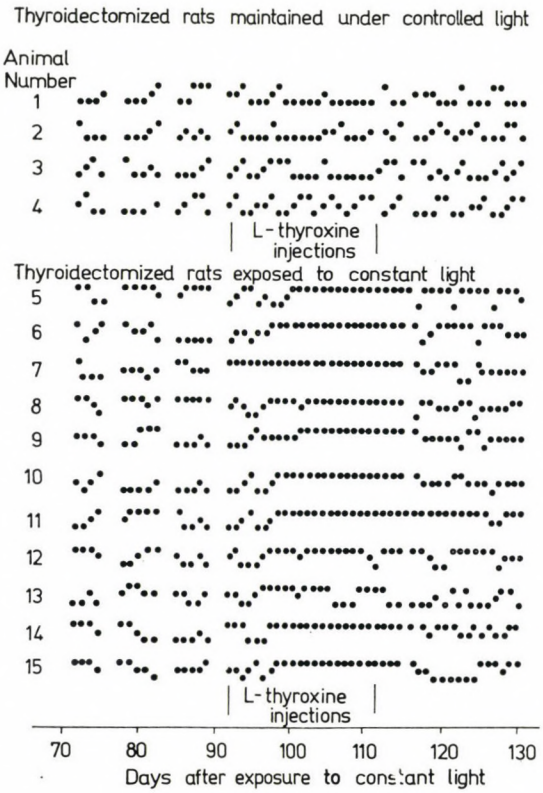

Fig. 1. Records of daily vaginal smears of the thyroidectomized rats. Basal dots (.) represent dioestrus, medial dots represent preoestrus(·) and upper dots illustrate oestrus(˙) (vaginal cornification). Above: 4 rats (Nos 1—4) maintained under controlled light, below: 11 rats (Nos 5—15) exposed to continuous illumination. Horizontal axis represents days after exposure to continuous illumination. After 90 days exposure to continuous illumination, the rats received daily injections of L-thyroxine for three weeks. Under controlled light, daily injections of L-thyroxine do not produce a prolonged oestrus, however, treatment with this regimen under continuous illumination produces a prolonged oestrus in the thyroidectomized rats

Table 3

Influence of thyroidectomy on ovulation in adult rats made persistent oestrus by exposure of continuous illumination

Treatment regimens	No. of rats	No. of rats showing persistent oestrus	No. of rats showing corpora lutea
Intact control rats	5	5	0
Thyroidectomized rats	11	11	0

Rats were exposed to continuous illumination at 40 days of age, and thyroidectomy was performed at 130 days of age (90 days after exposure of continuous illumination). Daily vaginal smears were taken and ovary was examined histologically at 30 days after thyroidectomy.

was performed at 24 days of age, only 2 of 23 rats developed polyfollicular ovaries and persistent oestrus; the other 21 rats had irregular oestrus cycles, while histology of the ovary showed primarily secondary follicles with no corpus luteum (Table 4).

Table 4

Influence of hypothyroid state in juvenile stage on ovulation and vaginal cycles in neonatal androgen treated rats

Treatment regimens	No. of rats	No. of rats showing persistent oestrus	No. of rats showing corpora lutea
Group 1.			
Intact control rats	14	12	2
Thyroidectomized rats	15	2	0
Group 2.			
Intact control rats	8	8	0
Thyroidectomized rats	8	0	0

Rats received 100 μg testosterone propionate in oil at 5 days of age and thyroidectomy was performed at 24 days of age. Daily vaginal smears were taken and corpora lutea were examined histologically at 130 days of age.

Experiment 5. In these thyroidectomized rats (neonatal androgen treatment), injections of 1.75 μg L-thyroxine were given daily for 24 days beginning at 130 days of age, producing a prolonged oestrus; the ovaries were polyfollicular, but with no corpus luteum. However, this prolonged oestrus ceased after cessation of L-thyroxine treatment (Table 5).

Experiment 6. Rats receiving 100 μg of testosterone propionate at 5 days of age became anovulatory and persistent oestrus. Removal of thyroid gland at 130 days of age did not interfere with persistent vaginal cornification, and the ovaries were polyfollicular with no corpus luteum (Table 6).

Table 5

Effect of L-thyroxine on ovulatory cycles in thyroidectomized rats

Treatment regimens				Onset of prolonged oestrus		No. of rats showing corpora lutea
Agent	Doses (μg)	Duration (days)	No. of rats	No. of rats	Initial days** (days ± S.E.)	
Saline	0.1 ml	24	8	0	—	0
L-thyroxine	1.75×2	24	12	12	9.4 ± 2.0	0

* L-thyroxine (1.75 μg) were given subcutaneously at 8 h and 16 h
** Initial days of the appearance of prolonged oestrus

Table 6

Influence of thyroidectomy on ovulation in adult rats made persistent oestrus by neonatal androgen treatment

Treatment regimens	No. of rats	No. of rats showing persistent oestrus	No. of rats showing corpora lutea
Intact control rats	5	5	0
Thyroidectomized rats	8	8	0

Rats received 100 μg of testosterone propionate at 5 days of age and thyroidectomy was performed at 130 days of age. Daily vaginal smears were taken and ovaries were examined histologically 30 days after thyroidectomy.

Discussion

In rats, the anterior hypothalamus, including the mPOA, is necessary for the regulation of thyroid function [2, 11]; in addition, the anterior hypothalamus has been implicated as a site of ovarian hormone feedback involved in the regulation of gonadotropin secretion [5, 12]. Lesioning of the anterior hypothalamus causes persistent oestrus and polyfollicular ovaries [5, 7].

In rats exposed to continuous illumination or neonatal androgen treatment, the capacity of oestrogen binding in the anterior hypothalamus is reduced [6, 10] and results in blockade of the cyclic release of gonadotropin and the development of persistent oestrus with polyfollicular ovaries [1, 3]. In such rats, thyroidectomy (hypothyroid state) in juvenile rats decreases hypothalamic responsiveness to oestrogen [9]. In addition, thyroidectomy prevents the development of persistent oestrus and polyfollicular ovaries. Replacement treatment with L-thyroxine brings about resumption of prolonged vaginal cornification; however, this prolonged oestrus ceases after termination of L-thyroxine treatment. Thyroidectomy is effective on reproductive cycles only when performed in the juvenile rats, and does not interfere with persistent vaginal cornification and polyfollicular ovaries when performed in adult persistent oestrous rats.

These data suggest that thyroidectomy in juvenile rats interferes with the functional maturation of oestrogen binding receptors in the anterior hypothalamus. Furthermore, an additional reduction of oestrogen binding capacity in the anterior hypothalamus by thyroidectomy in rats exposed to continuous illumination or neonatal androgen treatment may prevent the development of polyfollicular ovaries and persistent oestrus.

Therefore, it implies that circulation of a physiological level of thyroid hormone in juvenile rats is necessary in the functional maturation of oestrogen binding receptors in the anterior hypothalamus concerning regulation of gonadotropin secretion.

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CARBOHYDRATES FROM HYDROCARBONS

II. FREE AND BOUND SUGARS FROM YEAST CELLS GROWN ON *n*-HEXADECANE

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Abstract

Candida lipolytica (strain 10) was grown on an *n*-hexadecane medium with and without yeast extract. The harvested dry cells were weighed at various stages of growth. The free sugars from the cultures were obtained by Soxhlet extraction with 85% ethyl alcohol. Further qualitative and quantitative analyses of free monosaccharides in the concentrated alcoholic extracts were made by paper chromatography. Glucose was the only free monosaccharide that could be identified at various stages of growth. The chromatographic analysis of the acid-hydrolyzed yeast cells indicated the presence of glucose and mannose as dominant bound sugars; galactose and xylose were present in minor quantities. In the harvested dry cells from the yeast extract-containing medium, in general, greater amounts of bound sugars were present.

Introduction

The utilization of hydrocarbons as the sole carbon source by microorganisms have been the subject of many studies. Concerning hydrocarbon-assimilating yeast, in particular, the production of microbial cells from hydrocarbons has been studied by many authors [7, 12, 16, 17]. The production of organic acids from *n*-paraffins by yeasts was worked out by others [9, 11, 15, 18, 19, 20]. In addition, WATANABE and HIRAI [22] have reported on microbial production of fat and carotenoids, and NAOMICHI and TADASHI [10] studied the utilization of *n*-paraffins and the production of vitamin B_2 by yeasts.

Available knowledge about the production of carbohydrates by hydrocarbon-assimilating yeast is, however, still scanty. IIZUKA and co-workers [5] investigated the differences between the monosaccharide components of hydrocarbon-assimilated cells and those of carbohydrate-assimilated ones. Other studies on the composition of the polysaccharide contents of yeast cells grown on hydrocarbons and glucose were carried out by NABESHIMA and co-workers [8]. We have reported [6] on the extracellular polysaccharides obtained from a hydrocarbon-assimilating yeast.

The present work was undertaken to explore the possibility of producing free and bound sugars of commercial and clinical value by *Candida lipolytica* (strain 10) cells grown on *n*-hexadecane.

Material and method

Microorganism. The hydrocarbon-assimilating yeast used in the present study, *Candida lipolytica* (strain 10), was obtained through the courtesy of Prof. IVAN MALEK, Director of the Institute of Microbiology, Czechoslovak Academy of Sciences.

General culture conditions. The yeast was grown on a stock medium containing 2 g washed agar per 100 ml of liquid culture medium. The liquid medium contained per liter: 10 ml *n*-hexadecane (99% purity obtained from Phillips Co.), 3.4 g KH_2PO_4, 1.5 g $Na_2HPO_4 \cdot 12\ H_2O$, 0.7 g $MgSO_4 \cdot 7\ H_2O$, 4.0 g $(NH_4)_2SO_4$, 0.1 g $CaCl_2$, 1 mg $FeSO_4 \cdot 7H_2O$, 0.1 ml oligoelements, and 0.1 g yeast extract. The pH was finally adjusted to 4.

Fermentation was carried out in 500-ml Erlenmeyer flasks containing 100 ml of the liquid medium and inoculated with about 0.1 g of 36—48 cultures. Two fermentation media were used throughout; the first was the above-defined liquid medium, and the second was the same except that it did not contain yeast extract. The inoculated flasks were shaken at 30°C, and the yeast cells were harvested after different incubation periods, by filtration through Millipore H. A. membrane filter, followed by quick washing with distilled water and then ether. The collected cells were dried over P_2O_5 *in vacuo* to a constant weight.

Extraction of free monosaccharides from yeast cells. Dry yeast cells (2 g) were extracted with 85% ethanol for 20 h in a Soxhlet apparatus. The alcoholic extract was concentrated to 5 ml and the residual yeast cells were dried under reduced pressure over $CaCl_2$, and weighed.

Identification of extracted free monosaccharides. We examined the concentrated alcoholic extract of yeast cells by paper chromatography, using *n*-butanol–ethanol–water (40 : : 11 : 1 9, v/v) [13], *n*-butanol–pyridine–water (6 : 4 : 3, v/v) [21], and pyridine–ethyl acetate–acetic acid–water (5 : 5 : 1 : 3, v/v) [3]. The chromatographic papers were sprayed with aniline diphenylamine phosphoric acid, aniline oxalate and ammoniacal $AgNO_3$ reagent [2].

Quantitative determination of free monosaccharides. The method of WILSON [21] was used for estimation of the free monosaccharides in concentrated alcoholic extracts of yeast cells.

Identification of bound sugars of yeast cells. Alcohol-extracted yeast cells were hydrolyzed with 2 N H_2SO_4 [4] and the hydrolysis products were identified chromatographically. The same technique was used as for free monosaccharides.

Quantitative determination of bound sugars of yeast cells. The acid-hydrolyzed samples were subjected to a quantitative paper chromatography [21] to obtain each monosaccharide individually.

Results and discussion

Harvesting and alcohol extraction of yeast cells. The yeast cells were harvested after various incubation periods, then extracted with ethanol for 20 h. The weights (g/l) of the harvested cells before and after alcohol extraction are shown in Table 1. The maximum yield of the yeast cells grown on the yeast extract-free medium was reached after 120 h incubation; this was about 1/3 of the yield obtained after 96 h in the presence of yeast extract (0.1 g/l). Thus, from the economical point of view it is advisable to add yeast extract (0.1 g/l) to the medium instead of continuing the fermentation process over 120 h. Furthermore, Table 1 clearly shows that the cells grown in the presence of yeast extract had higher contents of ethanol-extractable materials than those grown without yeast extract. The production of the ethanol-extractable materials reached a maximum after 48 h of incubation in the presence, and after 120 h in the absence of yeast extract.

Table 1

Production of Candida lipolytica (strain 10) cells in a medium containing n-hexadecane, in the presence and in the absence of yeast extract

Incubation period (h)	Weight of cells grown in yeast extract-containing medium (g/l)		Weight of cells grown in yeast extract-free medium (g/l)	
	before alcoholic extraction	after alcoholic extraction	before alcoholic extraction	after alcoholic extraction
24	1.10	0.85	0.39	0.32
48	3.90	2.21	0.77	0.70
72	4.80	3.01	0.73	0.64
96	5.75	3.99	0.97	0.83
120	4.48	3.93	1.79	1.39

Table 2

Production of free glucose by Candida lipolytica (strain 10) cells grown on the n-hexadecane medium in the presence of yeast extract

Incubation period (h)	Free glucose produced	
	(per cent of cells)	(mg/l)
24	1.25	13.75
48	0.48	18.72
72	0.45	20.40
96	0.36	20.70
120	0.29	12.99

Free monosaccharide components of yeast cells. The paper chromatograms of all alcoholic extracts of yeast cells revealed one spot with migration distances and colour reactions characteristic of glucose. Table 2 shows the rate of production of free glucose by *Candida lipolytica* (strain 10) cells grown on the yeast extract-containing medium. The amounts of free glucose produced by the yeast cells grown in the absence of yeast extract were too small to be determined quantitatively.

The results of the chromatographic analysis of the free sugars in the yeast cells during various stages of growth indicate that the sole free monosaccharide was glucose. This monosaccharide did not exceed 5.50% of the total alcohol-extractable materials. This observation infers the presence of other alcohol-soluble materials, such as lipids and absorbed hydrocarbon, in the yeast cells.

The results also showed that free glucose was abundant on the first few days, then its amounts decreased with time, suggesting that the free sugars

change with increasing time into polymers which cannot be extracted by alcohol. The appearance of a high percentage (94.50%) of other alcohol-extractable materials may probably be due to the presence of the absorbed hydrocarbon in the cell wall. The results thus agree with those of RACHINSKII and co-workers [14], who stated that the alkane which had entered the cell wall was totally extracted in a Soxhlet extractor with ethanol–ether.

It is also evident from the results that the addition of yeast extract to the *n*-hexadecane fermentation medium stimulated the production of yeast cells and free glucose, as well as other alcohol-extractable materials and bound sugars. This stimulating effect can be attributed to the efficiency of the yeast extract as a nitrogen source and/or to its action as an essential factor for the growth and production of the above-mentioned materials.

Bound sugars of the yeast cells. The hydrolysis products of each of the alcohol-extracted cell samples were analyzed by paper chromatography. The R_f values and colour reactions of the sugar components of the hydrolysates were identical with those of authentic galactose, glucose, mannose and xylose.

To determine the mentioned sugars quantitatively, correction factors, viz., 1.056, 1.067, 1.208, and 1.147, were used for galactose, glucose, mannose, and xylose, respectively. The same correction factors were previously computed and used [6] to compensate for the breakdown effect of various hydrolysis conditions on these sugars. The quantitative analysis of each sample of the hydrolyzed yeast cells are given in Table 3.

Table 3

Bound sugars in the alcohol-exctracted Candida lipolytica (strain 10) cells grown on media containing n-hexadecane

Incubation period (h)	Yeast cells grown on the yeast extract-containing medium					Yeast cells grown on the yeast extract-free medium				
	glucose	mannose	galactose	xylose	Total	glucose	mannose	galactose	xylose	Total
	(per cent of the alcohol-extracted cells)									
24	6.53	6.12	2.50	0.88	16.03	8.10	7.94	2.59	0.84	19.47
48	9.14	6.77	2.63	0.62	19.16	6.66	5.65	2.16	0.86	15.33
72	7.81	6.55	2.73	0.53	1.762	5.60	5.73	2.00	0.68	14.01
96	8.13	6.18	2.43	0.48	17.22	6.56	5.00	2.31	0.69	14.45
120	12.98	8.63	3.24	0.55	25.40	9.41	6.61	2.65	0.61	19.28

In spite of the difference between the two fermentation media, all the hydrolysates of *Candida lipolytica* (strain 10) cells revealed a relatively high content of glucose and mannose and a low content of galactose and xylose.

But, whereas the cells grown in the presence of yeast extract showed their maximum contents of bound glucose, mannose, and galactose after 120 h of incubation, the maximum for bound xylose appeared after 24 h. In absence of yeast extract, on the other hand, the cells showed the peak of bound glucose and galactose after 120 h of incubation, while the peak of bound mannose and xylose appeared after 24 h and 48 h, respectively. These results, collectively, indicated that most of the bound sugars showed higher percentages after 120 h of incubation, and this can be attributed to the accumulation of the reserving and cell wall polysaccharides in the yeast cells at this late stage of growth.

The results shown in Tables 1 and 3 indicate that the increase in the production of yeast cells may not be accompanied by an increase in their bound sugar contents. Table 4 was thus so designed as to express the production of the endocellular bound sugars in mg/l. In both types of yeast cells, the maximum yield of endocellular bound glucose, mannose, galactose, and xylose occurred after 120 h of incubation.

Table 4

Endocellular combined sugars produced by Candida lipolytica (strain 10) grown on media containing n-hexadecane

Incubation period (h)	Cells grown on the yeast extract-containing medium					Cells grown on the yeast extract-free medium				
	glucose	mannose	galactose	xylose	Total	glucose	mannose	galactose	xylose	Total
	(mg/l)					(mg/l)				
24	55.74	52.24	21.34	7.51	136.83	25.68	25.16	8.21	2.66	61.71
48	202.01	149.62	58.13	13.70	423.47	46.69	39.61	15.14	6.03	107.47
72	235.42	197.44	8.229	15.98	531.13	35.78	36.61	12.78	4.35	89.52
96	324.57	246.72	97.01	19.26	687.56	54.58	41.60	19.22	5.74	121.14
120	509.75	338.91	127.24	21.59	997.49	131.18	92.14	36.94	8.50	268.76

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THE FERMENTATIVE PRODUCTION OF ACETONE–BUTANOL BY CLOSTRIDIUM ACETOBUTYLICUM

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Abstract

Fourteen different media were used in the fermentative production of acetone–butanol. The highest total yields were achieved in medium I. Potato starch and soluble starch were suitable as carbon sources. The best concentrations of potato starch and soluble starch were 500.0 and 10.0 g/l, respectively. Peptone was the most favourable nitrogen source. The best concentration of peptone was 4.0 g/l. Calcium carbonate in 3.6 g/l acted as buffering agent in the fermentation process. The best initial pH value of the fermentation medium was 6.0. The optimum temperature was 32—33°C. The fermentation process required 120 h to obtain maximum yields of acetone–butanol.

Introduction

Pasteur was the first to show that butyl alcohol is a direct product of fermentation. The acetone–butanol fermentations are true fermentations, in the sense that they are anaerobic. The fermentations are brought about by various strains of *Clostridium acetobutylicum* and closely related species or variants. The important neutral products formed by these organisms are *n*-butanol, acetone and ethanol. The microbiological production of acetone and butanol was one of the first large-scale microbiological processes. The process has been operated primarily as a source of butanol, although, during World War I, acetone produced by fermentation proved very useful in war factories for dissolving cordite.

The commonest of the carbohydrate raw materials used in the acetone–butanol fermentation are molasses and corn. Several types of molasses (invert, cane, beet) have been employed. It is, however, justified to supplement the molasses medium with ammonium sulphate and phosphate as sources of nitrogen and phosphorus, respectively. Grain was often used as a carbohydrate source during the early work on the acetone–butanol fermentation. Since the strains of *C. acetobutylicum* are able to hydrolyse starch and other polysaccharides, the grain mashes do not need to be hydrolyzed. The use of starchy mate-

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rials in the acetone–butanol fermentation has been discussed [2, 3]. The grain, which is usually maize, is screened, degerminated, and finally ground. An 8—10% mash is prepared and cooked, usually in a continuous cooker for one hour at ~130°C, to gelatinize starch. No further additions are necessary, since the mash (pH 6.0—6.5) contains sufficient quantities of nitrogenous nutrients to support growth of the bacterium. Some workers have, however, recommended to add stillage [3]. The sterile mash is transferred directly to the fermentor.

Several other carbohydrate materials have been examined as raw materials in this fermentation, including glucose from corn starch and wood sugars obtained by acid hydrolysis of wood. Wood sugars in the form of sulphite waste liquor have been used [10] as also corncob hydrolysates which contain xylose [4, 5, 9—13], but use of these materials on a commercial scale has not been reported.

The aim of this work is to utilize raw materials such as Egyptian black strap molasses and other carbon and nitrogen sources for the fermentative production of acetone–butanol by *C. acetobutylicum*.

Material and method

Maintenance of C. acetobutylicum. The microorganism producing acetone–butano was maintained in a medium containing the following ingredients: wet potato mash 250 g, glucose 5 g, calcium carbonate 2 g and $(NH_4)_2 SO_4$ 1.5 g per litre. The ingredients were thoroughly mixed and transferred into test tubes. The test tubes were plugged, and sterilized at 120°C for 25 min, cooled to room temperature and inoculated with *C. acetobutylicum*. The inoculated tubes were incubated at 37°C for three weeks to obtain luxuriant growth and sporulation. The sporulated tubes were stored at 5—8°C in a refrigerator.

Fermentation media. C. acetobutylicum was grown anaerobically in 14 different constitutive media. The composition of the media (ingredients in g/l) was as follows. Medium I = potato starch 250.0, glucose 5.0, $CaCO_3$ 2.0 and $(NH_4)_2SO_4$ 1.5. Medium II = soluble starch 250.0, glucose 5.0, $(NH_4)_2SO_4$ 1.5 and $CaCO_3$ 2.0. Medium III = pure starch 250.0, glucose 5.0, $(NH_4)_2SO_4$ 1.5 and $CaCO_3$ 2.0. Medium IV = pure flour (1) 250.0, glucose 5.0, $(NH_4)_2SO_4$ 1.5, and $CaCO_3$ 2.0. Medium V = glucose 10.0, $(NH_4)_2SO_4$ 2.0, $CaCO_3$ 2.0 and KH_2PO_4 1.0. Medium VI = Sucrose 10.0, $(NH_4)_2SO_4$ 1.5, KH_2PO_4 1.0 and $CaCO_3$ 2.0. Medium VII = corn steep liquor 250.0, glucose 5.0, $(NH_4)_2SO_4$ 1.5 and $CaCO_3$ 2.0. Medium VIII = wort 250.0, glucose 5.0, $(NH_4)_2SO_4$ 1.5 and $CaCO_3$ 2.0. Medium IX = fodder yeast (40.0% total protein) 250.0, glucose 5.0, $(NH_4)_2SO_4$ 1.5 and $CaCO_3$ 2.0. Medium X = fodder yeast (50.0% total protein) 250.0, glucose 5.0, $(NH_4)_2SO_4$ 1.5 and $CaCO_3$ 2.0. Medium XI = soybean meal 250.0, glucose 5.0, $(NH_4)_2 SO_4$ 1.5 and $CaCO_3$ 2.0. Medium XII = rice bran 250.0, glucose 5.0, $(NH_4)_2SO_4$ 1.5 and $CaCO_3$ 2.0. Medium XIII = maize bran, 250.0. glucose 5.0, $(NH_4)_2SO_4$ 1.5 and $CaCO_3$ 2.0. Medium XIV = stillage (slope) 250.0, glucose 5.0, $(NH_4)_2SO_4$ 1.5 and $CaCO_3$ 2.0. The initial pH value of the media was adjusted to 6.3—6.6 except when indicated in the text. The different media were distributed in 500 ml Erlenmeyer flasks, 250 ml in each. The flasks were plugged and sterilized at 120°C for 30 min. The tubes contained the organism in sporulated form; the organism was heat shocked in a water bath containing boiling water for three min and suddenly cooled to room temperature. Then the media were inoculated with *C. acetobutylicum*. The flasks were incubated at 31°C for 72 h. At the end of this period, the pH value of the fermented media, and the amounts of acetone, butanol and ethanol were estimated.

Carbon sources. The glucose in Medium I was replaced by an equal amount of soluble starch, sucrose, maltose, molasses, mannose, fructose, glycerol, lactose or citric acid to investigate the suitable ingredient favouring the fermentative production of acetone–butanol by *C. acetobutylicum*. The effects of different concentrations of potato starch and soluble starch on acetone—butanol fermentation were investigated.

Nitrogen sources. The ammonium sulphate in Medium I was replaced by an equal amount of ammonium thiocyanate, ammonium nitrate, ammonium chloride, sodium nitrate, peptone, ammonium hydroxide, urea, ammonium phosphate monobasic or ammonium oxalate. The effects of different concentrations of peptone on acetone–butanol fermentation were examined.

Calcium carbonate, initial pH value of the medium and incubation period. The influences of different concentrations of calcium carbonate (0.0 to 0.4 g/l), of the initial pH value of the medium (5.0 to 7.0), temperature (30 to 40°C) and incubation period (24 to 120 h) on the fermentative production of acetone-butanol by *C. acetobutylicum* were investigated.

Determination of butanol and ethanol. Johnson's method [8] was used.

Determination of acetone. Goddwin's method [6] was used.

Results

Fermentation media

Different fermentation media were used in the production of acetone-butanol. The amounts of acetone, butanol and ethanol produced depended on the constituents of the fermentation media used. The fermentation medium which produced maximum yield of total solvents was Medium I (Table 1). The final pH values of the different media ranged between 5.4 and 6.7, depending on the ingredients present in the medium. The amounts of butanol produced in the different fermentation media were higher than the corresponding

Table 1

The fermentative production of acetone–butanol by C. acetobutylicum in different media

Medium No.	Final pH*	Acetone, mg/100 ml potato	*n*–Butanol, mg/100 ml potato	Ethanol, mg/100 ml potato	Total solvent, mg/100 ml potato
I	5.4	295	1036	62	1393
II	5.7	37	96	20	153
III	6.7	49	104	4	157
IV	5.6	34	126	14	174
V	5.6	263	691	30	984
VI	5.2	281	725	44	1050
VII	6.7	77	106	18	201
VIII	5.9	29	51	35	115
IX	6.3	56	135	22	213
X	6.1	63	190	11	264
XI	5.3	101	109	28	328
XII	6.2	44	144	nil	188
XIII	6.4	24	110	24	158
XIV	5.3	10	39	10	59

* The initial pH value of the different media was adjusted to 6.3—6.6

amounts of acetone and ethanol. The fermentation medium supporting the production of acetone–butanol contained potato starch, glucose, calcium carbonate and ammonium sulphate. The descending order of the different fermentation media arranged according to the acetone–butanol yield was as follows: Medium I > Medium VI > Medium V > Medium XI > Medium X > Medium IX > Medium VII > Medium XII > Medium IV > Medium XIII > Medium III > Medium II > Medium VIII > Medium XIV.

Carbon sources

Medium I was selected to study the influence of different carbon sources on the fermentative production of acetone–butanol by *C. acetobutylicum* (Table 2). Soluble starch was the best carbon source for the fermentative production of acetone–butanol. It was followed by sucrose. The arrangement of the different carbon sources according to the descending production of acetone–butanol was as follows: soluble starch > sucrose > maltose > glucose > molasses > mannose > fructose > glycerol > lactose > citric acid. The final pH value of the fermentation medium was between 4.2 and 5.7.

Table 2

Influence of different carbon sources on the fermentative production of acetone–butanol by C. acetobutylicum

Carbon sources	Final pH*	Acetone, mg/100 ml potato	*n*–Butanol, mg/100 ml potato	Ethanol, mg/100 ml potato	Total yield, mg/100 ml potato
Soluble starch	2.5	361	940	45	1 346
Sucrose	5.4	378	871	68	1 317
Maltose	5.6	392	867	55	1 314
Glucose	5.7	336	858	43	1 237
Molasses	5.5	218	949	70	1 237
Mannose	5.5	348	870	10	1 228
Fructose	5.6	329	850	45	1 224
Glycerol	5.3	319	850	50	1 219
Lactose	5.4	339	820	45	1 204
Citric acid	4.2	12	17	1	30

* The initial pH value of the medium was adjusted to 6.3—6.6

The production of acetone–butanol was greatly affected by the amounts of potato starch added to the fermentation medium (Table 3). The total

Table 3

Effect of different concentrations of potato starch and soluble starch on the fermentative production of acetone–butanol by C. acetobutylicum

Potato starch (g/l)	Final pH*	Acetone mg/100 ml potato	*n*-Butanol, mg/100 ml potato	Ethanol, mg/100 ml potato	Total yield, mg/100 ml potato
0	5.4	94	415	5	414
50	5.4	119	619	55	793
100	5.4	121	766	50	937
125	5.6	227	808	50	1 085
150	5.6	268	880	38	1 186
175	5.7	242	960	40	1 242
200	5.8	304	942	39	1 285
225	5.3	300	1 027	37	1 364
250	5.6	325	1 000	51	1 375
275	5.4	329	1 023	40	1 392
300	5.4	353	1 095	42	1 490
350	5.6	369	1 121	101	1 591
400	5.7	396	1 113	101	1 610
450	5.7	428	1 177	40	1 645
500	5.7	441	1 262	31	1 734
Soluble starch (g/l)					
0	5.7	763	1 248	88	1 699
1	5.6	410	1 254	85	1 749
2	5.4	337	1 191	137	1 665
3	5.6	389	1 216	55	1 660
4	5.7	401	1 184	90	1 675
5	5.4	435	1 287	108	1 830
6	5.8	417	1 341	113	1 871
7	5.7	415	1 290	105	1 810
8	5.8	421	1 868	55	1 744
9	5.7	413	1 230	45	1 688
10	5.6	435	1 193	75	1 703

* The initial pH value of the medium was adjusted to 6.3—6.5

yield (acetone-butanol + ethanol) increased with the increase of potato starch concentration, reaching an optimum at 500.0 g/l. At the end of the fermentation process, the pH ranged between 5.4 and 5.7. The results obtained (Table 3) reflected that the total yield increased with the increase of the soluble starch concentration, reaching its optimum at 10.0 g/l. The final pH value of the fermented medium was 5.4—5.8.

Nitrogen sources

Peptone was the best nitrogen source favouring the production of acetone–butanol (Table 4). The yields of butanol were higher than yields of both acetone and ethanol. The order of the different nitrogen sources according to the descending total yield (acetone + butanol + ethanol) was as follows: peptone > ammonium nitrate > ammonium sulphate > ammonium chloride > sodium nitrate > ammonium thiocyanate > ammonium hydroxide > urea > ammonium phosphate monobasic > ammonium oxalate > ammonium citrate medium without addition of nitrogen source. By the end of the fermentation process, the pH had diminished to 5.3—5.7.

Table 4

Influence of different nitrogen sources on the fermentative production of acetone–butanol by C. acetobutylicum

Nitrogen sources	Final pH*	Acetone, mg/100 ml potato	*n*-Butanol, mg/100 ml potato	Ethanol, mg/100 ml potato	Total yield, mg/100 ml potato
Peptone	5.5	319	1 133	55	1 507
Ammonium nitrate	5.7	296	1 123	32	1 451
Ammonium sulphate	5.5	310	993	53	1 356
Ammonium chloride	5.5	283	959	70	1 312
Sodium nitrate	3.3	299	921	15	1 235
Ammonium thiocyanate	5.4	261	966	5	1 232
Ammonium hydroxide	5.4	249	938	30	1 217
Urea	5.5	242	927	42	1 21
Ammonium phosphate	5.5	276	873	60	1 209
Ammonium oxalate	5.3	281	689	39	1 189
Ammonium citrate	5.7	319	598	55	972
No nitrogen source	—	—	—	—	—

* The initial pH value of the medium was adjusted to 6.3—6.5

The yields of total solvents increased with the increase of peptone concentration, reaching an optimum at 4.0 g/l (Table 5). The final products of solvents were mainly acetone and *n*-butanol. The final pH value was 5.6—5.9.

Calcium carbonate, pH, temperature and incubation period

Calcium carbonate had some effect on the fermentative production (Table 6). The total yields increased with the increase of calcium carbonate concentration, reaching an optimum at 3.6 g/l, above which a slight decrease

Table 5

Effect of the concentration of peptone on the fermentative production of acetone–butanol by C. acetobutylicum

Peptone (g/l)	Final pH*	Acetone, mg/100 ml potato	*n*-Butanol, mg/100 ml potato	Ethanol, mg/100 ml potato	Total yield, mg/100 ml potato
0.0	5.8	348	1 365	0	1 713
0.4	5.9	416	1 321	4	1 741
0.8	5.7	384	1 352	52	1 788
1.2	5.6	425	1 338	57	1 820
1.6	5.9	394	1 430	0	1 824
2.0	5.8	440	1 382	10	1 832
2.4	5.8	420	1 411	9	1 840
2.8	5.8	428	1 408	19	1 850
3.2	5.8	432	1 425	19	1 876
3.6	5.8	423	1 470	17	1 900
4.0	5.8	442	1 487	2	1 931

* The initial pH value of the medium was adjusted to 6.3—6.6

Table 6

Effect of the concentrations of calcium carbonate on the fermentative production of acetone–butanol by C. acetobutylicum

Calcium carbonate (g/l)	Final pH*	Acetone, mg/100 ml potato	*n*-Butanol, mg/100 ml potato	Ethanol, mg/100 ml potato	Total yield, mg/100 ml potato
0	5.3	522	1 156	83	1 761
0.4	5.3	389	1 368	50	1 807
0.8	5.4	451	1 318	78	1 847
1.2	5.4	544	1 198	115	1 857
1.6	5.6	524	1 253	88	1 865
2.0	5.9	432	1 342	105	1 879
2.4	5.9	482	1 319	80	1 881
2.8	6.0	508	1 279	102	1 889
3.2	6.3	508	1 312	88	1 908
3.6	6.3	515	1 357	73	1 945
4.0	6.3	534	1 300	88	1 922

* The initial pH value of the medium was adjusted to 6.3—6.6

was shown. The final pH value of the fermented mash was 5.3—6.0. The presence of calcium carbonate (2.8—4.0 g/l) buffered the pH value of the medium.

As shown in Table 7, the final biosynthesis of acetone, butanol and ethanol depended on the initial pH of the medium. The yields increased with an increase in the initial pH, reaching its optimum at pH 6.0, above which a decrease in the total yields was recorded. Therefore, it is preferable to adjust the initial pH value of the medium at 6.0.

Table 7

Effect of the initial pH value of the medium on the production of acetone–butanol by C. acetobutylicum

Initial pH	Final pH	Acetone, mg/100 ml potato	*n*-Butanol, mg/100 ml potato	Ethanol, mg/100 ml potato	Total yield, mg/100 ml potato
5.0	5.0	131	483	0	611
5.2	4.0	203	610	0	813
5.4	5.2	237	760	65	1 062
5.6	5.3	329	1 040	24	1 383
5.8	5.7	503	1 293	53	1 849
6.0	5.8	668	1 344	15	1 927
6.2	5.8	707	1 282	15	1 904
4.6	5.8	486	1 319	33	1 838
6.6	5.9	546	1 125	81	1 758
6.8	6.1	435	915	85	1 435
7.0	6.4	334	660	169	1 163

Table 8

Effect of temperature on the fermentative production of acetone–butanol by C. acetobutylicum

Temperature, °C	Final pH*	Acetone, mg/100 ml potato	*n*-Butanol, mg/100 ml potato	Ethanol, mg/100 ml potato	Total yield, mg/100 ml potato
30	5.8	532	1 240	137	1 909
31	5.8	598	1 255	68	1 921
32	5.8	633	1 295	45	1 973
33	5.8	651	1 298	25	1 974
34	5.8	556	1 319	50	1 935
35	5.8	532	1 335	55	1 922
36	5.8	518	1 349	39	1 897
37	5.8	503	1 336	0	1 839
38	5.8	387	1 330	50	1 767
39	5.8	308	1 300	10	1 618
	5.8	319	1 280	0	1 599

* The initial pH value of the medium was adjusted to 6.0

The acetone–butanol fermentation was greatly affected by temperature (Table 8). The suitable temperature for the fermentation process was 32—33°C, above which a decrease in the total yield was recorded. The final products were mainly butanol and acetone. The initial pH value of the fermentation medium was adjusted to 6.0 and at the end of the fermentation process, it was found to be 5.8.

The acetone–butanol fermentation was greatly affected by the incubation period as well (Table 9). The total yields increased with the increase of the incubation period, reaching an optimum at 96—120 h. The initial pH value (pH = 6.0) of the fermentation medium fell to 5.4—5.8.

Table 9

Influence of the incubation period on the fermentative production of acetone–butanol by C. acetobutylicum

Incubation period (hr)	Final pH*	Acetone, mg/100 ml potato	*n*-Butanol, mg/100 ml potato	Ethanol, mg/100 ml potato	Total yield, mg/100 ml potato
24	5.4	208	730	55	993
48	5.5	324	969	50	1 343
72	5.7	556	1 349	30	1 936
96	5.8	663	1 300	10	1 973
120	5.8	643	1 325	10	1 978

* The intial pH value of the medium was adjusted to 6.0

Discussion

The acetone–butanol production by *C. acetobutylicum* was shown to be determined by certain factors, viz., the carbon and nitrogen sources in the fermentation medium, their suitable concentrations, calcium carbonate, initial pH value of the medium, temperature and the incubation period.

Fourteen media were used for the fermentative production of acetone–butanol by *C. acetobutylicum*. Medium I was suitable for the production. Any of soluble starch, sucrose, maltose, glucose, molasses, mannose, fructose, glycerol and lactose was a good carbon source for the production of acetone. Soluble starch was the most favourable carbon source for the total yield of solvents (acetone + butanol + ethanol). The best concentrations of potato starch and soluble starch favouring the total yield were 500.0 and 10.0 g/l, respectively. Peptone was the best nitrogen source favouring the production of acetone–butanol. The suitable concentration of peptone was 4.0 g/l. The presence of calcium carbonate in the fermentation medium buffered the acidic organic compounds liberated during the fermentative production of acetone–butanol and this led to an increase in the total yield, the best concen-

tration of calcium carbonate being 3.6 g/l. The best initial pH value and temperature of the fermentation of medium favouring the production of acetone-butanol was 6.0 and 32—33°C, respectively. The fermentation process required 120 h to obtain maximum yields of acetone–butanol.

Acetone-butanol fermentation by microorganisms is an economic procedure, especially in countries where the raw materials are available. Some authors claim that the microbiological production is more costly than the production of the two solvents (acetone and butanol) by petrochemical industries. Actually, the countries which have raw agricultural by-products can produce the two solvents economically by fermentation. In one day, however, the crude petroleum will be exhausted, while the microbiological production may be flourished and continuously used.

The *n*-butyl alcohol is used in a number of industrial processes, ranging from production of lacquers to extraction of antibiotics from media. Urea-formaldehyde resins and amines are also manufactured using butanol. Although the total annual output of butanol in the U.S.A. has increased steadily in recent years, the contribution made by butanol fermentation to this total output is diminishing. It is probable that little butanol is now produced by fermentation either in the U.S.A. or in Great Britain. In the first place, competition from butanol produced in the petrochemical industry has undoubtedly made deep inroads into a market hitherto held by fermentation butanol. A second argument against the fermentation industry has been the steep rise in the price of molasses during recent years. However, these remarks apply mainly to the production of butanol fermentation in countries of the western world, for it is possible that Asian and African countries which do not have large petrochemical industries may still produce butanol by fermentation at a price which can compete with the synthetic products. The economic position of acetone–butanol fermentation is somewhat similar to that of the alcohol fermentation industry.

Despite many years of study and research, no entirely satisfactory way has yet been found for increasing the low yields of butanol in this fermentation or for controlling the proportions of acetone and butanol in the product. Since the fermentation has been operated primarily as a means for producing butanol, many attempts have been made to reduce the production of acetone, in favour of butanol. Some success has been achieved by selecting strains of *C. acetobutylicum* that produce only 20—25% acetone. Attempts have also been made to reduce the ratio acetone to butanol in culture. Thus, addition of radox dyes has been claimed to change the solvent ratio, and adding 0.1% neutral red increases the proportion of butanol by about 25% at the expense of acetone.

One of the major disadvantages associated with the acetone–butanol fermentation is the low yield of solvents obtained, this being, to some extent,

a reflection of the inability of the bacterium to tolerate concentrations of butanol exceeding 1.3%. Using media containing 6% sugar, it is possible to obtain yields of approximately 2% mixed solvents. Attempts have been made to find ways of overcoming this by using bacterial strains tolerating higher concentrations of butanol. Selection of mutants after ultraviolet irradiation and adaptation of cultures have, on the whole, proved unsuccessful, but slightly greater tolerance was obtained by using inocula containing large numbers of actively growing bacteria. Bacterial cultures used in the industrial processes are invariably maintained in soil or sand. The inoculum for seeding the large batches of media is grown anaerobically in successive volumes of medium with the soil culture.

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EFFECT OF ENDOTOXIN ON THE PERITONEAL MAST CELLS IN MICE

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Abstract

Endotoxin administered intraperitoneally has been found to decrease the absolute and relative number of the peritoneal mast cells in mice. The number of peritoneal mast cells showed a dose-dependent biphasic response to endotoxin. Maximal and more consistent decrease in the peritoneal mast cell count was observed at about 24 h and lasted for at least 4 days. Mast cell damage also occurred. The possible mechanism of this phenomenon is discussed.

Introduction

In a previous paper [18] we reported on the endotoxin-induced cellular and permeability changes in the peritoneal cavity of mice. In this communication data are presented referring to the number of mast cells (MCs) after endotoxin (ET) administration.

Material and method

E. coli O 86 endotoxin prepared according to WESTPHAL's method [29] was injected i.p. to groups of randomly bred male BALB/c mice. Saline was given to control mice of the same age. At the time indicated, the mice were anesthesized with ether (5 min), then 3 ml of buffered salt solution (0.05 mol/l phosphate buffer pH 7.0; 0.38% Na-citrate, 0.58% NaCl, 1 mg/100 ml phenol red) was injected i.p. After gentle massage the peritoneum was exposed and the peritoneal fluid was sucked off with a Pasteur pipette.

For counting the MCs a fivefold dilution of the peritoneal fluid was prepared with buffered saline containing 10% inactivated normal rabbit serum. Five microliters of these cell suspensions were placed on microscope slides with an automatic micropipette. The drops were dried at 37°C, then fixed immediately in Carnoy's solution. Alcian blue–safranin staining [26] was performed according to BALOGH's modification as described by RÖHLICH and CSABA [22].

The absolute number of apparently intact MCs in the drop (5 μl) was counted at a magnification of 750, and calculated for 3 ml volume.

Of these cells only few (less than 10%) had alcian blue-positive granules, the great majority of cells was safranin-positive (mature).

The rate of MC damage was determined from Giemsa-stained smears. For this purpose the cells were centrifuged (100 *g* for 15 min), suspended in one drop of normal rabbit serum and smeared on microscope slides. The number of non-intact MCs (including predegranulation) as a percentage of total number of MCs was calculated.

Results

Figure 1 shows that after the administration of 25 μg ET, the peritoneal MC count decreased in the first five hours, then a rise was observed up to the 10th h. This was followed by a marked fall at 24 h, lasting for several days.

It is remarkable that in the early hours following ET injection an inverse dose-response relationship was observed, so that, e.g., 100 μg of ET may even increase the number of MCs in the peritoneal cavity (Table 1). A more

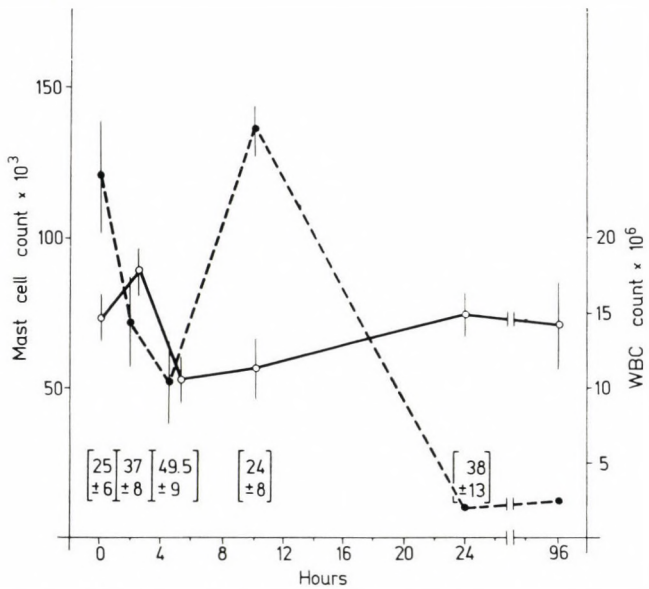

Fig. 1. The effect of 25 μg endotoxin on the peritoneal mast cell count. ●----● MC-count ○——○ WBC-count; the numbers in parentheses show the percentage of non-intact mast cells Mean ± SE; 5 mice per group

Table 1

*The effect of graded doses of endotoxin on the peritoneal mast cell count**

Treatment	5 hours		24 hours	
	MC × 10^3	WBC × 10^6	MC × 10^3	WBC × 10^6
Saline	128 ± 12	13.4 ± 0.9	118.5 ± 12	14.1 ± 1.8
ET 10 μg	77.5 ± 18	13.0 ± 2.9	83.2 ± 17	17.7 ± 2.7
ET 25 μg	94 ± 16	13.6 ± 1.3	53.6 ± 10	15.1 ± 2.5
ET 100 μg	167.5 ± 58	13.5 ± 2.7	43.8 ± 3	8.4 ± 2.1

* 6 mice per group: mean ± SE

consistent and pronounced fall in the absolute and relative number of the peritoneal MCs was observed at 24 h. This decrease seemed to be independent of age (Table 2).

Table 2

The effect of endotoxin on the peritoneal mast cell count in mice of various age*

Age in weeks	Treatment	Mast cell count $\times 10^3$	Total WBC count $\times 10^6$
3— 4	Saline	54.5 ± 7.9	4.06 ± 0.55
	ET	23.2 ± 13.0	8.07 ± 1.65
5— 6	Saline	83.1 ± 11.6	11.47 ± 1.52
	ET	26.7 ± 1.7	12.15 ± 1.23
11—12	Saline	157.0 ± 17.0	12.40 ± 0.80
	ET	45.3 ± 5.8	14.90 ± 1.50
16—17	Saline	196.0 ± 22.0	19.40 ± 2.30
	ET	36.1 ± 8.4	18.10 ± 2.70

* 24 h after the injection of 25 μg ET; mean ± SE; 5—6 mice per group

The progressive increase in the percentage of degranulated and non-intact MCs up to the 5th h following ET injection is shown in Fig. 1. The signs of degranulation compared with the controls are still visible at 24 h. Thus the decrease in the number of MCs is due first of all to MC damage.

Discussion

Our results are in good accordance with the data of URBASCHEK [27], who observed degranulation of the mesenteric MCs following i.v. administered ET in species other than mouse. It seems to be obvious that, unlike antigen–antibody reaction involving IgE antibody, ET has no direct effects [2, 3, 14] on MCs and its effect is prolonged in time.

The damage of MCs as a result of ET administration may be interpreted as follows.

ET activates complement [12, 13] thus generating a series of complement-derived and other factors, e.g., chemotactic [4, 25, 28], permeability [21] factors, and anaphylatoxins [10, 19, 20]. In consequence of these events membrane labilization [16] and cytotoxic effects [6, 15] occur. As MCs are known to be the most sensitive cell type to a variety of noxious agents, they may be the first to react with harmful stimuli, thus initiating or enhancing the inflammatory process. There is a number of substances in the organism

which are known to act selectively on MCs. Among these are some cationic macromolecules: the complement-derived anaphylatoxins [10, 19, 20], the cationic proteins form the PMN-lysosomes [17] and nuclear histones released from the destroyed cells.

The latter two substances might act mainly at a later stage, while anaphylatoxin formation is thought to be the primary event leading to MC damage. The release of biogenic amines and the visible MC damage may be two consecutive steps depending on the concentration of the disruptor agents [24].

The rather surprising finding that at 10 h after ET injection neither the MC number nor the peritoneal cell picture differ from the controls significantly, merits attention. The mechanism of the rise in MC count after the 5th hour is not entirely clear, but in all probability it is due to contraregulatory processes.

ET is a powerful stimulator of the secretion of glucocorticoids [1] and the latter are known to influence the generation, disribution and maturation of MCs [7, 8, 9]. Although some relevant data are still lacking, we suggest the following interpretation for the above phenomenon.

Besides the events leading to MC destruction, ET, or rather some tissue products, stimulate the ACTH-glucocorticoid secretion [1]. The raised glucocorticoid level would mobilize new MCs, presumably from the thymus [8, 9]. As at the same time permeability and/or lymph circulation is also increased [18], the cell population of the peritoneal fluid would exchange with mostly intact cells coming along with the blood stream or lymph about 10 h after ET administration. As this contraregulatory process is limited in duration, the great number of new MCs would also fall victim to the destroying effect of substances induced by ET.

As recirculation is a prerequisite of the above explanation, the decrease in the number of MCs in the peritoneal fluid may be a reflexion of the decrease in the total easily mobilizable MC pool.

Apart from the role in the inflammatory process, little is known about the significance of the ET-induced MC damage. It can only be hypothesized that the liberation of some MC constituents and the granule phagocytosis may have a role in the restitution, proliferation [11, 23] and increase in activity [5] of the mononuclear-phagocytic and lymphoid system after ET injection.

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SYNERGISTIC ANTIFIBRINOLYTIC ACTION OF THE POTATO PROTEASE INHIBITOR AND E-AMINOCAPROIC ACID

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Abstract

Fibrinolysis inhibition by a mixture of potato inhibitor and E-aminocaproic acid was greater than might be expected from the sum of the antifibrinolytic effects of these inhibitors investigated separately. This inhibition was observed in studies on the plasma euglobulin fraction and in a system containing isolated elements of the fibrinolytic system. The synergistic antifibrinolytic action of the potato protease in hibitor and E-aminocaproic acid is probably due to the fact that these inhibitors have different mechanisms of action and thus there is no competition between them for the effectors in the enzyme molecule.

Introduction

Two types of fibrinolysis inhibitors are distinguished by their different mechanisms of antifibrinolytic action. Synthetic fibrinolysis inhibitors, such as E-aminocaproic acid (EACA), trans-4-aminomethyl cyclohexane carboxylic acid (AMCHA) and *p*-aminomethyl benzoic acid (PAMBA), block only the specific binding site of plasmin with fibrin, without blocking the active centre of that enzyme and reducing its proteolytic effect [1, 7, 9]. Natural polypeptide protease inhibitors, which include the potato inhibitor, block the active centre of proteolytic enzymes, thus preventing their proteolytic and fibrinolytic action [5, 12].

It has been found that a mixture of different agents which bring about the same biological effect but differ in their mechanism of action frequently shows synergistic effects [3, 11].

It might therefore be presumed that a mixture of different fibrinolysis inhibitors which do not compete between themselves for the receptors of the enzyme would have a greater antifibrinolytic effect than the sum of the antifibrinolytic effects of each of the components of the mixture.

Material and method

The potato protease inhibitor (PPI) was obtained by the method previously described [12].

E-aminocaproic acid (EACA), produced by "Ziołolek", Poznan, Poland.

Dog plasma euglobulin fraction was obtained by the isoelectric precipitation method as described by Kowalski and co-workers [6].

Plasmin (EC 3.4.4.14), Novo Industry A/S, Copenhagen, Denmark.

Thrombin (EC 3.4.4.13), the Lublin Serum and Vaccine Laboratories, Lublin, Poland, expressed in NIH units [10].

Bovine fibrinogen, free from plasminogen and fibrin-stabilizing factor (factor XIII), was obtained by the method of KEKWICK and co-workers [4].

Palitsch's borate buffer at pH 7.6 was used as solvent for all the reagents.

The antifibrinolytic effect of the potato protease inhibitor and E-aminocaproic acid was investigated on a plasma euglobulin fraction (A) and by means of purified elements of the fibrinolytic system (B). The investigations were carried out with two systems. In system I the effect of single inhibitors was studied and in system II the effect of a mixture of inhibitors was determined.

(A) In the investigations on the euglobulins in system I, 0.25 ml PPI (20, 40, 80 and 160 μg/ml) or EACA (1.6, 3.2, 6.4 and 12.8 mM/l) was added to 0.5 ml euglobulin; in system II, 0.25 ml of a PPI and EACA mixture, in which the concentration of the single inhibitors was the same as in system I, was added to 0.5 ml euglobulin and in the control experiment 0.25 ml borate buffer was added to 0.5 ml euglobulin. After 3 min incubation at laboratory temperature, 0.25 ml $CaCl_2$ (0.025 mol/l) was added, and the fibrinolysis time in a water bath at 37°C was determined.

(B) In the experiments on the purified elements of the fibrinolytic system in system I, 0.25 ml PPI or EACA was added to 0.25 ml plasmin (0.004%), in system II, 0.25 ml mixture of these inhibitors, in the same concentrations as used in the experiments with euglobulins, was added to 0.25 ml plasmin and in the control experiments 0.25 ml of borate buffer was added. After 3 min incubation at laboratory temperature, 0.25 ml fibrinogen (0.2%) and 0.25 ml thrombin (10 u/ml) were added to both systems and fibrinolysis time was determined.

Results and discussion

The inhibition of euglobulin fibrinolytic activity by the concentrations of PPI and EACA used in these experiments was of the same order, whereas in the systems with purified elements greater antifibrinolytic effects were obtained with PPI (Fig. 1). The fibrinolysis inhibition by a mixture of PPI and EACA is much greater than might be expected from the sum of the antifibrinolytic effects of the same concentrations of both inhibitors investigated separately. This effect was observed in the euglobulin fraction and to a lesser extent in the purified systems, being particularly marked when high concentrations of these inhibitors were used.

The synergistic antifibrinolytic action of PPI and EACA probably occurs because these fibrinolytic inhibitors have different points on which they exert their effect, the former blocking the active centre of plasmin and the latter causing the formation of ineffective acceptors for plasmin binding-site on the peptide chains of the fibrin molecule. The presence of both inhibitors, PPI and EACA, at the same time probably raises the stability of complexes of these inhibitors with plasmin or causes deeper conformation changes in that enzyme.

The stronger antifibrinolytic effect of EACA, both alone and in mixtures with PPI, in the euglobulins than purified systems is in all probability due to the fact that EACA shields stabilized fibrin from the effects of plasmin better than it shields unstabilized fibrin [8, 9].

A synergistic antifibrinolytic effect has also been observed in investigations on a mixture of EACA and the trypsin inhibitor isolated from urine [2].

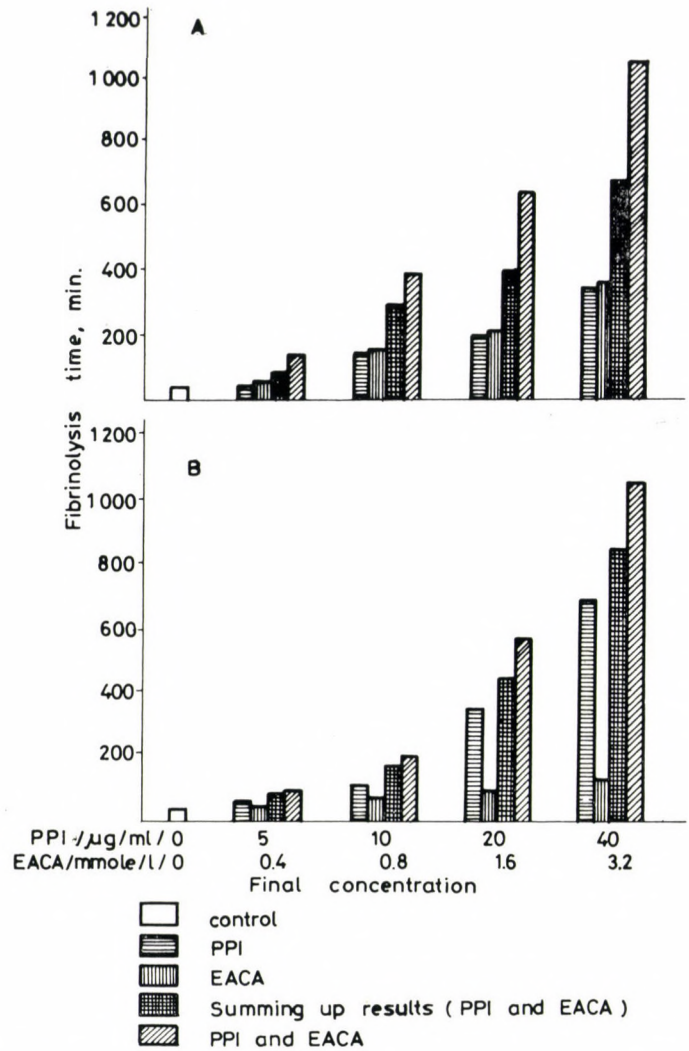

Fig. 1. The antifibrinolytic effect of the potato protease inhibitor (PPI) and E-aminocaproic acid (EACA) and a mixture of the two inhibitors tested on a plasma euglobulin fraction (A) and in a purified system (B). The mean values of three determinations are given

The fibrinolytic inhibitors are widely used in the treatment of fibrinolytic haemorrhagic diatheses. One of the chief disadvantages in their practical application is that large and frequently repeated doses are necessary in order to maintain an effective level of these inhibitors. It may therefore be presumed that the administration of a mixture of inhibitors, with its synergistic antifibrinolytic effect, would give a therapeutic result with smaller doses.

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EFFECT OF PREDNISOLONE ON THE GLYCOSAMINOGLYCAN COMPONENTS OF THE REGENERATING ARTICULAR CARTILAGE

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Abstract

Investigations were performed on the effect of prednisolone (0.5 mg/kg) on the regenerating femoral articular cartilage of the knee joint in dogs that had been subjected to semiarthroplasty. After 70 days of prednisolone treatment the dogs were killed and the regenerating articular cartilage was removed, minced, and dried with acetone. The acetone-dried material was used for the determination of galactosamine, glucosamine, uronic acid, sulphate, sialic acid and hydroxyproline. Prednisolone treatment elicited a quantitative increase in galactosamine (30.2 %), uronic acid (76.2%), and sulphate (9.1%), while no difference was observed in sialic acid content between the treated and untreated groups. From the molar ratio of the measured components it appears that prednisolone produced an increase in chondroitin sulphate and hyaluronic acid, and a decrease in the keratosulphate content of cartilage. By comparing the values measured in the regenerating articular cartilage of control and prednisolone-treated dogs with the values obtained in the mature articular cartilage, we may conclude that prednisolone — at least as regards the glycosaminoglycans of the ground substance — exerts an accelerating effect on cartilage regeneration.

Introduction

The effect of prednisolone on the regenerating articular cartilage was studied in previous works by histological and histochemical techniques [5] and by determining the hexosamine and uronic acid contents [6]. From these investigations we have concluded that 70 days after semiarthroplasty the regenerating cartilage tissue of prednisolone-treated animals contains mainly young chondrocytes embedded in a fibrous ground substance. Biochemically, the progress of regeneration may be characterized by a rise in the glycosaminoglycan (GAG) content of cartilage [10]. The hexosamine and uronic racid values were higher in the prednisolone-treated than in the control regenerating cartilages. In the present work we have performed investigations on other GAG components and on the hydroxyproline content of the regenerating articular cartilage.

Material and method

Semiarthroplasty according to KROMPECHER [7] was performed in the left knee joints of 32 dogs. The operation consisted of removal of the cartilaginous surface of the distal part of the femur including some mm of the spongiosa and moulding a new articular surface. After

operation the dogs were divided in two groups of 16 each. The animals of Group I were given prednisolone orally, in daily doses of 0.5 mg/kg. Group II comprised the controls.

Seventy days after the surgical intervention the dogs were killed and the newly-formed regenerating cartilage tissue was removed from the articular surface. The removed tissue was minced thoroughly, washed in acetone and dried. The acetone-dried material was used for the determinations. The same procedure was applied to the intact articular cartilage of untreated dogs.

Hexosamine content and the galactosamine/glucosamine ratio were determined as described by LUDOWIEG and BENMAMAN [9]. For the determination of uronic acid, sulphate and sialic acid DISCHE's [3], DODGSON and PRICE's [4] and SVENNERHOLM's [13] procedures, respectively, were used. Hydroxyproline was determined according to STEGEMANN and STALDER's [12] method and the N content by Kjeldahl's method.

The chemicals used for the determinations were products of different factories; viz., Fluka (glucosamine, acetylacetone, Dowex ion exchange resins); British Drug Houses Ltd. (galactosamine, carbazole and glucuronic acid); Merck (chloramine T), and Reanal (all the other compounds).

Results

The quantitative changes due to prednisolone in the GAG components of the regenerating articular cartilage as compared with the controls are shown in Figs 1 and 2. The total hexosamine content increased by 20.6% (control: 1.06 ± 0.08 g/100 g; prednisolone-treated: 1.28 ± 0.13 g/100 g).

Within the total hexosamine, the galactosamine content augmented by 30.2% (control: 0.63 ± 0.08 g/100 g; prednisolone-treated: 0.82 ± 0.11 g/100 g). In the glucosamine content the increase was 14.2% (control: 0.43 ± ± 0.04 g/100 g; prednisolone-treated: 0.49 ± 0.04 g/100 g). The greatest increase was shown in the uronic acid content: 76.2% (control: 0.76 ± 0.07 g/100 g; prednisolone-treated: 1.34 ± 0.18 g/100 g). The rise in sulphate content

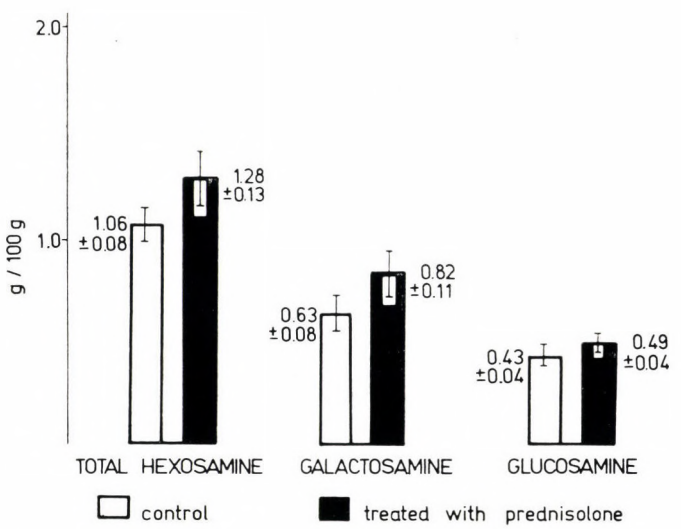

Fig. 1. Total hexosamine, galactosamine and glucosamine contents of the regenerating articular cartilage in control and prednisolone-treated dogs (g/100g dry tissue)

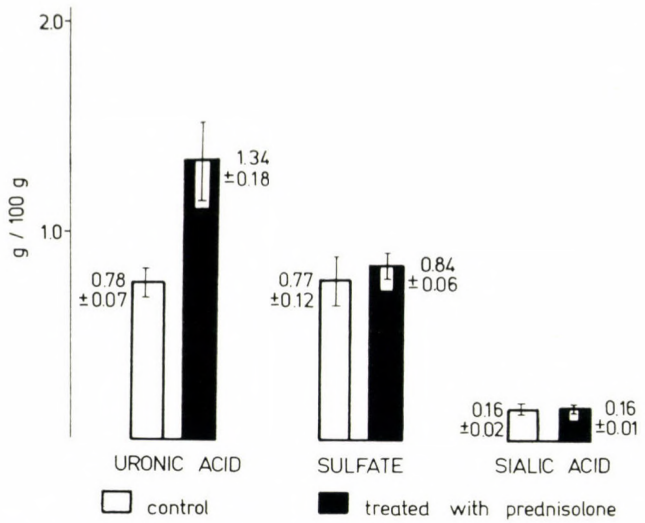

Fig. 2. Uronic acid, sulphate and sialic acid contents of the regenerating articular cartilage in control and prednisolone-treated dogs (g/100 g dry tissue)

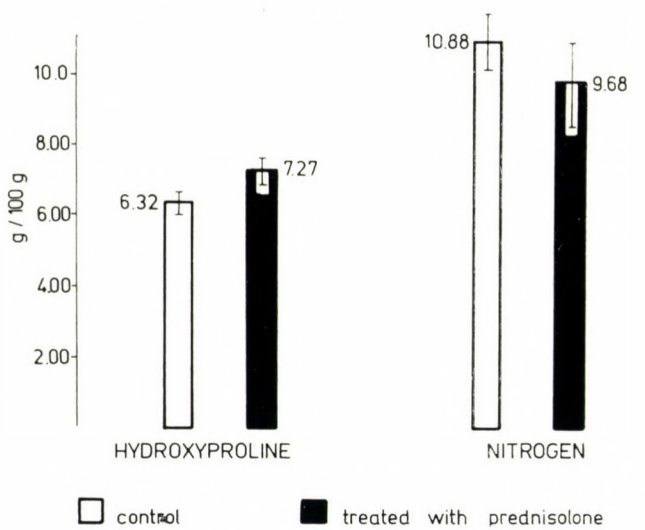

Fig. 3. Hydroxyproline and nitrogen content of the regenerating articular cartilage in control and prednisolone-treated dogs (g/100 g dry tissue)

was only 9.1% (control: 0.77 ± 0.12 g/100 g; prednisolone-treated: 0.84 ± 0.06 g/100 g). No change was noted in the sialic acid content: a value of 0.16 ± 0.02 g/100 g was measured in both groups.

The values of hydroxyproline and nitrogen are shown in Fig. 3. Hydroxyproline augmented by 16.6% in the treated material (control: 6.32 ± 0.27

g/100 g; prednisolone-treated: 7.27 ± 0.35 g/100 g), while nitrogen decreased by 11.3% (control: 10.88 g/100 g; prednisolone-treated: 9.68 g/100 g).

On prednisolone treatment the sulphate/hexosamine ratio decreased from 0.73 to 0.66, and the sulphate/uronic acid ratio from 1.01 to 0.63.

Discussion

From the results we have concluded that prednisolone treatment elicited a rise in the GAG content of the regenerating articular cartilage. From the changes of the molar ratio of the different GAG components we may infer to the change in the disribution of the different GAGs. On this basis, we have determined the distribution of hexosamines in the different GAGs measured in control and prednisolone treated regenerating cartilages and in the intact articular cartilage (Table 1).

The galactosamine corresponding to chondroitin sulphates was found to be 3.5 μmol in the untreated regenerating, 4.6 μmol in prednisolone-treated regenerating, and 9.2 μmol in the intact mature cartilage. The uronic acid non-

Table 1

Distribution of glycosaminoglycan components of regenerating articular cartilages in control and prednisolone-treated dogs, and in intact articular cartilages of untreated dogs

Glycosaminoglycan components	Regenerating articular cartilage				Intact articular cartilage	
	control		prednisolone-treated			
	g/100 g	μmol/100 g	g/100 g	μmol/100 g	g/100 g	μmol/100 g
Total hexosamine	1.06	5.9	1.28	7.1	2.76	15.4
Galactosamine	0.63	3.5	0.82	4.6	1.65	9.2
Glucosamine	0.43	2.4	0.49	2.5	1.11	6.2
Uronic acid	0.76	3.9	1.34	6.9	2.34	12.1
Sulphate	0.77		0.84		2.36	
Hydroxyproline	6.32		7.27		7.20	
Sialic acid	0.16		0.16		0.43	
Sulphate/uronic acid	1.01		0.63		1.01	
Sulphate/hexosamine	0.73		0.66		0.85	
Galactosamine+uronic acid, CS		3.5		4.6		9.2
Uronic acid+glucosamine, HY		0.4		2.3		2.9
Other glucosamines, Ks, GP		2.0		0.2		3.3

The values are calculated for 100 g acetone-dried tissue; CS = chondroitin sulphate, HY = hyaluronic acid, Ks = keratosulphate, GP = glycoprotein

bound to galactosamine, probably corresponding to hyaluronic acid, was 0.4 μmol, 2.3 μmol, and 2.9 μmol, respectively. Thus, on prednisolone treatment the hyaluronic acid content increased about sixfold. At the same time glucosamine non-bound to uronic acid fell to about the tenth of the original amount. One part of glucosamine is the component of keratosulphate and the other part, together with the sialic acid, may contribute to the formation of glycoproteins. According to our measurements no change occurred in sialic acid (and presumably in glycoprotein), therefore the marked fall in the glucosamine content seems to be attributable to a considerable decrease in keratosulphates. This may also explain the increase in per cent of the sulphate content which was less than that of chondroitin sulphate. It is unlikely that sulphatation of keratosulphate would have decreased to such an extent, therefore we are inclined to think that while in the control cartilage the sulphate content derives from chondroitin sulphate and keratosulphate in almost equal amounts, in the regenerating cartilage of prednisolone-treated animals the somewhat higher total sulphate content belongs almost entirely to the accumulated chondroitin sulphate, since the keratosulphate content of the tissue is very low.

Concerning the effect of glycocorticoids on the GAG-sulphate metabolism there are divergent data in the literature. Some authors found a decreased sulphate uptake [1, 8], others an increased sulphate incorporation [2]. According to our observations prednisolone treatment elicited a slight rise in the sulphate content. At the same time chondroitin sulphate and hyaluronic acid increased, whereas keratosulphate decreased in quantity. From this it may be concluded that here a transformation of sulphates occurs. In agreement with the data of SCHILLER and co-workers [11] this seems to indicate that prednisolone acts diversely on the different GAGs, even in the same tissue. The divergencies in the results obtained by the different authors are probably due to differences in the experimental material, preparations employed, dosage, methods and other conditions.

From our results it may be concluded that on prednisolone treatment the chondroitin sulphate and hyaluronic acid content of the regenerating articular cartilage increases and — at least as far as the GAG components of the ground substance are concerned — it exerts an accelerating effect on cartilage regeneration.

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HISTOMORPHOLOGY AND PROTEOLYTIC ACTIVITY IN THE GASTRIC APPARATUS OF FRUGIVOROUS, CARNIVOROUS AND OMNIVOROUS SPECIES OF BIRDS

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Abstract

The histomorphology of the gastric apparatus, the pepsin level and the optimum pH for pepsin were investigated in *Psittacula krameri* (frugivore), *Lanius schach* (carnivore) and *Acridotheres tristis* (omnivore) species of birds. The proventricular glands were found to be made up of oxynticopeptic cells. The lobules of the oxynticopeptic cells are polyhedral; they are the largest in *P. krameri*, and the smallest in *A. tristis*. However, their greater number in *A. tristis* enables a higher secretion of hydrochloric acid and pepsin. The villi are more developed in *A. tristis* than in *L. schach* and *P. krameri*. The gizzard is larger in *A. tristis* than in *P. krameri* and *L. schach*. The circular muscle in gizzard is more developed in *P. krameri* and *A. tristis* than in the carnivore *L. schach*. Koilin lining is beset with horny cones, which were well developed in *A. tristis*, moderately developed in *P. krameri* and absent in *L. schach*.

The pepsin activity is higher in the proventriculus of the carnivorous *L. schach* and the omnivorous *A. tristis* than in the frugivorous *P. krameri*. Slight pepsin activity was also observed in gizzard tissue extracts in all the three species. The optimum pH for pepsin was found to be 1.5 for *P. krameri* and 1.8 for both *L. schach* and *A. tristis*.

Introduction

CLARA [4] discovered the oxynticopeptic cells in the proventriculus of thrushes. The proventicular glands are multilobular in galliform birds [3] and unilobular in ducks [22]. The variability in form and size of gizzard has shown a close relationship with the type of food [18]. Histology of stomach has been studied by STEINBACHER [21] in *Psittaculadae*, passerines [17] and in *Gallus domesticus* [24]. The influence of diet on musculature in gizzard of granivorous and omnivorous species has been investigated by LENKEIT [15] and BROUSSY [1]. Their koilin lining has been the subject of thorough investigations.

Information on the pH value and pepsin activity in the gastric apparatus of birds is very limited. Presence of acid and pepsin in gastric juices was demonstrated in pigeons by TIEDEMANN and GMELIN [23] and in chicken by HEWITT and SCHELKOPF [13]. HERPOL [9] observed higher pepsin levels in the proventriculus of carnivores than in the granivorous birds.

Since comparative account on this particular aspect of the digestive system of herbivorous (frugivorous), carnivorous and omnivorous birds

does not seem to be sufficient to draw definite conclusion, an attempt was made to investigate the histomorphological peculiarities and pepsin level with its pH optima in the gastric apparatus of three species of birds having diverse food habits.

The birds selected for this study include *Psittacula krameri* SCOPOLI (fam: Psittaculidae), *Lanius schach* LINN. (fam: Lanidae) and *Acridotheres tirstis* LINN. (fam: Sturnidae). *P. krameri* (parrot) is a very common Indian frugivorous (herbivorous) bird and *L. schach* (rufous-backed shrike) is a carnivorous bird, feeding upon lizards, young amphibians and large insects. *A. tristis* (desi myna) is an omnivorous bird, feeding mainly upon grains, earthworms and kitchen scraps [20].

Material and method

Specimens of *P. krameri*, *L. schach* and *A. tristis* were collected, and maintained in the laboratory on their normal diets. For the morphological and histological studies, a few specimens were dissected and the proventriculus and gizzards were collected. For histological studies the proventriculus was fixed in alcoholic Bouin's (Duboscq—Brasil) fluid [14]. The gizzard was fixed in a slightly modified aqueous Bouin's fluid (formalin, 60.0 ml; 5% potassium hydroxide, 15.0 ml; and aqueous 1% picric acid, saturated, 15.0 ml).

Dehydration, clearing and embedding were carried out as usual. Serial sections of 5—7 μm thickness were cut, and stained with Heidenhain's iron hematoxylin or Delafield's hematoxylin and counterstained with eosine or orange G. The stained sections were microphotographed.

Preparation of tissue extracts for enzyme assay. From another set of starved birds the proventriculus and the gizzard were removed immediately after dissection and washed thoroughly with double glass-distilled water. Each tissue was homogenized in 5.0 ml of triple glass-distilled ice-cold water in a mortar and pestle with the aid of acid- and alkali-free sand under cold conditions. The homogenate was then centrifuged for 10 to 15 min at 3 000 rev/min. The supernatant fluids were collected in test tubes and kept at 0°C.

Assay of pepsin and its optimum pH. We estimated the activity of pepsin by the method of HERRIOTT [12] using haemoglobin as substrate, with some modification. 2% solution of haemoglobin was adjusted to pH 1.0, 1.5, 1.8, 2.0, 2.5 and 3.0 by 0.005 to 0.08 N HCl; 2.5 ml of substrate and 0.5 ml of tissue extract were pipetted in a tube and incubated at 37°C for 10 min. After incubation, 5 ml 5% trichloroacetic acid (TCA) was added and the precipitated protein was removed by filtration. To 2.5 ml of the filtrate 5 ml 0.5 N NaOH and 1.5 ml diluted Folin phenol reagent were added, slowly with constant agitation. The blue colour that had developed was read after 5 min in a Bausch and Lomb Spectronic—20 at 660 nm. The colour value was expressed in milliequivalents of tyrosine, using 10^{-3} mol/l tyrosine in 0.2 N HCl as standard. A haemoglobin-enzyme blank was prepared by adding TCA before the tissue extracts.

Unit. One unit of proteinase was defined as "the amount which digests haemoglobin under the standard conditions, at an initial resulting in the liberation, per minute, of an amount of split products, not precipitable with TCA, which gives the same colour with the phenol reagent as 1 mEq of tyrosine".

One, 2, 3, 4, 5 and 8×10^{-4} pepsin units correspond to the release of 1.8, 3.5, 5.0, 6.3, and 11×10^{-4} mEq tyrosine, respectively, into 5 ml of TCA filtrate over and above the blank.

Estimation of protein and calculation of specific activity. The procedure followed for protein estimation was that of Lowry et al. [16]. Bovine serum albumin (Sigma Chem., U. S. A.) was used as standard. The proteins in the tissue extracts were estimated by the above method and specific activity was expressed in terms of units/mg of protein.

Results

The gastric apparatus lies immediately behind the sternum in all the three species.

Proventriculus

In *P. krameri* (Fig. 1) proventriculus is a large bulbous glandular structure of about 3.0 cm length and 0.4 cm diameter. The posterior widest region, measuring about 0.8 cm in diameter, passes into the prominent gizzard through an orifice.

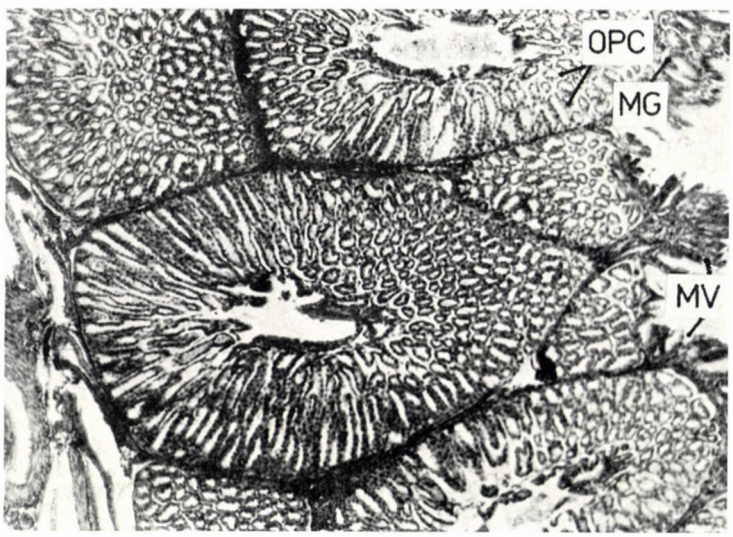

Fig. 1. Transversal section of the proventriculus of *P. krameri* oxynticopeptic cells (OPC) mucosal glands (MG), and mucosal villi (MV); ×68

The mucosa is composed of a single layer of columnar epithelial cells, projected into numerous microscopic villi. The proventricular glands are composed of lobules, which are tubular alveoli having oxynticopeptic cells, and extend deeply in the lamina propria communicating with the lumen through the primary and secondary ducts between the villi. In general appearance, these lobules seem polyhedral in shape, and measure 0.29—1.5 mm in length and 0.2—0.6 mm in width. They ranged from 21 to 25 in number, grouped together in the form of lobules around a common central lumen.

Its outermost layer (about 0.03 mm thick) is a serosa membrane of mesothelial cells. It is followed by the feebly developed muscularis externa, differentiated into an outer thin layer of longitudinal muscle fibres (about 0.068 mm thick) and an inner layer of circular muscle fibres (about 0.136 mm thick). The former was found in the form of small interrupted muscle bands. The muscularis mucosa is a thin and discontinuous layer of connective tissues that bind the lamina propria. The submucosa was very much reduced in volume.

In *L. schach* (Fig. 2) the proventriculus is a bulbous and glandular organ about 1.5 cm length and 0.5 cm in diameter. The mucosal epithelium is made

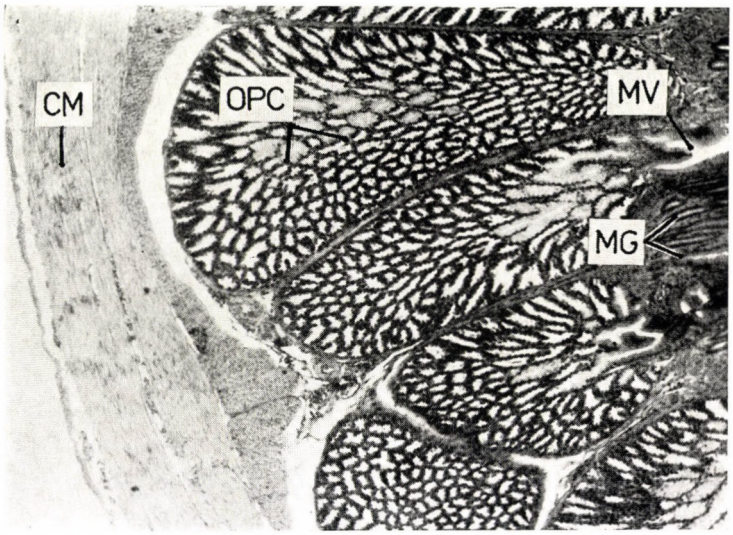

Fig. 2. Transversal section of the proventriculus of *L. schach.* Oxynticopeptic cells (OPC), mucosal glands (MG), mucosal villi (MV) and circular muscle layer (CM); ×68

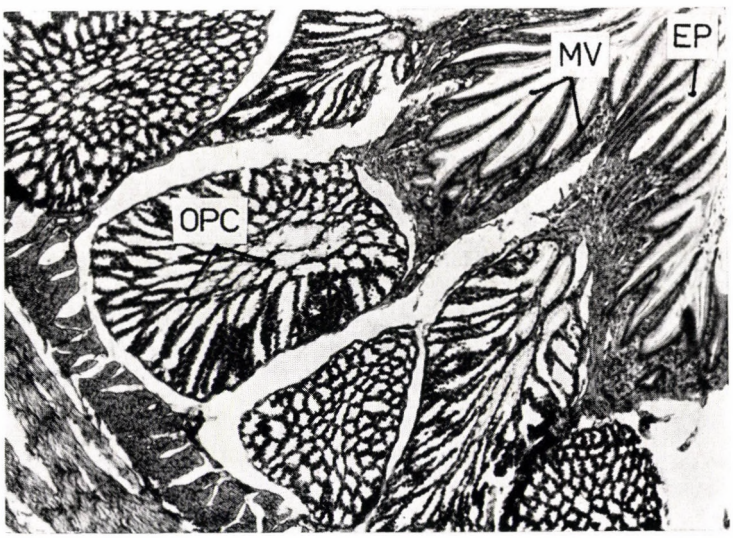

Fig. 3. Transversal section of the proventriculus of *A. tristis.* Oxynticopeptic cells (OPC), mucosal villi (MV) and epithelium (EP); ×68

up of a single layer of columnar epithelial cells, followed by a well developed lamina propria (about 1.5 mm thick), which is studded with elongated proventricular glands. The villi are moderate in size. The muscularis externa is more developed than in *P. krameri.* It is differentiated into an inner thick layer of circular muscle fibres (0.2 mm thick) and a poorly developed outer

layer of longitudinal muscle fibres (0.7 mm thick). The submucosa is distinct, having lymphatics and blood vessels.

The proventricular glands are compound tubuloalveoli of variable size and about 30 in number. The lobules are lined cuboidal epithelial (oxynticopeptic) cells. The collecting ducts of the lobular glands open into the central lumen.

In *A. tristis* (Fig. 3) the proventriculus is bulbous and glandular about 1.4 cm in length. The anterior region is 0.5 cm, while the posterior is about 0.7 cm in diameter.

The inner mucosal membrane is made up of single-layered cuboidal epithelial cells and thrown into numerous large villi of highly mucogenous nature. The well-developed lamina propria is packed with proventricular glands. These lobules are somewhat spheroidal, varying from 0.2 mm to 0.7 mm in length and are composed of oxynticopeptic cells. They are about 35 in number. Their ducts open into the central lumen. The muscularis externa, like in *L. schach*, is well differentiated in an inner (0.4 mm thick) layer of circular muscles and an outer layer of longitudinal muscles (0.07 mm thick). The submucosa is reduced in amount. The serosa (about 0.05 mm thick) was as usual.

Gizzard

In *P. krameri* the gizzard is a large blind muscular sac of about 1.9 cm diameter and 1.0 cm thick, lying on the right side at the junction of proventriculus and duodenum.

Histologically (Fig. 4), the mucosal layer is composed of columnar epithelial cells and projected into numerous folds. It has simple comb-shaped tubular glands of variable size, made up of cuboidal epithelial cells, which possess fine granules and a large nucleus. The elastic and collagen fibres were found near the bases of these glands. These glands are known to secrete the koilin lining. This layer including the glands was about 0.28 mm thick. The koilin is dense and provided with horny cones. The lamina propria (about 0.03 mm thick) is followed by poorly developed muscularis mucosa and submucosa. The outer longitudinal muscle layer of muscularis externa is reduced while the inner circular muscle layer is enormously developed (2.8 mm thick). The outermost covering is the serosa (about 0.05 mm thick) that has the thick tendons.

In *L. schach* (Fig. 5) the gizzard is a sac-like muscular structure (length 1.7 cm), measuring 1.3 cm in diameter and 0.9 cm in thickness. The koilin lining (0.15 mm thick) is dense and dark greenish with linearly arranged hard streaks. It is followed by a well-developed zone of elongated simple tubular (about 0.25 mm thick) glands, lined by cuboidal epithelial cells.

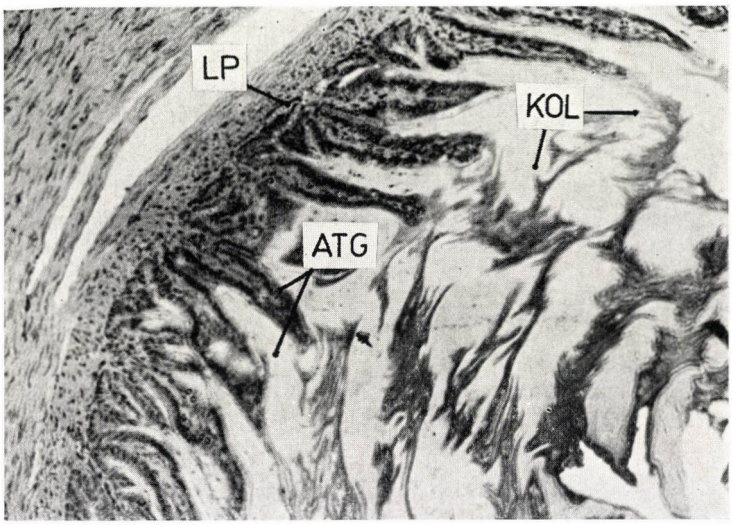

Fig. 4. Transversal section. Gizzard of *P. krameri.* Koilin (KOL), alveolo-tubular glands (ATG), lamina propria (LP) and circular muscle layer; ×170

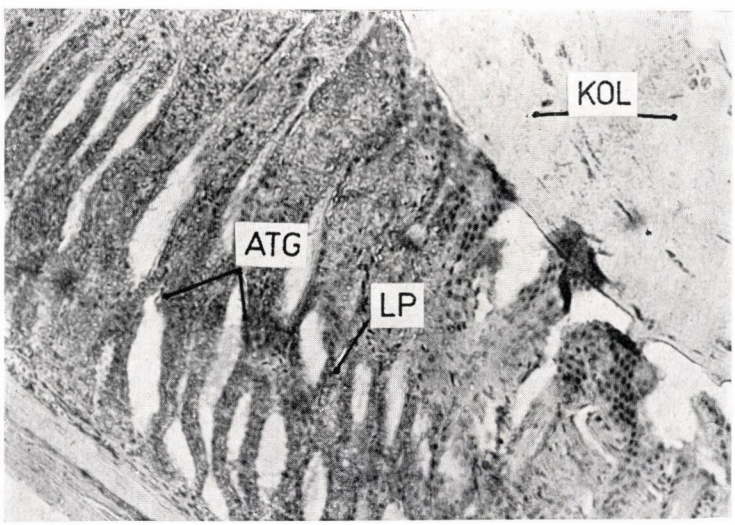

Fig. 5. Transversal section. Gizzard of *L. schach.* Koilin (KOL) without cones, alveolo-tubular glands (ATG), lamina propria (LP) and circular muscle layer; ×170

The lamina propria is poorly developed and muscularis mucosa is absent. The longitudinal muscle layer (2.0 mm thick) occupies its major portion.

In *A. tristis* (Fig. 6a) the gizzard is a long (2.4 cm in length), highly muscular sac measuring 1.8 cm in diameter and 1.2 cm in thickness. Its koilin lining (about 0.2 mm thick) is characterized by well-developed horny cones

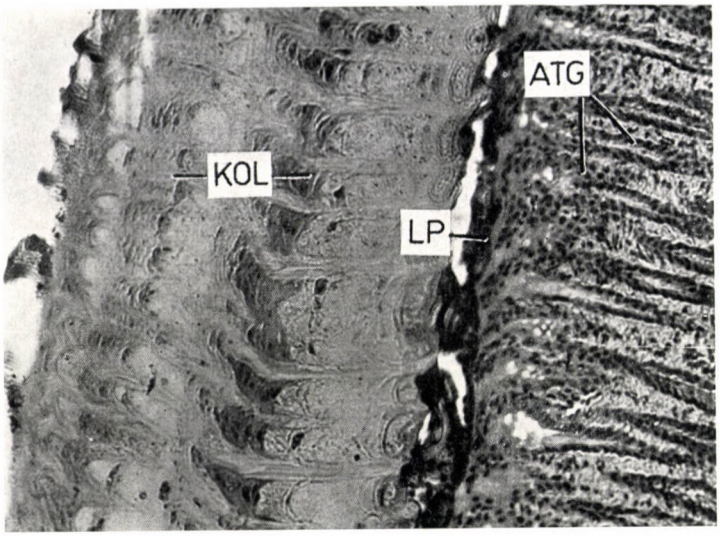

Fig. 6a. Transversal section. Gizzard of *A. tristis.* Koilin (KOL) with horny cones, alveolo-tubular glands(ATG) and lamina propria (LP); ×170

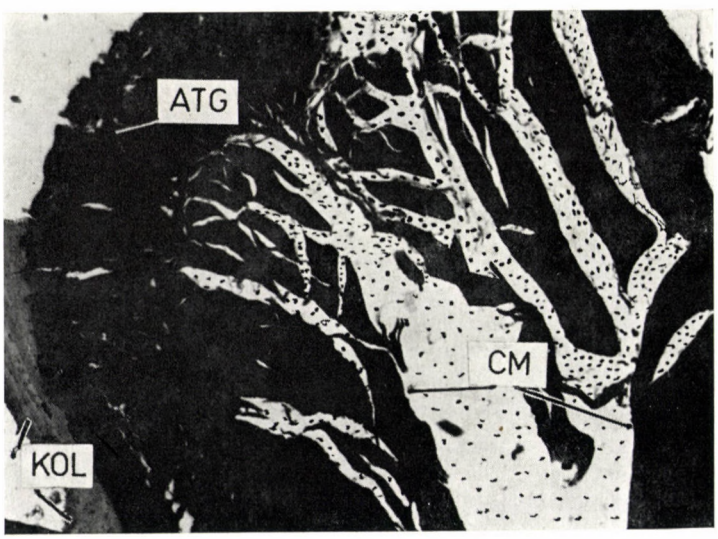

Fig. 6b. Transversal section. Gizzard of *A. tristis.* Well-developed circular muscle layer (CM): ×68

and perpendicular striations with alternate darker striae. The cones are arranged in two or three rows providing support and strength to the koilin layer.

A well-marked zone of elongated simple comb-shaped tubular glands lies beneath the koilin layer. These glands of about 0.25 cm length are lined by glandular cuboidal epithelial cells. The lamina propria is in the form of

connective tissues, but the muscularis interna and submucosa are lacking. The inner circular muscle layer (2.8 mm thick) of muscularis externa is well developed, constituting the major part of the gizzard (Fig. 6b). The outer layer is bound by the outer serosa, having thick tendons.

Pepsin: Fig. 7 shows the pH activity curve for pepsin. The optimum pH for proteolytic breakdown of haemoglobin by proventriculus tissue extracts was 1.5, 1.8 and 1.8 in *P. krameri*, *L. schach* and *A. tristis*, respectively.

The pepsin activities in terms of pepsin units are shown in Table 1. Maximum specific activity was observed in *A. tristis* and least activity in *P. krameri*.

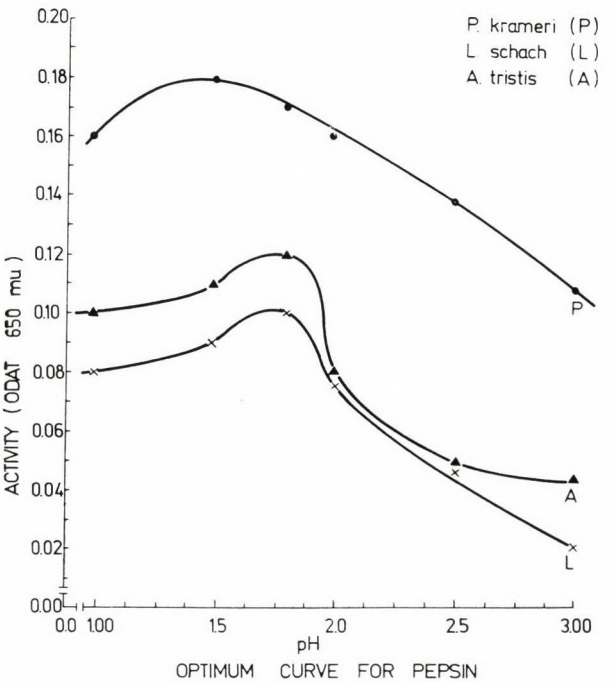

Fig. 7. pH optimum for pepsin: *P. krameri* (P); *L. schach* (L); *A. tristis* (A)

Table 1

Pepsin activity in proventriculus of P. krameri, L. schach and A. tristis

Species	Optimum pH	Activity per ml of tissue extract (pepsin units)	Specific activity (pepsin units/mg of protein)
P. krameri	1.5	1.37×10^{-4}	0.778×10^{-4}
L. schach	1.8	1.67×10^{-4}	1.5×10^{-4}
A. tristis	1.8	2.49×10^{-4}	3.11×10^{-4}

Discussion

During the present investigations the proventricular glands were found to be made up of oxynticopeptic cells arranged in the form of lobules in lamina propria. In *P. krameri*, each lobule is large and polyhedral in shape, which may be responsible for elaboration of greater quantities of hydrochloric acid, to maintain a relatively higher hydrogen ion concentration necessary for optimum activity of pepsin in this particular species (pH 1.5 vs. 1.8 for the other two species) (Table 1 and Fig. 7). In spite of the fact that *A. tristis* which being an omnivorous bird is capable of digesting a more complex group of proteins (both plant and animal), the lobules are the smallest in this species. However, the small size of lobules in this species is compensated by the presence of the greater number of these lobules, resulting in secretion of more gastric pepsin and hydrochloric acid. In *L. schach* the lobules are moderate in size, slightly elongated, fewer in number, and the pepsin activity per 1 ml tissue extract was lower than in *A. tristis*. This might be explained on the basis of the smaller body size and lesser quantity of food consumed by *L. schach*.

The mucosal villi are poorly developed in *P. krameri* and *L. schach*. This obviously suggests that the food does not stay in this region for digestion, but is mixed with the gastric juice. In *A. tristis*, the glandular mucosal villi are developed. The reason may be to increase the mucosal surface of glandular epithelium for higher mucus secretion in order to lubricate the food. Secretion of mucus by this layer has been reported by FARNER [6] in other birds.

Gizzard, the crushing and digestion site of food, showed remarkable variations and modifications in accordance with the feeding habits of the bird. The circular muscle layer of muscularis externa, in *P. krameri* and *A. tristis*, is very well developed for crushing the fruits, seeds and grains by its rhythmic movement. In *L. schach* this musculature is less developed; it may not be needed for grinding soft animal food. Similar adaptive features were observed by REED and REED [19] in hawks and owls. LENKEIT [15] and BROUSSY [1] pointed out that the modification in musculature of gizzard depends on the hardness of food in granivorous, herbivorous and omnivorous birds.

As *A. tristis* feeds on hard grains and cutenoid insects, koilin lining is provided with well-developed horny cones and perpendicular striations. This type of tough lining was well suited for fine crushing or grinding of hard grains and insects with the help of stone pieces which are casually taken during feeding. Similar type of cones was found in grain-eating pigeon [25] and striations in other birds [3].

With respect to *P. krameri* and *L. schach* the koilin lining is dense, provided with moderately developed cones in *P. krameri*, but has no cones

in *L. schach*, whose food, i. e., flesh of fruits, fresh seeds and animals, respectively, are sufficiently broken by the help of beaks and palatal folds. FARNER [8] has reported the lack of striae in koilin of birds which feed on soft food, and conspicuous striae in herbivorous and granivorous birds.

The gizzard is much larger in *A. tristis* than in *P. krameri* and *L. schach*, because due to the absence of crop, it performs both the functions of storage and crushing of food. In *P. krameri* the storage capacity of food is compensated by the presence of crop. DESSELBERGER [5] and CADOW [2] have reported a similar type of gizzard in other frugivorous birds. The moderate gizzard in *L. schach* was characterized by its thin-walled and bag-like nature, which clearly indicates its accommodating nature in relation to the food habit. Similar conditions in other carnivores (hawks and owls) have been reported by FARNER [8].

The optimum pH for pepsin was 1.5 for *P. krameri*, and 1.8 for both *L. schach* and *A. tristis*. HERPOL and GREMBERGEN [11] observed similar optimum pH for this enzyme in carnivorous and granivorous birds. FARNER [7] also found optimum peptic activity of less than 2 pH units.

The present results (Table 1) have shown that pepsin activity is higher in the proventriculus of the carnivorous and omnivorous birds *L. schach* and *A. tristis* than in the frugivorous *P. krameri*. HERPOL [9, 10] found higher pepsin content in the proventriculus of carnivorous than granivorous birds and indicated its correlation with the high protein diet. The present results are in conformity to these results.

The slight pepsin activity observed in the tissue extracts of gizzards in all three experimental birds may be attributed to the gastric pepsin contamination because histologically the epithelial linings of this region do not show the presence of oxynticopeptic cells. But HERPOL [9] has reported the presence of pepsin in the muscular stomach of carnivore birds. These results were, however, not supported by histological studies.

In view of the results discussed above it may be concluded that gizzard mainly serves as site of action for the gastric enzymes secreted in the proventriculus in all the three species of birds.

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FUNCTIONAL DIFFERENTIATION OF THE FSH-SYNTHESIZING CELLS IN THE PARS DISTALIS OF THE FETAL PITUITARY GLAND OF THE RAT*

AN IMMUNOHISTOCHEMICAL STUDY

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Abstract

The onset of FSH synthesis in the embryonic pituitary gland of the rat was studied by a peroxidase-labelled antibody method. The first FSH-containing cells appeared on the 20th day of embryonic life. From that day onwards, FSH cells increased rapidly in number. It was found that in adult animals some pituitary cells reacted with both anti-HCG and anti-FSH sera, indicating the simultaneous presence of LH and FSH in the same cell.

Introduction

In order to determine when the fetal pituitary gland starts to influence target organs and establishes a functional relationship with the hypothalamus, one has to know first the onset of hormone synthesis in the cells of the pars distalis. Determination of the functional differentiation of the troph hormone-producing cells in the pituitary gland of the rat has been attempted in many laboratories and in different ways.

Light microscopical search for hormone-containing cells in the fetal pituitary gland based on tinctorial, dimensional and histochemical principles has failed to give accurate information about the beginning of hormone synthesis in the cells of the pars distalis. JOST and TAVERNIER [2] and PHILLIPS and SCHMIDT [10] have been able to demonstrate earliest the presence of basophilic cells in 15.5- or 14-day-old fetal rat pituitaries, respectively, but no further information could be obtained from their work.

Electron microscopic investigations gave but limited information on the hormone content of the earliest granulated cells of the pars distalis because of the ambiguous character of these cells and their granules [13, 16].

Demonstration of the presence of troph hormones in the fetal pituitary gland of the rat with bio- or radioimmunoassay methods [1] is limited by the sensitivity of these methods and by the very low concentration of the hormones in the pituitary at this age.

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The most accurate data about the functional differentiation of the cells in the fetal [11] and early postnatal [12] anterior pituitary gland of the rat have been obtained with the peroxidase-labelled antibody method [6]. These studies determined the onset of hormone synthesis in all cell types of the pars distalis except in FSH cells.

The aim of the present work was to complete our knowledge about the functional differentiation of pituitary cells in the fetal adenohypophysis with the determination of the onset of FSH synthesis.

Material and method

The onset of hormone synthesis in the LH and FSH cells of the pars distalis of rat embryos (originally Wistar Strain) was followed daily between the 14th day of gestation and birth. The presence of sperms in the vaginal smear of the mothers was used to calculate the age of the embryos. The day when sperms were found in the smear was considered to be the first gestational day. After Cesarean section, pituitary glands of the embryos were excised and immersed in cold picric acid–formaldehyde solution [14] for 1—4 h, depending on the size of the gland. At least 6 fetuses from each developmental stage collected from different mothers were examined. The fixative was washed out with several changes of cold 0.1 mol/l phosphate-buffered saline (PBS), pH 7.6. The glands were dehydrated in graded alcohols and embedded into polywachs 1 000 (Chemische Werke, Hüls) [4]. To remove the embedding medium, sections of the glands, 5 μm in thickness, were floated on PBS overnight. Sex of the embryos was identified by histological investigation of the gonads up to the 18th gestational day or, in older embryos, by inspection. Morphological features of the differentiated FSH and LH cells were examined also in postnatal animals. Luteinizing hormone (LH) or follicle-stimulating hormone (FSH) was localized on sections by reacting them initially with appropriate rabbit antisera* and followed by peroxidase-labelled sheep anti-rabbit gamma globulin.** The reacted sections were developed for peroxidase using hydrogen peroxide and either 3,3'-diaminobenzidine (DAB) (Sigma) or α-naphthol [7]. Some sections were used to localize the two hormones simultaneously [7]. Sections were mounted on glass slides after staining.

Control sections failed to give colour reaction when normal rabbit serum was used instead of the specific one. Suitable controls to check nonspecific binding of the peroxidase-labelled rabbit antibody and the substrate itself, gave also negative results. Endogenous peroxidase activity could be detected in red blood cells only and was negligible under the conditions of our staining procedure.

Results

LH-containing cells appear first on the 18th day in the cell cords situating closest to the lumen of the former Rathke's pouch. At this developmental stage the pars distalis seems to grow by apposition of new cell cords to the cell mass already existing. Differentiation of LH cells in these peripheral cell cords begins only on the subsequent days.

The LH-containing cells rose significantly in number on the 19th gestational day (Fig. 1). They are frequently found in groups and their stronger staining indicates increased hormone content compared with the cells from the

* Rabbit antiserum against human chorionic gonadotropin (Serono), which cross-reacts with rat luteinizing hormone [5] and against rat follicle stimulating hormone (NIAMDD--A-RAT FSHS-6) were used at different dilutions with PBS.

** Peroxidase-labelled sheep anti-rabbit gamma globulin was prepared according to the method of NAKANE and KAWAOI [8].

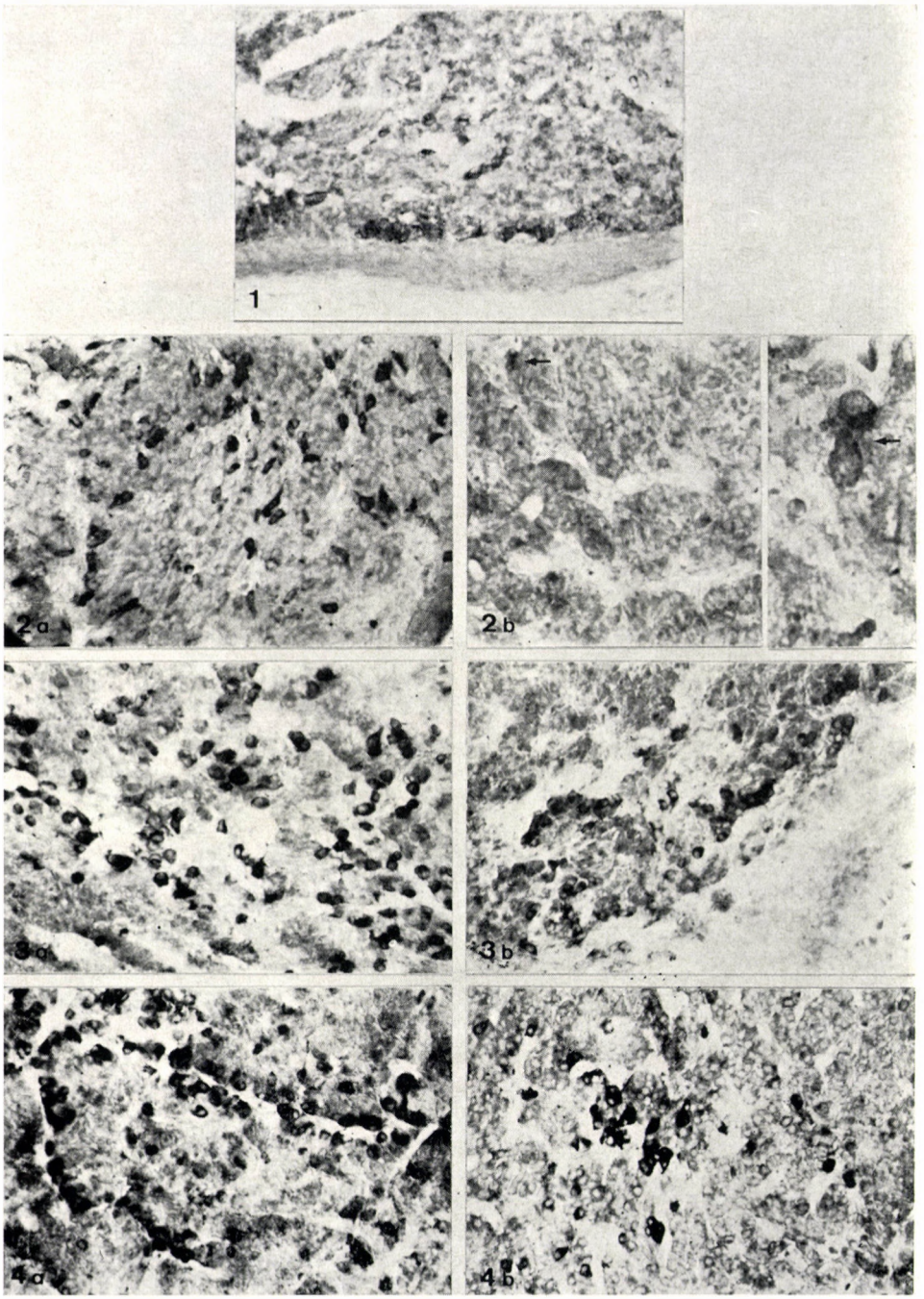

Fig. 1. LH-containing cells in the pituitary gland of a 19-day-old rat embryo. ×160
Figs 2a, 3a and 4a. LH cells in the pituitary gland of rat embryos from the 20th and 21st days of intrauterine life and from a newborn rat, respectively. ×160
Figs 2b, 3b and 4b. FSH-containing cells in the pituitary gland of rat embryos from the 20th and 21st days of intrauterine life and from a newborn rat, respectively. Arrows in Fig. 2b indicate identical areas; ×160. Inset in Fig. 2b; ×640. For details, see text

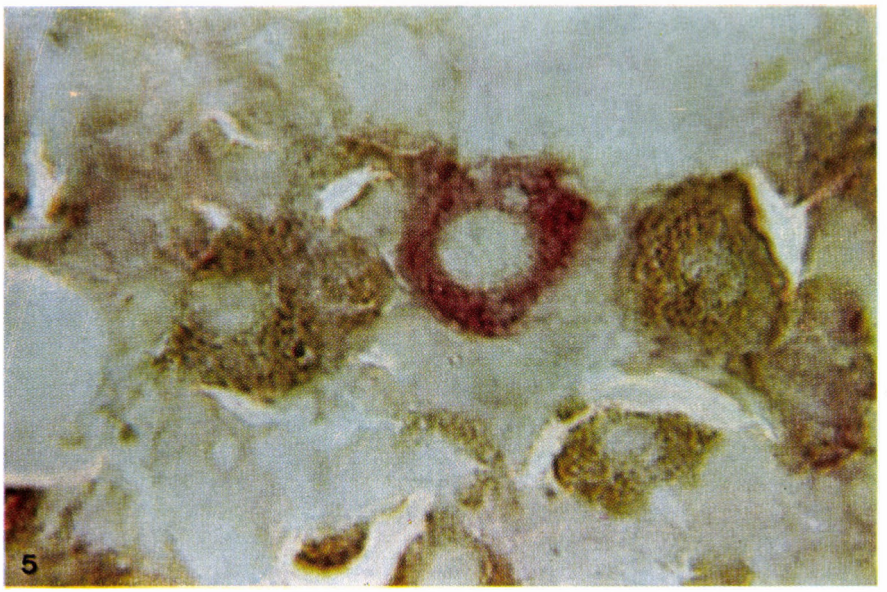

Fig. 5. LH (brown) and FSH (pink) cells in the pituitary gland of an adult rat. For details, see text. ×1 680

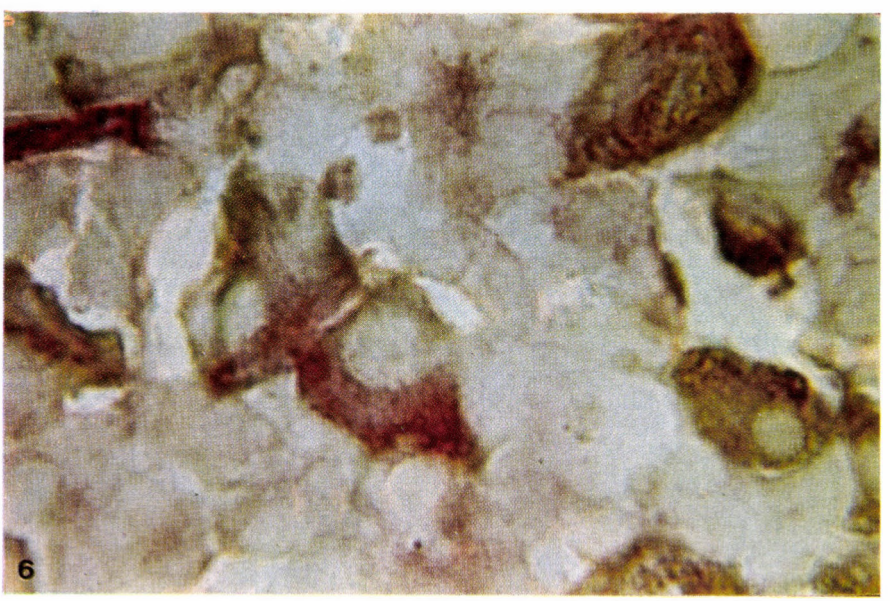

Fig. 6. LH- (brown) and LH- and FSH- (reddish-brown) containing cells in the pituitary gland of an adult rat. ×1 680

previous day. No FSH-positive cells can be demonstrated at this developmental stage even by prolonged incubation time.

It was the 20th day of intrauterine life when both LH and FSH cells could be stained in our material. Distribution of the LH cells is similar at this age to that of the adult's, e. g., LH cells have a tendency to occupy the peripheral region of the cell cords, making contact with the sinusoids of the pituitary gland (Fig. 2a). On this day, some pituitary cells bind small amounts of FSH antibodies, indicating the onset of FSH synthesis in the gland (Fig. 2b).

Regarding LH cells, only quantitative change can be observed in the embryos from the 21st day of intrauterine life (Fig. 3a). Changes are more pronounced in connection with the FSH cells. They increase in number and tend to take a position at the periphery of the primitive cell cords (Fig. 3b). Their deep staining indicates larger amount of the stored hormone. Distribution of FSH cells in the pituitary gland of the 21-day-old embryos shows the same picture as LH cells on the 19th gestational day.

Distribution of the LH cells in the newborn rat was practically the same as that characteristic of the pituitary gland of an adult animal. The round or oval LH cells formed rows along the sinusoids of the gland (Fig. 4a). In newborn rats, a further increase can be recognized in the number and hormone content of the FSH cells. They are frequently found in groups and have peripheral positions in the cell cords (Fig. 4b).

In order to test the specificity of our LH and FSH antisera, we attempted simultaneous staining of LH and FSH cells in the same section from embryonic pituitary. We failed, however, this test because of the high background staining of the sections. We will return to this problem in the discussion.

Simultaneous localization of the LH and FSH cells was successful on adult pituitaries. Some cells, situating mainly in the central region of the pars distalis, showed pure brown or pink colour, indicating that only one of the antisera was bound by these cells (Fig. 5). Other pituitary cells, having preferentially more peripheral position, showed mixed coloration. The mixed brown and pink colour indicated that these cells bound both antisera, consequently, these cells reacted with both substrates (Fig. 6).

No differences could be observed between the sections of male or female pituitaries regarding LH and FSH cells at these ages.

Discussion

Determination of the earliest day when different troph hormones appear in the pars distalis of the pituitary gland of rat fetuses has been attempted with various methods. As already mentioned in the introduction, light microscopic investigations have given only limited information about cellular differentiation of fetal pars distalis.

Electron microscopic approach of the same problem has been performed in several laboratories [3, 13, 16]. The earliest granulated cells have been demonstrated in the rat either on the 16th day [13], or on the 17th day [16] of gestation. However, all of the authors failed to identify these granulated cells concerning the hormone content of the granules. YOSHIMURA and HARUMIYA [15] termed these earliest granulated cells as "ambiguous", referring to the unknown developmental orientation of these cells. "Ambiguous cells" contain small granules, 50 nm in diameter, which gradually grow in size to 100—160 nm during late gestational days. The cells at this developmental stage are called "intermediate cells" by YOSHIMURA and HARUMIYA [15] and seem to develop further along the acidophil and basophil axis. SVALANDER [13] introduced somewhat different classification in which the "ambiguous cell" of YOSHIMURA and co-workers [16] was termed type I cell. He was able to make further classification within "intermediate cells" and distinguished type II, III and IV cells in this class. According to his proposal, type II, III and IV cells corresponded to thyrotropic, somatotropic and gonadotropic cells, respectively. The timetable of the differentiation of these cells would be the 18th day of intrauterine life for thyrotropic cells, and the 19th day for both somatotropic and gonadotropic cells. No further differentiation has been attempted between gonadotrops by this author (Table 1).

Regarding the gonadotropic cells of the pituitary, the works of SÉTÁLÓ and NAKANE [11, 12] give the most detailed information. Accordingly, LTH cells appear in the embryonic pituitary gland on the 17th day of intrauterine life. The next day, LH cells are also present in the gland.

This timetable of differentiation of the gonadotropic cells can be completed now with our results on FSH cells. FSH cells appear in the pars distalis as the last troph hormone-producing cells on the 20th day of intrauterine life.

One can raise the question whether the antisera used in our investigation were specific of not? Having been unable to obtain antisera against the β-chains of LH and FSH, we decided to test our antisera with double staining. The fact that many cells in the adult pituitary gland bound clearly only one of the antisera (this was indicated by the appearance of pure brown or pink colour) indicates that our antisera gave no cross reaction with the other antigen. The observation that some gonadotropic cells (situating mainly at the periphery of the pars distalis) appeared in mixed colours, indicating the simultaneous presence of both LH and FSH in the same cell, is in agreement with literary data. NAKANE [7] and PHIFER and co-workers [9] observed that some gonadotropic cells contain really both LH and FSH. The data of PHIFER and co-workers [9] are especially valuable in this respect since they used antisera against β-LH and β-FSH.

The problem that we failed to perform a double staining on embryonic pituitary can be explained on the basis of our observation on other embryonic

tissues. Sections of embryonic tissues show always higher background staining than adult material, and high background interferes with any double staining. Probably, embryonic tissues are less differentiated in respect of antigene city and, therefore, they have a tendency to bind non-specifically more antibodies than the tissues of adult animals. The specificity of our FSH antiserum is supported also by the observation that with this antiserum no cells could be stained before the 20th day of intrauterine life, though there were many LH and TSH cells in the pituitary gland of younger embryos.

The result of our study on the differentiation of FSH cells serves as additional data about the functional differentiation of the pituitary gland of the rat. Table 1 summarizes the results obtained in this subject with various methods and at the same time indicates the higher "resolving power" of immunohistological methods in such investigations.

Table 1

Functional differentiation of the cells in the pars distalis of the rat pituitary according to the data of different authors

Authors	Days after copulation								Cell types
	15.	16.	17.	18.	19.	20.	21.	22	
YOSHIMURA et al. (1970)			——	——	——	——	——	——	Ambiguous cells
				——	——	——	——	——	Thyrotropic cells
				——	——	——	——	——	Gonadotropic cells (male)
						——	——	——	Acidophil cells
SVALANDER (1974)			——	——	——	——	——	——	Type I (ambiguous)
				——	——	——	——	——	Type II (TSH)
					——	——	——	——	Type III (STH)
					——	——	——	——	Type IV (gonadotropic)
SÉTÁLÓ and NAKANE (1972)			——	——	——	——	——	——	ACTH
			- - - -	——	——	——	——	——	LTH
			- - - -	——	——	——	——	——	TSH
				——	——	——	——	——	LH
					——	——	——	——	STH
Present authors (1976)					- - - -	——	——	——	FSH

—— present in all embryos
- - - - present in some embryos

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THE HYPOPHYSIS OF THE MARINE TELEOST FISH, MUGIL AURATUS RISSO

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Abstract

Owing to its lack of a hypophysial stalk, the hypophysis of the marine fish, *Mugil auratus*, is of the platybasic type. Its differentiation into adenohypophysial and neurohypophysial regions well conforms with the situation already recognized in other fishes. But, although the adenohypophysis shows distinction into pro-, meso- and meta-adenohypophysis, the lineo-vertical arrangement which they assume is not of common occurrence. By contrast, the neurohypophysis forms the usual central nervous core, surrounded by the adenohypophysial regions. The acidophils constituting the pro-adenohypophysis, having a deep staining affinity, resemble those in the meso-adeno-hypophysial region. The acidophils of the meso-adenohypophysis occupy a small region, whereas their associating cyanophils occupy a larger ventral region. The meta-adeno-hypophysis consists of an integral convoluted sac of epithelial amphiphilic cells. Such encloses the bulk of the neurohypophysis. It is remarkable that the neurohypophysial fibres never interdigitate among the meta-adenohypophysial cells, so they are more closely related in arrangement to tetrapods than to fishes.

Introduction

Anatomical and histological descriptions of the teleostean hypophysis or pituitary gland have been given by BELL [1], KERR [4], SCRUGGS [21], POTTS [10], MILLER [7] and SATHYANESAN [18].

More recently, detailed studies on the pituitary gland of some Indian freshwater teleosts have been carried out by SATHYANESAN [19, 20] and SINGH and SATHYANESAN [23], of some Nile teleosts by RIZKALLA [11, 13], YOAKIM [24] and KAMEL and co-workers [3] and of some marine teleosts by SAGE and BERN [17].

It has been revealed that the hypophyses of teleost fishes exhibit a remarkably varied structural pattern. Therefore, the marine teleost fish, *Mugil auratus*, has been selected for the present investigation in order to show to what extent its hypophysis resembles, to or differs from, that of *Mugil capito* [12].

Material and method

Adult specimens (about 30 cm in total length) of *Mugil auratus* were fished off the Libyan coast, at Tripoli, during April, May and June. The brains with the attached hypophyses were carefully dissected, and fixed either in Zenker's formol or Gomori's fixative. The fixed brains were trimmed to separate the hypothalami with the intact hypophyses. The material was dehydrated, cleared and embedded in the usual way. Serial longitudinal and transverse sections of 5—6 μm thickness were cut. Heidenhain's azan stain as modified by RIZKALLA[14], and Halmi's aldehyde fuchsin stain as modified by DAWSON [2], were used.

Results

The pituitary gland of *Mugil auratus* has a vertical or dorso-ventral position, but it is slightly inclined towards the anterior side of the body. It is more or less conical in shape and its broad basal or dorsal part is closely attached to the infundibular region of the brain (Fig. 1).

The gland is differentiated into two main divisions, the adenohypophysis and the neurohypophysis. The adenohypophysis consists of three glandular regions (the pro-, meso- and meta-adenohypophysis), whereas the neurohypophysis is formed of a core of nervous tissue from which numerous neurohypophysial sheets or processes interdigitate into the surrounding adenohypophysial regions (Fig. 1).

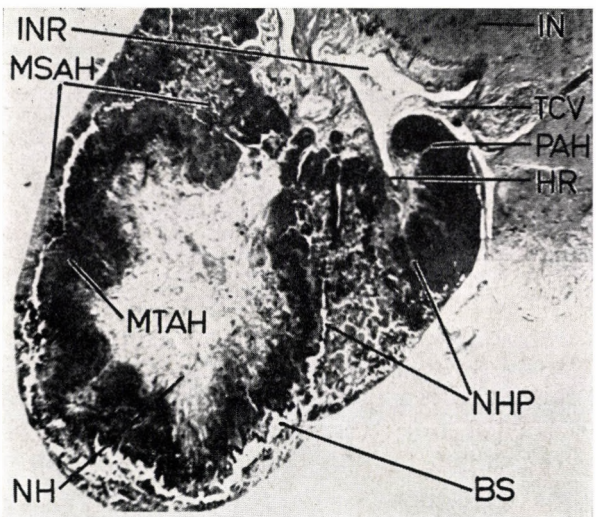

Fig. 1. Photomicrograph of a sagittal section of the pituitary gland of *Mugil auratus*, showing its general structure; ×50.
Abbreviations: AC = acidophils; BC = blood capillary; BS = blood sinusoid; CM = colloidal material; CY = cyanophils; HR = hypophysial recess; IN = infundibulum; INR = infuudibnlar recess; MSAH = meso-adenohypophysis; MTAH = meta-adenohypophysis; NH = neurohypophysis; NHP = neurohypophysial process; NSM = neurosecretory material; PAH = pro-adenohypophysis; TCV = third cerebral ventricle; V = vacuole

The adenohypophysial glandular regions

The pro-adenohypophysis

The pro-adenohypophysis is the smallest adenohypophysial region and occupies an area at the anterior side of the dorsal part of the gland (Fig. 1). It consists mainly of solid masses of deeply-stained acidophils which are separated by vascular neurohypophysial sheets. However, faintly-stained acidophils, approaching the chromophobic condition, are confined to the inner part of the pro-adenohypophysial region adjacent to the main mass of neurohypophysis. In general, the acidophils are either rounded or polygonal in shape, but with ill-defined cell outlines. They possess nuclei of a large proportional size with prominent central nucleoli (Fig. 2).

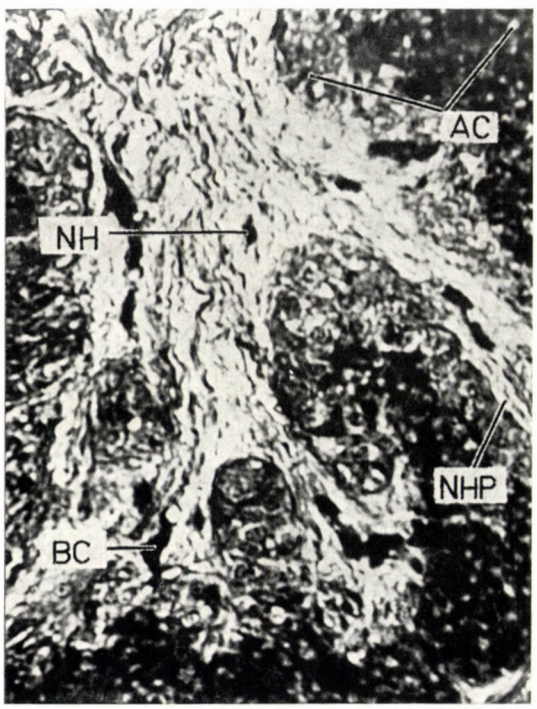

Fig. 2. Photomicrograph of part of a longitudinal section of the pituitary gland of *Mugil auratus*, showing the structure of the pro-adenohypophysis; ×500

The meso-adenohypophysis

The meso-adenohypophysis constitutes the largest adenohypophysial region and occupies an area at the posterior side of the dorsal part of the gland. It extends anteriorly and comes to lie ventral to the pro-adenohypophysis. Also, it continues ventrally all around the gland and forms a thin layer which

envelops completely the meta-adenohypophysis. This thin layer appears loose as it is highly penetrated by a plexus of blood sinusoids (Fig. 1).

The meso-adenohypophysis consists of deeply stained acidophils and cyanophils which exhibit a definite pattern of arrangement. The dorsal portion of the meso-adenohypophysis is almost always formed of acidophils, while its ventral portion as well as the thin layer, surrounding the meta-adenohypophysis, consist mostly of cyanophils (Figs 1 and 3).

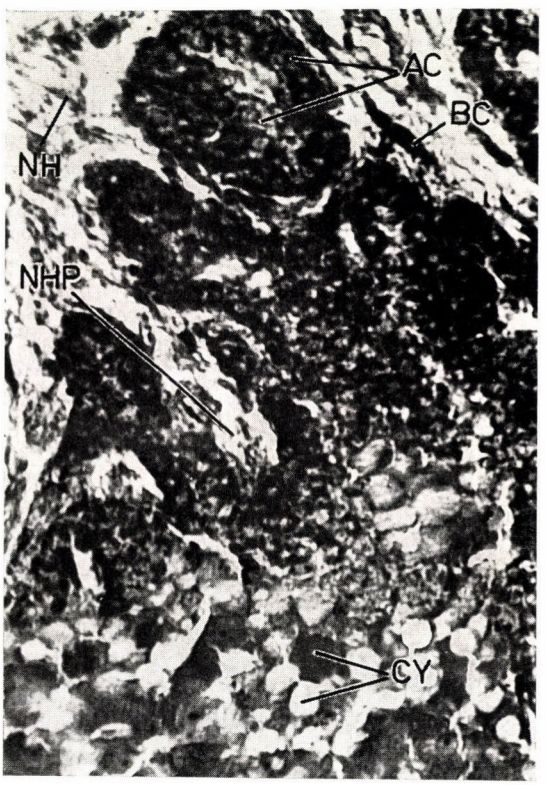

Fig. 3. Photomicrograph of part of a longitudinal section of the pituitary gland of *Mugil auratus*, showing the structure of the meso-adenohypophysis; ×550

The acidophils are usually arranged into solid masses of rounded, polygonal and oval cells, separated by vascular neurohypophysial sheets. Not all the acidophils are deeply stained, but some of them, especially those which do not lie in close contact with the neurohypophysial tissue, show a faint colouration. In general, the acidophils are smaller than the cyanophils, but

slightly larger than the pro-adenohypophysial acidophils and they are apparently resembling the latters, too (Fig. 3).

The region of the meso-adenohypophysial cyanophils is larger in size than that of the acidophils (Fig. 1), and thus the cyanophils are the prevailing meso-adenohypophysial cell type. The cyanophils are usually arranged into solid masses of cells, separated by highly vascular neurohypophysial sheets. Some cyanophils appear faintly stained or even vacuolated, depending, probably, on their secretory activity. In general, the cyanophils are rounded, oval or pyramidal in shape, with rounded eccentric nuclei. The vacuolated cyanophils, however, possess flattened and chromatic nuclei which are seen pressed against the cell membrane (Fig. 3).

The meta-adenohypophysis

The meta-adenohypophysis occupies an area at the ventral part of the gland and appears as a convoluted epithelial sac which encloses the bulk of the neurohypophysis. It is completely surrounded by the meso-adenohypophysis, but is partially separated from them by blood sinusoids and neurohypophysial sheets (Fig. 1).

The meta-adenohypophysis is made up of amphiphils which show a striking variation in their staining affinity for acidic and basic dyes. They are also variable in shape, being polygonal, oval or elongated and with prominent larger nuclei, compared with those of the other adenohypophysial cells. Vacuoles of variable size are usually seen within meta-adenohypophysial cells and they contain a highly refractive collodial material which, most probably, represents the hormonal secretory product of these cells (Fig. 4).

The neurohypophysis

The neurohypophysis is mainly formed of nerve fibres (Fig. 2); it forms the central core of the gland around which the three adenohypophysial regions are arranged. It gives off very few neurohypophysial processes which accompany the blood vessels penetrating the pro-adenohypophysis, but it arborizes extensively into the meso-adenohypophysial region. The bulk of the neurohypophysis, however, is enclosed within the meta-adenohypophysis and it is worthy of mention that none of the neurohypophysial processes are ever seen invading the meta-adenohypophysial epithelial layer (Figs 1 and 4).

The hypophysis of *Mugil auratus* is of the platybasic type, viz., it is closely and broadly connected with the hypothalamic floor of the brain and lacks the hypophysial stalk. The narrow third cerebral ventricle expands at the hypothalamic floor of the brain to form the infundibular recess which continues into the pituitary gland as a narrow hypophysial recess (Fig. 1).

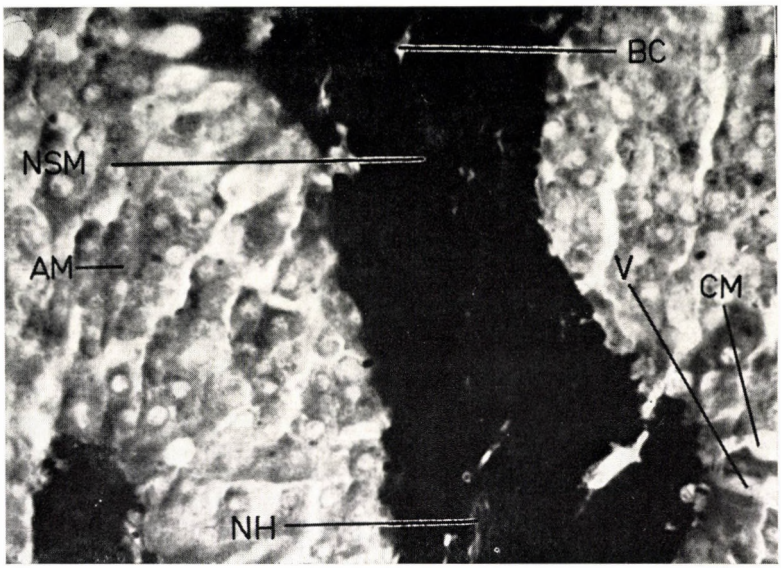

Fig. 4. Photomicrograph of part of a longitudinal section of the pituitary gland of *Mugil auratus*, showing the structure of the meta-adenohypophysis; ×500

Discussion

As far as known, the adenohypophysial regions display three modes of arrangement. In the first type, the pro-, meso- and meta-adenohypophyses are arranged in an antero-posterior direction (linear type), such as in some Indian fresh-water teleosts [20, 23] and in some Nile silurid fishes [3, 11, 24]. This linear type of arrangement represents the most primitive condition [6]. In the second type, the pro-, meso- and meta-adenohypophyses show a dorso-ventral arrangement (vertical type), such as in *Cirrhina*, *Rita* and *Rohtee* [18] and in *Mugil capito* [12]. This vertical type of arrangement has been considered by BELL [1] to be the highly evolved condition. In the third type, the pro- and meso-adenohypophyses show an antero-posterior arrangement, while the meta-adenohypophysis comes to lie ventral to them (lineo-vertical type), such as in *Salmo* and *Oncorhynchus* [15, 16]. This lineo-vertical type of arrangement may represent an advanced condition over the primitive linear type [6]. The arrangement of the adenohypophysial regions in the pituitary gland of *Mugil auratus* of the present investigation may be considered as the nearest condition to the lineo-vertical type, and thus differs from that recorded for *Mugil capito* [12], in which the adenohypophysial regions show the vertical type of arrangement.

While the pro-adenohypophysis represents the smallest adenohypophysial region of the pituitary gland in *Mugil auratus*, it was found to be the largest region and constitutes about half of the gland in *Mugil capito* [12]. The pro-

adenohypophysial cells of these two *Mugil* species are almost always arranged into compact masses. This cell arrangement is considered by POTTS [10] to be an advanced condition over either the cordal cell arrangement found in *Carassius* [1] and *Cyprinus* [22], or the follicular arrangement recorded for some silurid fishes [3, 7, 11, 13, 24].

The meso-adenohypophysis of *Mugil auratus* represents the largest adenohypophysial region of the pituitary gland, whereas it constitutes the smallest region of the gland in *Mugil capito* [12]. In both *Mugil* species, it consists of acidophils and cyanophils, a finding which differs from what has been recorded for most teleosts where the meso-adenohypophysis consists of acidophils, cyanophils and chromophobes [4, 10, 21]. In *Mugil auratus* the acidophils constitute the dorsal portion of the meso-adenohypophysis, while the cyanophils form its ventral portion. On the other hand, in *Mugil capito* [12] the acidophils are abundant at the centre of the gland, whereas the cyanophils show a remarkable increase towards its periphery.

In *Mugil auratus*, the meta-adenohypophysial cells are of the amphiphilic type, while in *Mugil capito* [12] they are of the acidophilic type. The meta-adenohypophysis forms a convoluted epithelial sac which encloses the bulk of the neurohypophysis. The endings of the neurohypophysial fibres come in close contact with the inner layer of the meta-adenohypophysial cells, a condition which simulates that found in *Lepidogobius* [5] and tetrapod pituitaries. Thus, such a finding contradicts the typical piscine character in which the neurohypophysial tissue is intimately associated with that of the meta-adenohypophysis to the extent that these tissues form a complex structure (see reviews in [8, 9]).

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THE HYPOTHALAMIC NEUROSECRETORY SYSTEM OF THE MARINE TELEOST FISH, MUGIL AURATUS RISSO

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Abstract

The hypothalamic neurosecretory system of the marine fish, *Mugil auratus*, consists of two nuclei, viz., the nucleus praeopticus and the nucleus lateralis tuberis. Both are paired, and while those of the nucleus praeopticus are vertically arranged as L-shaped bodies, their strand-like counterparts in the nucleus lateralis tuberis extend in an antero-posterior direction. The two constituent bodies of the nucleus praeopticus lie on both sides of the third cerebral ventricle. Each is differentiated into a dorsal pars magnocellularis and a ventral pars parvocellularis. A nervous tract, the hypothalamo-hypophysial tract, extends posteriorly from each body, but it is not until after they penetrate the pituitary gland that they fuse into one structure, the neurohypophysis. Many neurosecretory granules accumulate in the neurohypophysis adjacent to the meta-adenohypophysial region, and fewer scattered granules of varying sizes are also present along the hypothalamo-hypophysial tracts. Inner to these hypothalamo-hypophysial tracts extend the two bodies of the nucleus lateralis tuberis along the infundibular region. Axons from this nucleus extend sideways, and as they merge with those adjacently disposed of the hypothalamo-hypophysial tracts, they enter the pituitary gland as a unified structure.

Introduction

The discovery of the hypothalamic neurosecretory system in teleost fishes [16] and the subsequent detailed work in the same field [10] have called the attention of the neuroendocrinologists. Thus, additional studies were subsequently carried out on *Salmo* [1], on some chondrostean fishes [11], on *Lepidogobius* [7], on some marine teleosts [18], on *Anguilla* [8, 9] and on some Indian freshwater teleosts [12, 14, 15].

It has been revealed that the hypothalamic neurosecretory system of teleost fishes shows a striking variability as regards its structural pattern. Therefore, the marine teleost fish *Mugil auratus* has been selected for the present investigation in order to reveal the topographical position and structure of its hypothalamic neurosecretory system and to clarify the relationship between this system and the pituitary gland, a subject about which very little is known as regards teleost fishes.

Material and method

The origin and length of *Mugil auratus* specimens and the applied histological methods were the same as described in the accompanying paper.

After fixation, the fore-brains with the attached pituitary glands were separated for further processing. Gomori's chrome alum haematoxylin — phloxin stain was also used.

Results

The hypothalamic neurosecretory system of *Mugil auratus* consists of two nuclei, viz., the nucleus praeopticus and the nucleus lateralis tuberis. Hypothalamo-hypophysial axonal tracts extend from these nuclei to the pituitary gland, where they form the neurohypophysis.

The nucleus praeopticus is located at the hypothalamic floor anterior to the pituitary gland and just in front of the origin of the optic nerves from the brain. It is formed of a pair of L-shaped bodies which are situated on both

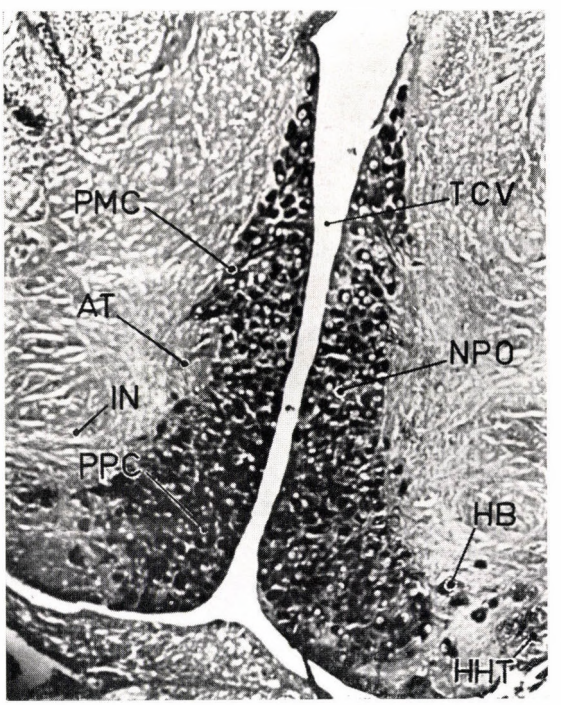

Fig. 1. Photomicrograph of part of a transverse section of the hypothalamic region of *Mugil auratus*, showing the topography of the nucleus praeopticus. ×85.

Abbreviations. AT = axonal tract; BC = blood capillary; EL = ependymal lining; HB = Herring bodies; HHT = hypothalamo-hypophysial tract; HR = hypophysial recess; IN = infundibulum; INR = infundibular recess; MSAH = meso-adenohypophysis; NB = neuronal body; NGT = neuroglia tissue; NH = neurohypophysis; NLT = nucleus lateralis tuberis; NPO = nucleus praeopticus; NSG = neurosecretory granules; NU = nucleus; PAH = pro-adenohypophysis; PMC = pars magnocellularis; PPC = pars parvocellularis; SM = secretory material; TCV = third cerebral ventricle

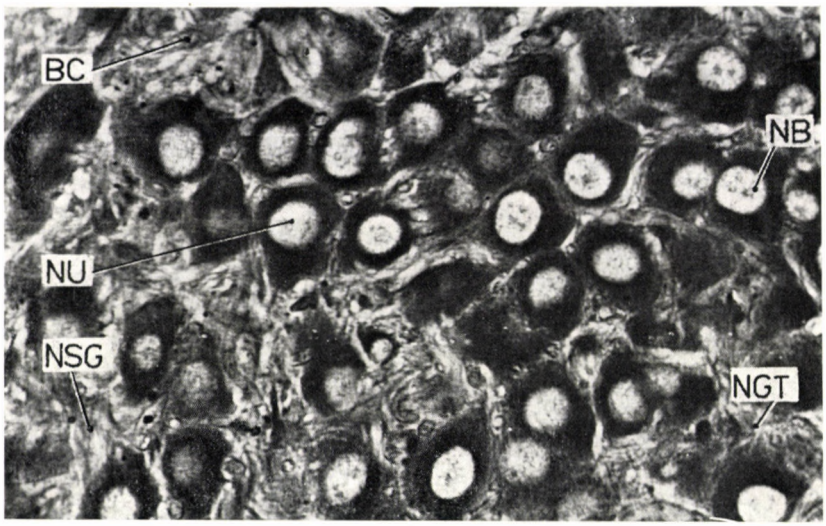

Fig. 2. Photomicrograph of part of a longitudinal section of the hypothalamic region of *Mugil auratus*, showing the structure of the nucleus praeopticus; ×545

sides of the third cerebral ventricle. The horizontal limb of each L-shaped body extends antero-posteriorly, i.e. superficially or ventrally along the infundibular region, while its vertical limb extends ventro-dorsally close to the third cerebral ventricular wall (Fig. 1).

The nucleus praeopticus consists of more or less compact neuronal bodies which are bound together by a highly vascular neuroglia tissue (Fig. 2). The neuronal bodies of the vertical limb, and especially those located at its dorsal part, are predominantly large and represent the pars magnocellularis, whereas the neuronal bodies of the horizontal limb are mostly small and represent the pars parvocellularis. These two regions are continuous with each other and are similar in structure (Fig. 1).

The neuronal bodies of the nucleus praeopticus are mostly polygonal in shape and possess large prominent nuclei. The neuroplasm contains coarse basophilic granules staining with aldehyde fuchsin and chrome alum haematoxylin. These granules vary in amount in the different neuronal bodies and even in one and the same neuronal body. In the latter case, the perinuclear area contains a large amount of basophilic granules, and thus appears more deeply stained than the peripheral one (Fig. 2).

The pars magnocellularis and pars parvocellularis, of either side, contribute to the formation of an independent right or left hypothalamo-hypophysial tract. These axons arise diffusely, but soon they aggregate and become consolidated to form a number of closely set bundles, constituting a definitive hypothalamo-hypophysial tract (Figs 1, 3 and 4). Each tract extends along

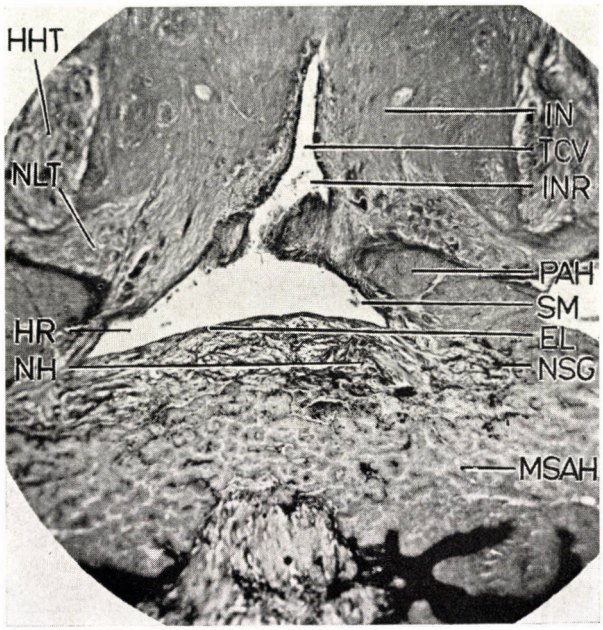

Fig. 3. Photomicrograph of part of a transverse section of the pituitary gland of *Mugil auratus* showing the hypothalamo-hypophysial tracts, the nucleus lateralis tuberis and the infundibular and hypophysial recesses; ×75

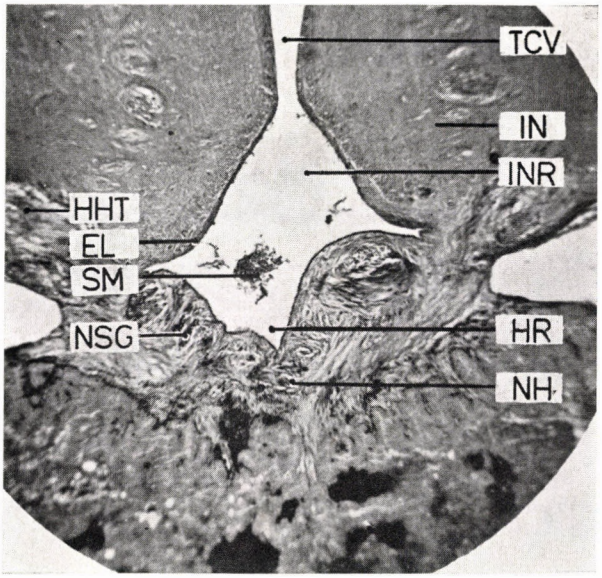

Fig. 4. Photomicrograph of part of a transverse section of the pituitary gland of *Mugil auratus*, showing the infundibular and hypophysial recesses and the hypothalamo-hypophysial tracts; ×70

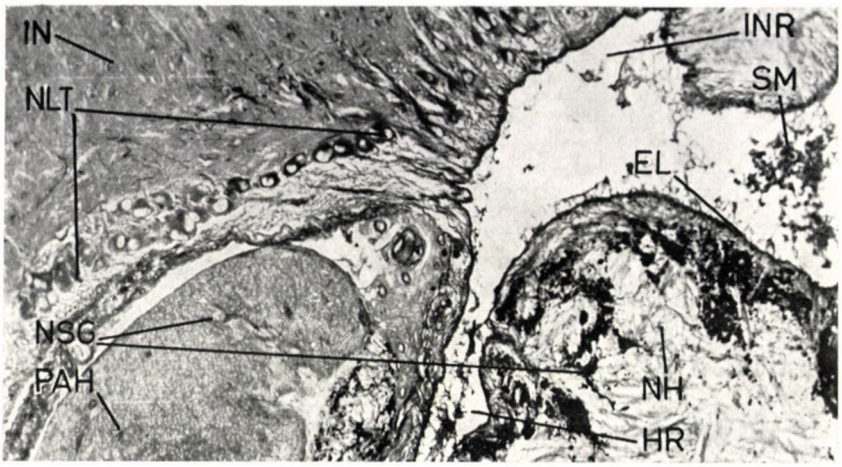

Fig. 5. Photomicrograph of part of a longitudinal section of the pituitary gland of *Mugil auratus*, showing the nucleus lateralis tuberis and the infundibular and hypophysial recesses; ×135

the hypothalamic floor towards the pituitary gland and penetrates it independently (Fig. 4). At the dorsal region of the gland, the right and left hypothalamo-hypophysial tracts join each other (Fig. 4), and then extend ventrally forming a neurohypophysial core, around which the pro-, meso- and metaadenohypophysial regions are arranged.

The axons of the hypothalamo-hypophysial tracts are either typically beaded or uniformly stained. The beads are of variable size and represent the neurosecretory material or Herring bodies and their presence may be taken as a phenomenon of the hormonal secretion of the nucleus praeopticus. Scarce neurosecretory material is only found around the blood vessels ramifying in the pro-adenohypophysial region (Fig. 5). But, a moderate amount of this material is seen in the meso-adenohypophysis, both between the cell masses and between the individual cells. An enormous amount of neurosecretory material, however, occurs at the meta-adenohypophysial region. This may be attributed to the fact that the neurosecretory material is stored at the perivascular axonal endings close to the meta-adenohypophysial epithelial layer (Fig. 3). Although the neurosecretory material is found in dense aggregations to the extent that it forms a continuous layer lining the meta-adenohypophysial epithelial region, yet this material is never seen within this region.

Some of the axons of the hypothalamo-hypophysial tracts have extra-hypophysial perivascular endings through their course along the hypothalamic floor. Besides, other axons terminate on the ependymocytes lining the in-

fundibular and hypophysial recesses. These recesses contain varying quantities of a secretory substance, stainable with aldehyde fuchsin and chrome alum haematoxylin (Figs 3, 4 and 5). Most probably, this substance is the neurosecretory material of the nucleus praeopticus. It seems to reach the infundibular and hypophysial recesses *via* their lining ependymocytes.

The nucleus lateralis tuberis is another paired structure. It lies posterior to the nucleus praeopticus, extending along the infundibular region which faces the pituitary gland. This nucleus consists of two longitudinally or antero-posteriorly arranged strands of neuronal bodies which occupy an inward position to the hypothalamo-hypophysial tracts of the nucleus praeopticus (Figs 3 and 5).

The neuronal bodies of the nucleus lateralis tuberis are loose and less vascularized than those of the nucleus praeopticus. They show a reduction in size in the antero-posterior direction, and the majority of them have an acidophilic staining affinity, appearing light green or phloxin positive. Some neuronal bodies, and especially those located posteriorly, however, show a basophilic staining affinity as they are aldehyde fuchsin or chrome alum haematoxylin positive, similar to the neuronal bodies of the nucleus praeopticus (Fig. 5). But, the neuronal bodies of the nucleus lateralis tuberis are less abundant in basophilic neurosecretory material than those of the nucleus praeopticus. Most probably, the axons which arise from the nucleus lateralis tuberis merge with those constituting the hypothalamo-hypophysial tracts of the nucleus praeopticus before their entrance into the pituitary gland.

Discussion

Mugil auratus possesses two hypothalamic neurosecretory centres, viz., the nucleus praeopticus and the nucleus lateralis tuberis, similar to what has been recorded for other teleost fishes by BREHM [3] and GABE [5]. In some teleosts, however, a single hypothalamic neurosecretory centre, the nucleus praeopticus, has been described [6, 17].

The nucleus praeopticus may assume various shapes in teleost fishes, as it either consists of horizontally placed elongated bodies such as in *Carassius* [13], vertically set bodies such as in *Rita* and *Heteropneustes* [14, 15], U shaped bodies such as in *Clarias* [12] or L-shaped bodies such as in *Mugil auratus* of the present investigation.

The nucleus praeopticus of *Mugil auratus* contributes to the formation of the right and left hypothalamo-hypophysial tracts which penetrate independently the pituitary gland where they fuse and form the neurohypophysis. In *Clarias* [12] and *Heteropneustes* [15], however, the right and left hypothalamo-hypophysial tracts give rise posteriorly to a number of lateral tracts

which join to form a very closely approximated pair of median tracts. The right and left tracts and the median tracts join to form a common tract at the hypophysial stalk.

Some axons of the hypothalamo-hypophysial tracts of *Mugil auratus* separate from their main course and terminate on the hypothalamic blood vessels or the ependymocytes lining the infundibular and hypophysial recesses. Similar axonal extrahypophysial terminations have been described in *Anguilla* [19], *Lepidogobius* [7], *Clarias* [12] and *Rita* [14].

The axons of the nucleus lateralis tuberis of *Mugil auratus* seem to merge with those constituting the hypothalamo-hypophysial tracts of the nucleus praeopticus, and thus the bulk of the neurosecretory material of these nuclei reaches the pituitary gland by an axonal transport. However, the close proximity of the neuronal bodies of the nucleus lateralis tuberis to the hypothalamic blood vessels and the infundibular recess suggests the possibility of the diffusion of their neurosecretory material into these cavities. These findings coincide with those recorded for *Salvelinus* [2]. BREHM [3] stated that the neurosecretory material of the nucleus lateralis tuberis is released directly into the third cerebral ventricle, whereas PALAY [10] opined that this material reaches the pituitary gland by an axonal transport. STAHL and LERAY [18], however, suggested a tubero—ependymo—hypophysial pathway and vascular transport as alternative methods for conduction of the neurosecretory material of the nucleus lateralis tuberis.

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PROTEOLYTIC ACTIVITY OF SUBCELLULAR FRACTIONS FROM STREPTOMYCES GRISEUS NO. 45-H

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Abstract

Subcellular fractions were prepared from *Streptomyces griseus* No. 45-H at different stages of life cycle, and their proteolytic activity was examined. The highest proteolytic activity was found in the 24- and 72- h-old vegetative hyphae, the lowest in the resting spores. Spores contained about 9—30% of the proteolytic activity of vegetative cells. At the age of 16 h about 80%, at 26 h 70%, at 72 h 40%, and in spores about 60% of the proteolytic activity was particulate. The greatest part of the proteolytic activity could be inhibited by EDTA, lower levels of serine and sulfhydryl protease activities were detected in the cell-free extracts of vegetative cells.

Introduction

The aim of these experiments was to study the intracellular proteolytic activity of *Streptomyces griseus* No. 45-H during the life cycle in order to find the circumstances favourable for obtaining intact functional ribosomes.

In a previous work we reported [7] that spore ribosomes of *S. griseus* No. 45-H were stable during the isolation procedure, but in the case of vegetative cell ribosomes it was necessary to use protease inhibitor to get a reproducible ribosomal protein profile in one-dimensional polyacrylamide gels. We also found that washed ribosomes from vegetative hyphae had higher proteolytic activity than ribosomes from spores [8].

Material and method

The experiments were carried out with a streptomycin-non-producing variant of *S. griseus*, No. 45-H. The characterization of the strain was published elsewhere [9].

Mycelia were cultured in filtered soybean medium in 500 ml Erlenmeyer flasks at 27°C, and harvested at stated intervals.

Spores and old vegetative hyphae were produced from a 72-h culture as follows: the culture was harvested, washed twice with sterile bidistilled water and once with 0.01 mol/l Tris-HCl buffer, pH 7.6, containing 0.003 mol/l magnesium acetate (this buffer was used throughout the experiments), resuspended in cold buffer and sonicated for 5 min in ice bath with an MSE ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., Power Unit No. 3 000) at an intensity of 1.4—1.5 A, Ammeter position. Under these circumstances hyphae were disrupted, whereas there were no morphological alterations in the spores. After sonicating

the suspension was centrifuged at 10 000 *g* for 10 min, the supernatant served as a cell-free extract of 72-hour-old vegetative mycelia, the pellet contained the spores. The spores were washed twice with cold bidistilled water and once with buffer, and stored at −20°C until used.

Spores were free of vegetative cells and cell debris as judged in the phase-contrast microscope.

Cell-free extracts from mycelia (except the 72-hour culture) and from spores were prepared by breaking in a Braun cell homogenizer (model MSK). Ten ml suspensions of mycelia or spores were shaken with 50 g of 0.10—0.11 mm diameter glass beads for 1 or 2 min, respectively. Glass beads were separated by centrifugation for 20 min at 5000 *g*, and washed twice with buffer.

Supernatants were combined, treated with 3 μg/ml DNase (Worthington Biochemical Co., RNase-free) for 15 min in ice bath, then fractionated by the method of ORLOWSKI and WHITE [6] with some modifications. After treatment with DNase, the suspension was centrifuged in an MSE Highspeed 18 centrifuge at 10 000 *g* for 10 min, and a portion of the supernatant (S I) was centrifuged at 27 000 *g* for 60 min to yield a particulate fraction (P I) and a supernatant fraction (S II). A portion of S II was centrifuged in an MSE Superspeed TC 50 centrifuge at 140 000 *g* for 90 min to yield a second particulate fraction (P II) and a supernatant fraction (S III).

Protein in fractions was determined by the method of LOWRY and co-workers [3], using bovine albumin as a standard, then the protein concentration of fractions was uniformly adjusted to 2 mg per ml with buffer.

Protease activity of the fractions was determined as descibed by MATHESON and MIKULIK [4], using 2% casein (Serva, vitamin free) in 0.1 mol/l Tris-HCl (pH 7.9) as substrate [5]. Four ml casein and 0.4 ml fractions were incubated at 30°C. At 0, 3, 6 and 20 h 0.6, ml digests were removed, precipitated with 1 ml 6% TCA and stored at 0°C for 30 min. Samples were centrifuged and 1 ml supernatant was mixed with 2 ml 0.5 mol/l NaOH, then 0.6 ml phenol reagent was added and extinction was read at 650 mμ in a Unicam SP 1800 spectrophotometer.

Neutral metal proteases were inhibited with EDTA [2], alkaline serine proteases with PMSF [1, 2] and thiol proteases with PCMB, each reagent at a concentration of 10^{-3} mol/l.

Abbreviations used: EDTA = ethylenediaminetetraacetic acid, PMSF = phenylmethylsulfonylfluoride, PCMB = para-chloromercuribenzoate.

Results and discussion

Cell-free extracts of vegetative cells of *S. griseus* No. 45-H in all examined stages of development had higher proteolytic activity than spores had (Fig. 1).

During the life cycle the changes in the proteolytic activity of vegetative cells are in good correlation with the morphological changes of the culture. During the first 24 h of vegetative growth the intracellular proteolytic activity increased very quickly, then rapidly decreased in the following two hours (Fig 1). During this period the extracellular proteolytic activity of the culture broth attained a high level, which was accompanied by a great lysis of vegetative hyphae [9]. After lysis a secondary growth phase began and from this period of the life cycle both vegetative and reproductive elements were seen in the culture [9].

At 72 h of age, the old vegetative hyphae and the reproductive elements were separated from each other and their intracellular proteolytic activities were determined. Fig. 1 shows that the old vegetative hyphae were more active than the spores.

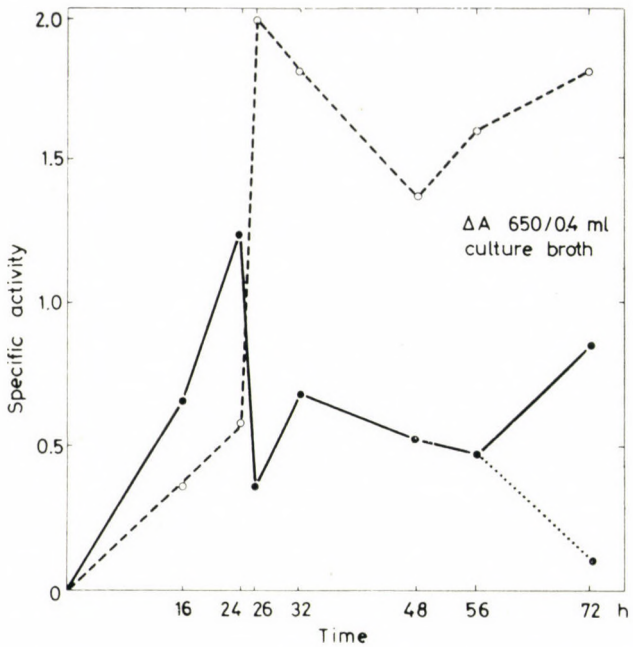

Fig. 1. Proteolytic activity in cell-free extracts (fractions I) from different phases of development of *S. griseus* No. 45-H. The solid line at the age of 72 h represents the proteolytic activity of old vegetative hyphae, the dotted one the proteolytic activity of spores. Details are given in the text. Intracellular specific activity was expressed in ΔA_{650} per 0.8 mg protein per 20 h. The dashed line shows the proteolytic activity of the culture broth during the life cycle; values are expressed in ΔA_{650} per 0.4 ml culture broth per 20 h

The highest proteolytic activity was found in the 24- and 72-hour-old vegetative cells, the lowest one in the resting spores.

Table 1 shows the distribution of proteolytic activity between soluble and particulate fractions of cell-free extracts from different stages of development. In young vegetative cells (16 h) 80% of the proteolytic activity was particulate, presumably membrane- and ribosome-bound, and at the end of the life cycle, in the old vegetative hyphae, this value decreased to 40%; in spores to 60%.

We found that 60—80% of the proteolytic activity, tested at pH 7.9 in cell-free extracts from either vegetative cells or from spores, can be inhibited by EDTA which was effective in every subcellular fraction of vegetative cells and of spores.

The inhibitory effect of PMSF varied between 0 and 40% in the different fractions. The proteolytic activity of fraction P I isolated from a 26-h culture could be inhibited more effectively by PMSF than that of fraction P I from

Table 1

Values of particulate proteolytic activity of cell-free extracts from different stages of development of Streptomyces griseus No. 45-H

Age of mycelia (hours)	Particulate activity (per cent)
16	80
26	73
72	40
spores	60

16-h mycelia. The proteolytic activity of fractions P II, obtained from vegetative cells, was inhibited by PMSF between 20 and 35%. PMSF was found without effect on the activity of fraction P II from spores.

The inhibitory effect of PCMB was examined with subcellular fractions of 26- and 72-h cultures. The soluble fractions from both cultures have higher PCMB sensitivity than particulate fractions have.

When a combination of EDTA, PMSF and PCMB was used, each at 10^{-3} mol/l, the activity of the soluble fraction of a 26-h culture was completely inhibited, but approximately 5—10% of the proteolytic activity of particulate fraction (P I and P II) was found to be insensitive to the treatment.

The above data agree with our earlier results [7, 8] that spore ribosomes were found more "stable" during the isolation procedure, as spores have much weaker intracellular proteolytic activity than vegetative cells have. In other respects the knowledge of these results will help us to elaborate the conditions under which intact, functional ribosomes can be obtained from *S. griseus* No. 45—H.

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IN VITRO INVESTIGATION OF BIOLOGICAL SPECIMENS BY ELECTRON MICROSCOPY

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Abstract

A microchamber was developed for the examination of biological specimens in nearly natural environments, in an electron microscope, at ~ 70kV accelerating voltage. The chamber can be supplied continually with the sample and with the reagents, which makes it suitable for the study of biochemical reactions, too. Temperature and vapour pressure in the chamber can be controlled and the thickness of the specimen can be varied. Transmission electron micrographs of biological specimens, such as human blood cells, bull gametes and *Bacillus subtilis* have been obtained. Mobility of microorganisms, which is regarded as a criterion of the wet state, has been observed.

Introduction

Since the introduction of the electron microscope, the investigation of wet biological specimens has been a permanently recurrent problem, namely, drying changes the specimen and may destroy its ultrastructure [1, 2]. The investigation by electron microscopy of biological specimens in nearly natural environments would offer new possibilities in many fields of research.

Material and method

Studying wet biological specimens in electron microscope meets many difficulties, as the wet state must be kept in high vacuum (10^{-4} — 10^{-5} torr). A great deal of effort has been expanded to overcome these difficulties and "environmental chambers" have been designed to maintain specimens in the wet state [5, 11, 15]. These chambers are divided into two types: static [1—4, 9, 18] and dynamic [6, 10, 13, 14, 16, 18, 19] chambers. In the former one, the specimens are kept in a closed space and are separated from the vacuum space of the electron microscope by thin electron-transparent windows. In the latter one, the specimen-space is directly connected to the column-space and the pressure in the chamber is controlled by allowing a gas to flow around the specimen.

The presence of windows leads to a serious reduction in resolution and contrast. The first "sealed" type chamber was constructed by MARTON [12]. Application of static chambers was facilitated by the introduction of high voltage electron microscopes (HVEM, bigger penetrating power). The first HVEM experiment was carried out by DUPOUY [2]. The use of HVEM involves some additional problems. Although the radiation damage is reduced, the elastic scattering in the specimen is decreased with increasing inelastic scattering, accordingly the contrast is reduced. Photographic emulsions are less sensitive to 1000 kV than those of lower energy and this results in a loss in sensitivity, at a HVEM.

It is no easy task to develop an appropriate wet chamber for biological investigations. Using the wet-technique the following three problems are to be solved:

(1) It is highly important to keep the specimens in thin layer and in the wet state indeed. A previous drying out or freezing cannot be alowed. A later introduction of water vapour does not help any more, because most of the livinlg processes are not reversible.

Motion of specimens (see e. g. Fig. 7) under electron microscopical investigations has been recorded in the "Hygro-Elmi" microchamber (Fig. 1) for the first time. These movements are the conclusive proof for the wet state of the biological specimen.

(2) Radiation damage of the sample (by ionizing and warming effects of the electron beam, as well as the problem of "death" of living specimens [7]) must be reduced.

(3) Image contrast and resolution must be improved (which are degraded by the thickness of the specimen and distortion caused by the scattering of the surrounding gas).

Results and discussion

A new experimental technique using the microchamber "Hygro-Elmi" (dynamic type) has been developed. It allows satisfactory conditions for the *in vitro* state: the wet state of specimens is illustrated by observation of movement; radiation damage is reduced by very short exposure time, by cooling the sample, by continuous fresh specimen supply, by low beam current; contrast is increased by using thin specimen layers [19]. The chamber is attached to a Zeiss EF-4 electron microscope (accelerating voltage 67 kV, electron beam current 10^{-3} to 10^{-2} A cm^{-2}). It can of course be adapted to any type of commercial electron microscope.

The inside space of the chamber, i.e., the specimen space is connected to the column space of the electron microscope through entrance and exit apertures of 100 μm diameter. The pressure in the chamber nearly corresponds to the equilibrium vapour pressure at the given temperature of the sample. This is achieved by supplying vapour from buffer systems while the streaming out is damped by turbulent currents developed at the narrow apertures. The chamber is continually supplied with specimens and the temperature and pressure are controlled. Furthermore the specimen may be treated *in situ* with chemical reagents and the subsequent reaction monitored simultaneously. The electron micrograph can be recorded on a film with a camera.

Figure 1 shows the scheme of the chamber. The pressure inside the chamber is maintained by water vapour coming from a buffer system. Cooling traps filled with liquid nitrogen are mounted around the chamber thus preventing the vapour from entering the electron-optical column. An instrument regulates and measures the temperature.

Transmission electron micrographs on objects such as human red and white blood cells, bull gametes and *Bacillus subtilis* have been obtained in physiological solutions, without any contrast material, under near equilibrium water vapour pressure, at 10°C and with exposure of $\sim 10^{-4}$ C cm^{-2}. These are illustrated in Figs 2—7 (low magnification, $\times$1 000 to 4 000). In Figs 2 and 5 the film was exposed directly to the transmitted electrons. In Figs 3. 4. 6 and 7 the film was exposed from the fluorescent screen with a 35 mm

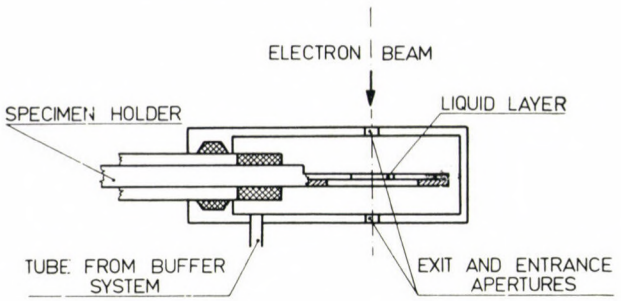

Fig. 1. Scheme of the microchamber "Hygro-Elmi"

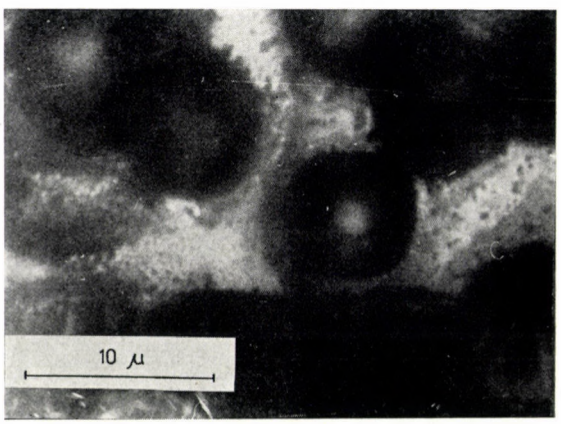

Fig. 2. Erythrocytes

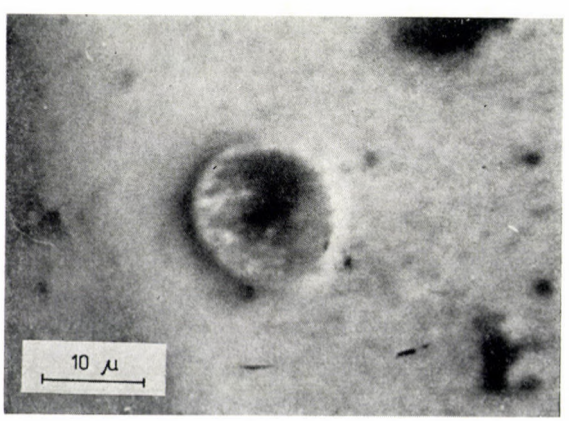

Fig. 3. Human white blood cell

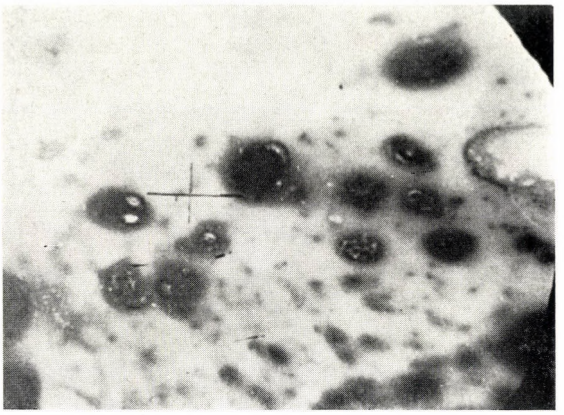

Fig. 4. Components in human blood

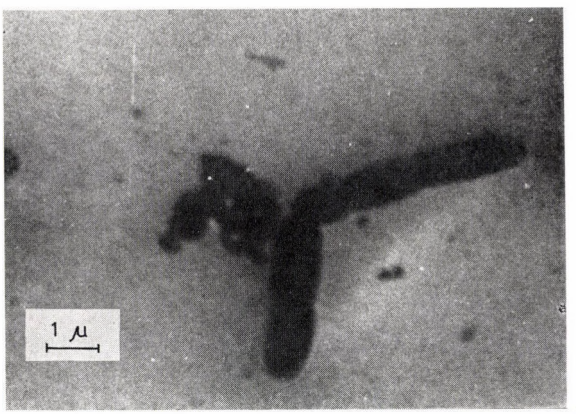

Fig. 5. Bacillus subtilis

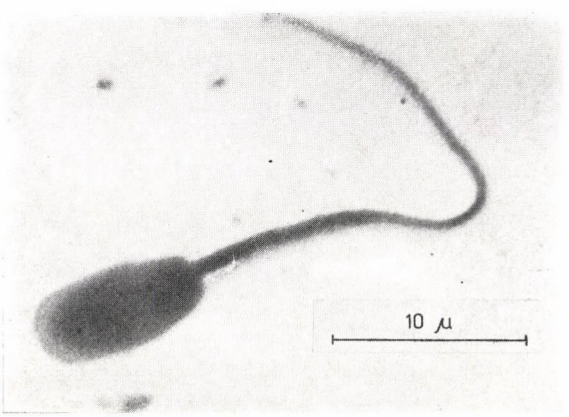

Fig. 6. Bovine spermatozoa

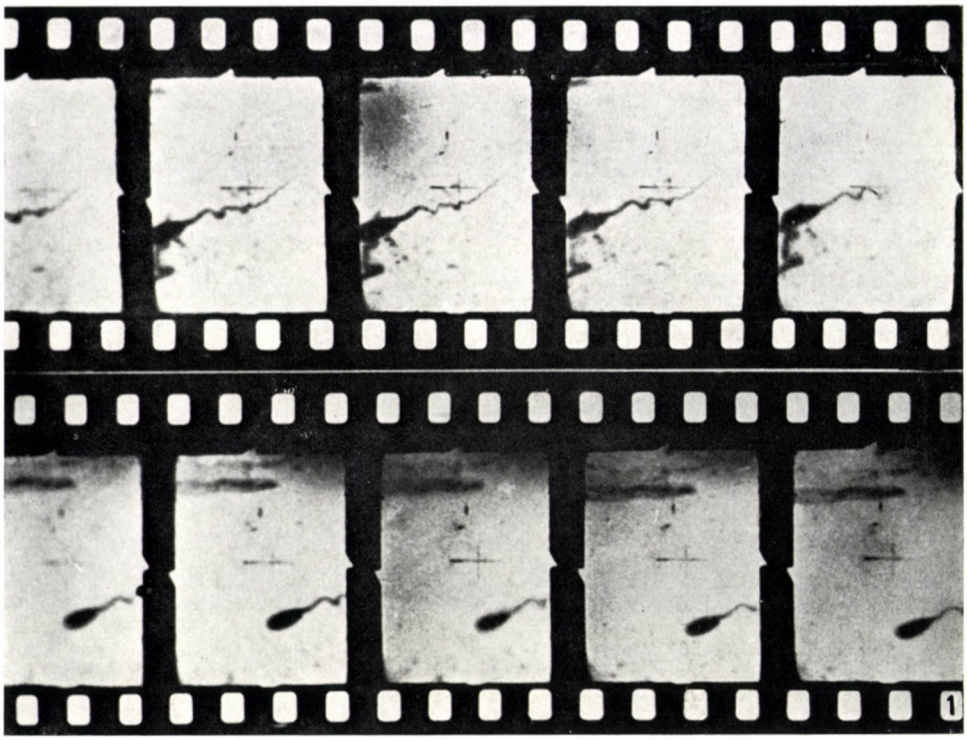

Fig. 7. Displacement of bull gamete. (There is a 1/24 sec interval between consecutive shots)

cine-camera mounted outside the electron-optical device (the crosses indicate the screen centre).

The micrographs 3 and 4 show cytoplasmic structures. The joined *Bacillus subtilis* is to be seen in the liquid as a triangle; Fig. 5 shows its shadow, too. In the TEM of bovine spermatozoa (see Fig. 6) the nucleus can be distinguished which is darker than the surrounding cytoplasma. Cells float and tumble in the chamber indicating a depth of liquid at least equal to the cell thickness, as shown in Fig. 7. True active cell mobility was observed but has not yet been recorded.

Further development of the chamber and the recording apparatus is in progress and study of various biological specimens is being pursued.

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ELECTRON MICROSCOPIC AUTORADIOGRAPHY OF SEROTONIN UPTAKE IN THE GANGLIA OF THE FRESH-WATER MUSSEL (ANODONTA CYGNEA L.)

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Abstract

After *in vitro* incubation, the uptake of labelled serotonin was investigated by electron microscopic autoradiography in the ganglia of fresh-water mussel (*Anodonta cygnea* L.). The labelled serotonin was primarily taken up by the axons of the neurons. The silver grains could always be localized over axons containing eccentric dense-core vesicles with a diameter of 100—200 nm. The results suggest (a) the possibility of the electron microscopic identification of serotonin-containing neurons, and (b) the direct role of the eccentric dense-core vesicles in the storage of serotonin.

Introduction

The general occurrence of the dense-core vesicles in the central nervous system of fresh-water mussel has been proved by several electron microscopic (EM) investigations [9, 17, 28]. Following glutaraldehyde-osmium tetroxide fixation, 5 types of vesicles were distinguished in the central neurons of *Anodonta*; clear (60—80 nm), small dense-core (70—120 nm), large dense-core (120—180 nm), eccentric dense-core (100—200 nm) and peptidergic neurosecretory (100—190 nm) ones [7]. According to density-gradient centrifugation, the serotonin (5HT) content of the *Anodonta* ganglia is mainly bound to the synaptosome fractions and the EM investigations showed that these synaptosomes contained dense-core vesicles [10]. After *in vitro* incubation the uptake of ^{3}H-5HT was unequivocally localized in the axon profiles of the ganglia [6]. Contrary to former results [29, 31], the above findings suggest the intra-axonal localization of 5HT in the ganglia. The aim of the present work was the ultrastructural localization of the storage site of 5HT in the central neurons of the fresh-water mussel.

Material and method

Adult specimens of the fresh-water bivalve *Anodonta cygnea* L. were used in our experiments. The dissected ganglia of the animals were incubated in 3 ml Anodonta saline [16] containing ^{3}H-5HT in a concentration of 0.5 and 1.5 nmol/ml (specific activity 500 mCi/mmol, Amersham) for 20, 50 and 120 min at 20 and 25°C, respectively. For EM autoradiography, materials of 50 and 120-min incubation were used because of their more intensive labelling detected

by light microscope. Following a short washing in Anodonta saline, the ganglia were fixed in 3% glutaraldehyde (diluted in Anodonta saline) for 2 h. Postfixation was performed in 2% OsO_4 buffered with s-collidine [1] for 30 min at 0°C. After dehydration, the ganglia were embedded in Araldite (Durcupan, ACM, Fluka). After polymerization, 2 μm thick sections were cut, deposited on glass slides and coated by dipping [15] in Ilford K-5 emulsion diluted 1 : 1. After 10 or 15 days of exposure, the radioautographs were processed in Kodak D19, stained with toluidine blue and examined with light microscope. Areas showing the most intensive radioactivity were chosen for EM autoradiography. Ultra-thin sections cut on an LKB Ultrotome III ultramicrotome were deposited on glass slides bearing a celloidin film [8], double stained with uranyl acetate and lead citrate, slightly vaporized with carbon [24] and coated by dipping in emulsion Ilford L-4 diluted 1 : 4. After 2 or 5 months of exposure the radioautographs were developed in Microdol-X (Eastman Kodak Co.). The celloidin films were floated on distilled water and the areas carrying the sections were picked up on copper grids. Sections were examined in a TESLA BS 413A electron microscope.

Results

According to earlier light-microscope investigations [6] the ^{3}H-5HT could characteristically be localized in axon profiles in the *Anodonta* ganglia, while the perikarya of the cortical layer seemed only very rarely to be labelled. The labelled 5HT was not taken up at all by the glial cells.

The present EM autoradiographic observations prove also unequivocally the axonal uptake of ^{3}H-5HT (Figs 1, 2). The greatest part of the axons incorporating exogenous 5HT contained vesicles. Among the vesicle types found in the central nervous system of *Anodonta cygnea* [5, 7] the clear (60—80 nm), and mainly the eccentric dense-core (100—200 nm), vesicles were observed in the labelled axons (Figs 3, 4, 5a). The grains were very frequently localized over eccentric dense-core vesicles (Figs 4, 5b). In other cases, the silver grains were found over axons containing also numerous dense-core vesicles of smaller diameter (70—120 nm) (Figs 5b, 6).

Sometimes, in the course of our EM analysis silver grains were also seen over a few perikarya. In all cases, the somata contained dense-core vesicles. Because of the rare occurrence of grains the statistical analysis of their distribution was not performed at all.

Discussion

Similarly to light microscopic autoradiographic results [6], the EM ones showed also the primary axonal uptake of exogenous ^{3}H-5HT in the ganglia of fresh-water mussel. This is in good agreement with the experimental observations obtained on the ganglia of *Helix pomatia* [20]. In the course of the light microscopic autoradiography of the ganglia [6], we supposed the macromolecular binding of the exogenous 5HT on the basis of the comparison of the glutaraldehyde-fixed materials with those fixed with paraform-

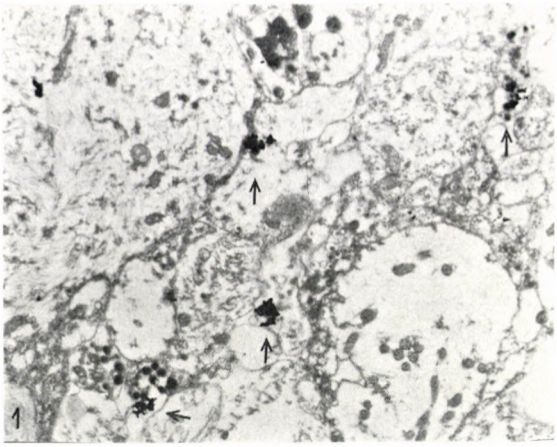

Fig. 1. Low-power magnification electron micrograph taken from the visceral ganglion of the fresh-water mussel. Silver grains are seen over axon profiles (arrows); ×12 000

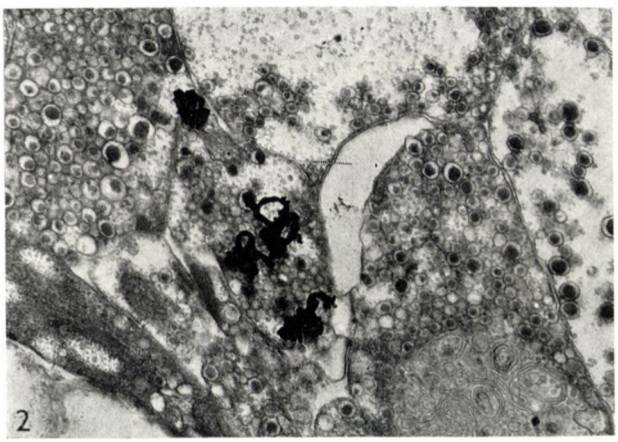

Fig. 2. Labelled axon in the neuropile of the pedal ganglion containing clear and eccentric dense-core vesicles; ×28 600

aldehyde [22]. According to DESCARRIES and DROZ [3] as well as DUCROS [4] the increased axonal incorporation of the exogenous noradrenaline and 5HT, respectively, may be explained by the presence of a macromolecular complex at the terminals. At the same time, the EM autoradiography clearly proved the view [30] that the 5HT content of the neuropile of the *Anodonta* ganglia could not be detected fluorescence-histochemically because of the lower sensitivity of the Falck-Hillarp method.

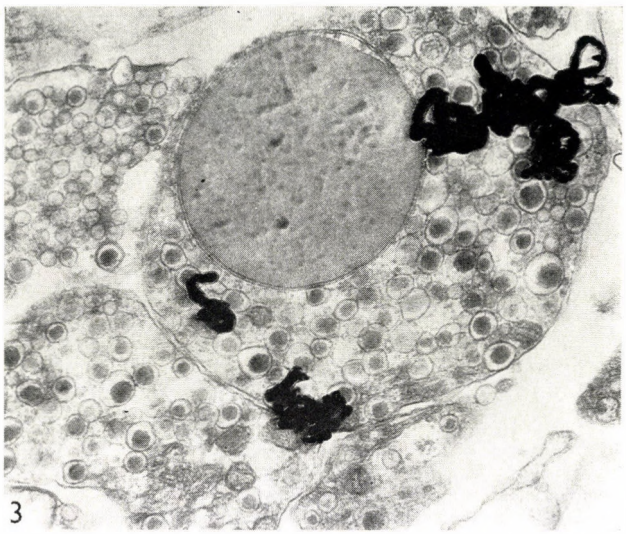

Fig. 3. High-resolution radioautography of a preterminal axon profile containing many eccentric dense-core vesicles; ×42 000

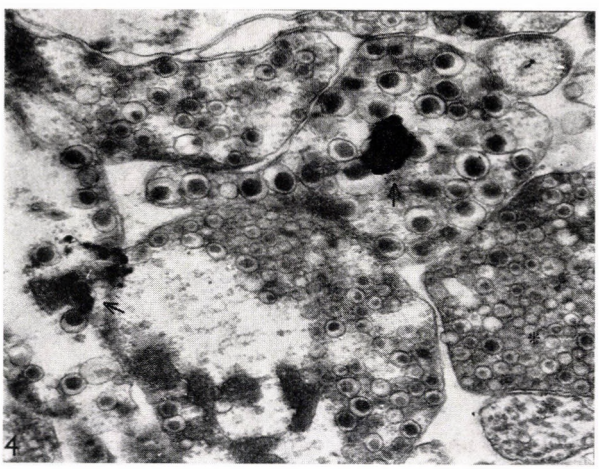

Fig. 4. Silver grains over eccentric dense-core vesicles (arrows). An axon (asterisk) showing only small dense-core vesicles is unlabelled; ×38 500

The ^{3}H-5HT uptake itself might be considered a specific phenomenon on the basis of the amine concentration (10^{-6} — 10^{-7}) M/ml employed. In the central nervous system of both vertebrates [18, 25] and invertebrates [11, 19] the uptake system, being characteristic of the 5HT incorporation exists, between these concentration values.

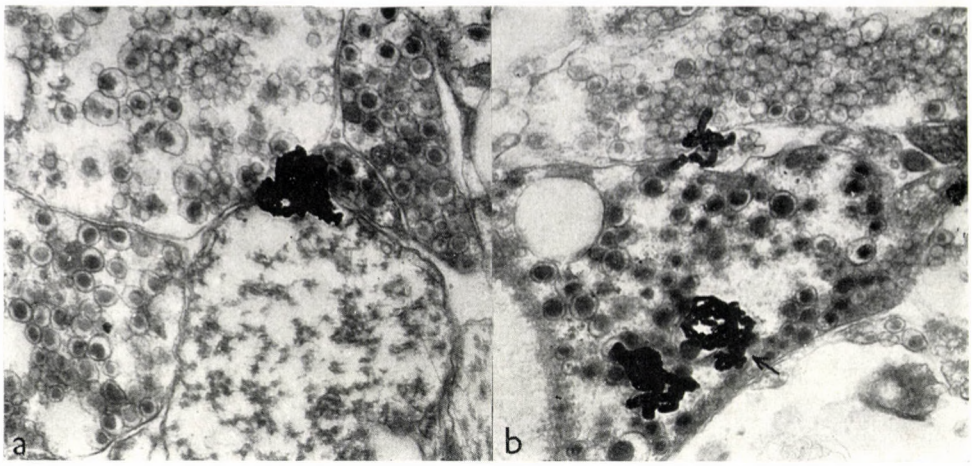

Fig. 5a—b. (a) Presynaptic area showing clear and eccentric dense-core vesicles. The labelling can be observed over the synaptic connection though another axon containing small dense-core vesicles is also in the vicinity of the grain; ×28 600
(b) Electron micrograph of an axon profile with small dense-core and eccentric dense-core vesicles. A silver grain is exactly seen just over an eccentric dense-core vesicle of large diameter (arrow); ×28 600

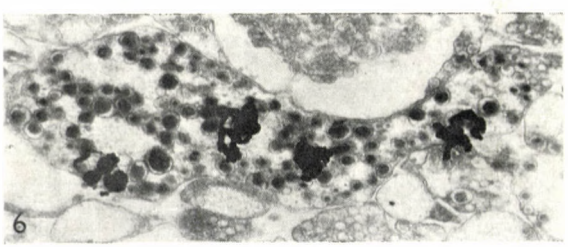

Fig. 6. Labelled axon in the neuropile of the pedal ganglion. Eccentric dense-core as well as small dense-core vesicles are also seen; ×26 400

As regards the subcellular localization of 5HT, the present results support former ones obtained after differential and density gradient centrifugation [10], since the labelled 5HT is characteristically bound to axons containing dense-core vesicles. The electron micrographs show that the silver grains can most frequently be localized over axons containing many eccentric dense-core vesicles with a diameter of 100—200 nm. Frequently, silver grains were directly seen over such vesicles. Several attempts have been made in order to localize 5HT electron microscopically in the nervous system of molluscs and other invertebrates. The storage of 5HT was shown to be bound to dense-core vesicles [14, 20, 21] among which eccentric dense-core

vesicles occurred with more or less frequency [2, 12, 23, 26, 27]. At the same time, however, EM investigations after reserpine treatment [29] and EM autoradiographic findings [18] refer to the dopamine content of the dense-core vesicles in the mussel ganglia. So, the presence of the eccentric dense-core vesicles seems to be the only and characteristic morphological feature of certain axons of the central nervous system of *Anodonta cygnea*. These axons also show the binding of labelled 5HT. Therefore, the results of HOWES and co-workers [12] and our own ones suggest the possibility of the ultrastructural identification of serotonin-containing axons by the presence of the eccentric dense-core vesicles in the ganglia of the freshwater mussel. Furthermore, the direct labelling of the eccentric dense-core vesicles may indicate also their direct role in the storage of 5HT. This hypothesis needs support by EM autoradiographic investigation of ^{3}H-dopamine and ^{3}H-noradrenaline uptake.

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^{3}H-GABA UPTAKE AND ACETYLCHOLINESTERASE ACTIVITY IN DISSOCIATED CELL CULTURES OF THE MEDIAL HYPOTHALAMUS

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Abstract

Medial hypothalamic tissue of 1 to 4 days old rats was dissociated and cultured *in vitro* for 8—10 days. Neuronal perikarya were demonstrated by supravital methylene blue staining and electron microscopy. Synapses with typical vesicles and subsynaptic thickening were also observed. ^{3}H-GABA was taken up into a small percentage of the cells in the cultures. Neuron-like perikarya and long processes accumulated the label while many neurons contained much less activity. Some astroglial and oligodendroglial cells and processes also accumulated GABA. A few neurons in these cultures contained acetylcholinesterase. It is concluded that neurons concentrating GABA and containing acetylcholinesterase are present in the hypothalamus of rats of 1 to 4 days of age and can be maintained in dissociated cell culture.

Introduction

Tissue culture methods have recently been developed that can be used in the study of various aspects of central nervous function, and the hypothalamus has been maintained in organ culture [2, 3, 6, 9, 10, 11]. An *in vitro* method for maintaining hypothalamic cells could be especially useful since the various types of hypothalamic neurosecretory cells and interneurons are known to be influenced by a large number of neuronal, hormonal and humoral factors and thus their basic properties are very difficult to study *in vivo*. We now present a method for obtaining an *in vitro* dissociated cell culture from the hypothalamus together with evidence for the existence of synaptic connections and at least two types of neurons in these cultures. During the preparation of this manuscript descriptions of dissociated cell cultures from hypothalamic tissue were published [1, 12, 13].

Material and method

One to four days old rats derived from our colony of CFY origin were used. After decapitation the brain was removed under sterile conditions. Two shallow transverse cuts were made at the anterior ends of the optic chiasma and the mamillary body, and about 1 mm deep and wide portion of medial hypothalamic tissue between these cuts was excised with fine scissors.

The hypothalami were washed several times with Eagle's medium enriched with antibiotics (200 iu/ml penicillin, 100 μg/ml of streptomycin and 100 μg/ml of neomycin), 20% fetal calf serum and 6 mg/ml glucose, cut to pieces of about 1 mm^2 and then the cells were dissociated either by trituration through a Pasteur pipette or by incubation at 37°C in a medium containing 0.25% trypsin (DIFCO) for 10 min followed by 10 min stirring in the same medium with a magnetic stirrer. The dissociated cells were sedimented by centrifugation at 600 g for 10 min and resuspended in a 4 : 1 mixture of Eagle's medium containing 6 mg/1 ml glucose and 20% fetal calf serum. The cells were plated at a concentration of 10^6/ml into Falcon plastic Petri dishes either on the inert surface or on a coverslip covered by a thin layer of coagulated plasma. Incubation was carried out at 37°C in an atmosphere containing 5% carbon dioxide. The medium was changed at 48 or 72-h intervals.

Some of the cultures were supravitally stained with 0.002% methylene blue [8], or impregnated with silver according to Holmes' method. Acetylcholinesterase (AChE) activity was demonstrated using Karnovsky's thiocholiniodide method [4]. ^{3}H-GABA (Gamma-aminobutyric acid—2, 3—^{3}H, Amersham, specific activity: 10 Ci/mM) uptake was studied by incubating the cultures in a medium containing 1.25—10 μCi/ml ^{3}H-GABA. After 15-min incubation the cultures were washed 3 times for 5 min with medium containing no GABA and fixed with 2.5% glutaraldehyde, washed and dried. Autoradiographs were prepared by exposing Kodak AR 10 stripping film for 1—7 days.

A few cultures were processed for electron microscopy by fixation in 2% glutaraldehyde in cacodylate buffer of pH 7.4 for 2 h, washing overnight, postfixing in 1% OsO_4 for 1 h and embedding in Durcupan ACM. Ultra-thin sections were stained with lead citrate and uranyl acetate and examined in a JEM 6-AS electron microscope.

Results

The dissociated hypothalamic cells were round and variable in size. They attached to the plastic or the plasma-covered coverslip in about 24 h and were seen to send out processes after having been cultured for 24—48 h. After a week the bottom of the dish was covered by an almost continuous layer of cells. Some of the cells in this layer were neurons as shown by supravital staining with methylene blue, silver impregnation and electron microscopy, but most of them proved to be of non-neuronal (ependymal and glial) cells. A few synapses have also been observed by electron microscopy (Fig. 1).

Some of the neuron-like cells and long processes strongly accumulated radioactivity in the presence of low concentrations of ^{3}H-GABA (Figs 2 and 3). The uptake proved to be saturable, since no uptake was observed in cultures exposed to ^{3}H-GABA in the presence of 10^{-4} M non-labelled GABA. Only less than about 2% of the cells concentrated GABA; but exact counts were not made, since the number of GABA-concentrating cells varied between dishes as well as at different sites in the dishes. This parallels the *in vivo* finding that only a minority of hypothalamic neurons take up ^{3}H-GABA after infusion into the third ventricle [4].

A few AChE-containing cells (less than 1% of all cells present) were also observed in a number of hypothalamic cultures. Most of the cells sent out at least one AChE-positive process (Fig. 4) and were seen in clusters containing 2—5 cells.

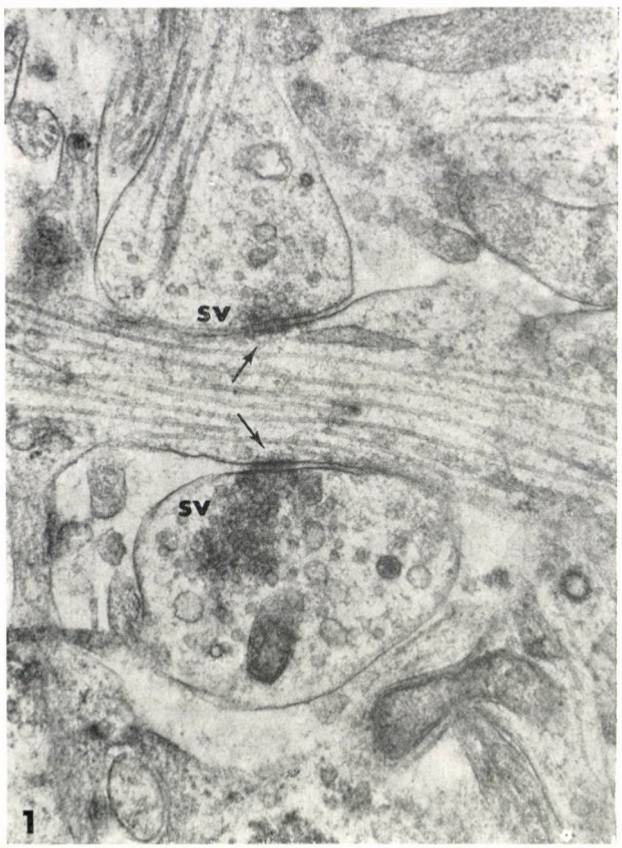
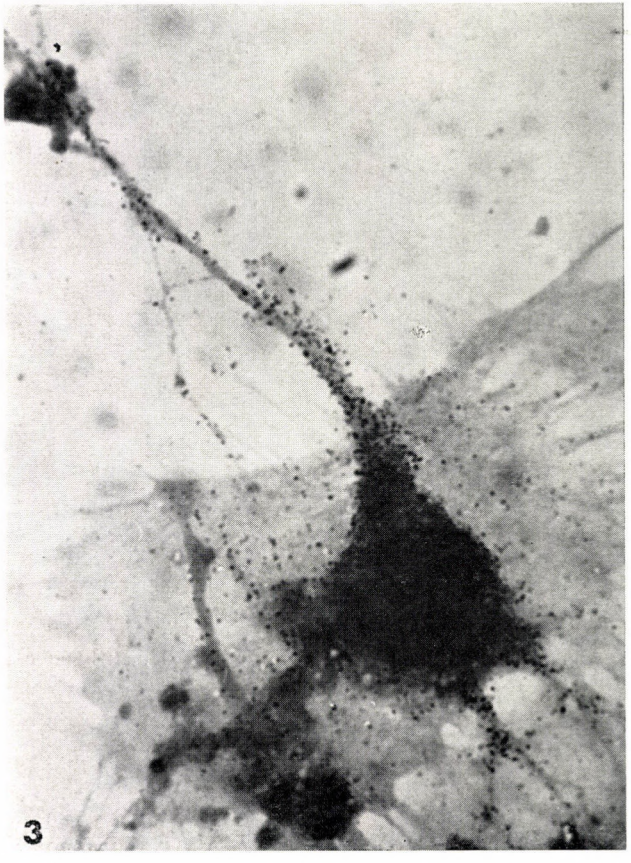


Fig. 1. Electron micrograph showing synapses in a hypothalamic cell culture maintained for 10 days *in vitro*. Arrows point to the synaptic contacts. SV = synaptic vesicles. ×31 500



Figs 2—3. Light microscopic autoradiographs from a hypothalamic cell culture exposed to ^{3}H-GABA. Note the cells covered by accumulations of silver grains over the cell body and processes. Giemsa stain, ×2 960

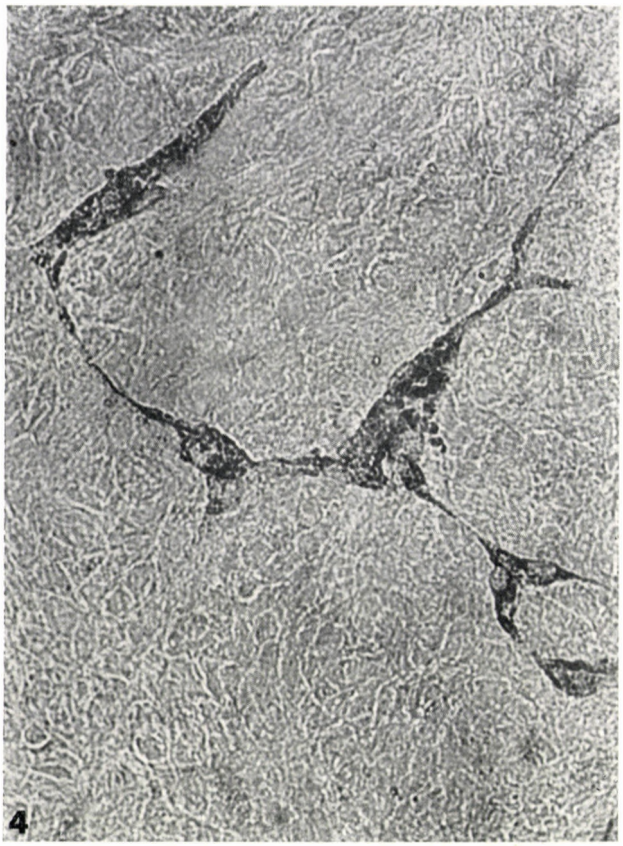


Fig. 4. Cholinesterase-positive cells with long processes in a hypothalamic cell culture. Note the absence of the reaction product in the background cells. Thiocholine method [4], ×250

Discussion

Our results as well as those published by WILKINSON and co-workers [13], BENDA and co-workers [1] and VITRY and co-workers [12] show that it is possible to obtain and culture dissociated hypothalamic neurons. However, the yield is low at present and better culture conditions as well as other manipulations (e.g. addition of nerve growth factor) might be necessary to obtain cell cultures with more hypothalamic neurons. Our electron microscopic results suggest, although do not prove, that synapses between the neurons develop in these cultures, since some synapses in a few cell clumps might have been preserved during the dissociation process. For proving *in vitro* synaptogenesis in hypothalamic cell cultures, longer survival times and/or the study of larger number of cultures with the electron microscope might be necessary.

The presence and growth of neurons in our cultures are indicated by the following findings: (1) some cells resembled multi- or bipolar neurons, (2) synapses with the characteristic ultrastructural features could be identified, (3) networks of long processes were impregnated with silver by the Holmes method, and (4) some bi- and multipolar cells contained AChE. Moreover, cells with long tortuous processes, strongly concentrating ^{3}H-GABA, have also been found. These cells seem to concentrate specifically GABA since labelling with other amino acids, such as glutamate, revealed a widespread and almost uniform labelling of all cells.

If neurosecretory cells and hypophysiotrophic releasing and inhibiting factors can be demonstrated under these conditions, such cultures may serve as a useful model system for studying a wide variety of problems, such as neurotransmitter action on neurosecretion, interrelationships of electrical and secretory phenomena, hormonal and humoral effects on neurosecretion, etc. The advantages of this model include the experimental control over many basic variables of the system, the easy penetration, and even distribution, of substances. Moreover, cells may be studied first by electrophysiological techniques and then the same cells might be studied by light or electron microscopy after applying specific histochemical staining methods.

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RECENSIONES

H. HAYDEN: **Laboratory physics for the life sciences**
Saunders Publishers, Philadelphia (1975), pp. 428.

The aim of this book is not involved in the title. It is presumably meant as a handbook for University preparatory courses. The style and arrangement of the volume are typically American, in a positive sense. The selection of curriculum does not agree entirely with our similar courses and secondary-school practice. Exercises starting like this: "take a 25 cm in length mathematical pendulum..." or, "measure acceleration by direct observation of the free falling of a body..." do not seem to be the most important from the aspect of life sciences. Nevertheless, these exercises are pedagogically valuable. The rather high-sounding title, the American book-cover and the elaboration of the book are extremely suitable for arousing and keeping up interest in methods of measuring in physics, which are in themselves rather dry, but certainly develop precise thinking. In this beautiful handbook, the protocoe formulas are a credit especially worth mentioning. Various coordinate papers and functions necessary during exercises are also presented. Thus, besides the 179 pages of text the volume of the book is increased to 428 pages. After the 22 practicals teachers are given very useful instructions.

A Hungarian translation of the book would certainly enrich our secondary-school education literature.

GY. TURCHÁNYI (Budapest)

Biokybernetik (Biocybernetics). Vol. 5
Materialien des IV. Internationalen Symposiums "Biokybernetik", Leipzig, 19—22, September, 1973.
VEB G. Fischer Verlag, Jena (1975), pp. 368.

This volume contains the material of the fourth International Symposium "Biocybernetics". Nowadays we frequently witness the aim which, concerning a problem, sets to obtaining a high-level integrated knowledge instead of a mosaic-like description given by the different branches of science. A fair example of this aim is given by this symposium, where results of this type were presented and which gave possibilities for researchers of different previous training to get into contact with or to strengthen these contacts. In biological systems many phenomena are related to the problems of communication and control, and cybernetics as an approach has proved very fruitful in the research of these processes. The book contains about two-third of the papers delivered at the symposium (about 50 papers), and consists of the following six chapters:

— Processing of information in the central nervous system
— Receptors and receptor mechanisms
— Processing of information in the visual system
— Pattern recognition in the visual system
— Pattern recognition (general and technical aspects)
— Regulation and adaptation

The papers give an accurate account of the relation characterizing the present stage of the progress of biology and cybernetics. Biocybernetics is a complex science, which can progress in several ways, viz., (a) biology and cybernetics may proceed on different paths and meet only at the synthesis of the material or (b) the exploration of a given problem proceeds from the beginning along the lines of this complex approach.

In the volume we can find numerous papers for both cases, but for the sake of illustration we mention examples from the chapter on receptors. In the work "Comparative study of the ultrastructure of arthropodal mechanoreceptors" (H. FÜLLER, A. ERNST) the authors describe electron microscopic morphology of three different types of mechanoreceptors and give their comparative analysis. In this way, they contribute to the data on a biological structure the synthesis of which will be made possible just by the aid of the cybernetical approach.

On the other hand, the paper "On the transduction mechanism in biological receptors with special emphasis on mechanoreceptors" contains mainly theoretical cybernetical considerations. The author explains the transducing action of receptors by the aid of a general transducer model and uses the mathematical solution of the model for interpretation of the experimental results in the literature. Works of such type may suggest new problems for experimental workers.

As an example of the third of papers we mention the work of B. BROMM "On the impulse formation in the receptor in the view of the ionic theory of stimulation", in which the author makes an experimental study on the formation of the rhythmical responses and for the interpretation of his results he applies the ionic theory of stimulation. He describes the model by a partial differential equation and compares its computer-made solution to his own results and, based on this comparison, draws conclusions on the formation mechanism of action potential.

It may be stated in general that in its six chapters the volume gives a number of valuable results for the study of phenomena listed as the main problems. The experimental parts contain very accurate, up-to-date electrophysiological measurements, and in the mathematical model construction we find about whole armory of modern mathematics (graph theory, matrix arithmetics, processes, etc.). The book seems to be a useful attitude forming information source for physiologists, biologists, researchers of theoretical medical sciences, as well as for physicists, mathematicians and engineers interested in biological problems.

I. TARJÁN (Budapest)

VALKOVIC, V.: **Trace element analysis**
Taylor and Francis Ltd., London (1975), pp. 229, £ 7.—.

Knowledge of trace elements is now indispensable in all the domains of life, science and technics. Nowadays the term "trace elements" is used to denote elements found in concentration of the order of one part per million (ppm) or smaller, in different systems. The book deals with the occurrence in nature of trace elements and with the modern analytical techniques by which concentrations at ppm levels can be measured. The book opens with the description of the genesis of the cosmic matter under the physical conditions existing in stars, and the gradual formation of heavier elements of the periodic system from lighter ones, from the nuclear "fuel" of stars, i.e. from hydrogen and helium. The author gives a detailed description of trace elements and of their relative abundance in the soil, water and air. Owing to man's activities, the distribution of trace elements can be affected. In fact, environmental pollution results from the introduction of different substances into the marine, atmospheric and soil environment. The author calls attention to the biological consequences of the pollution of the air, water and soil as it is the environmental equilibrium of trace elements that ensures the viability and health of the biological systems on the earth.

The book gives an overall view of the role played by trace elements in the biological systems, the importance of the essential elements in catalytic processes at the molecular level, in the maintenance of DNA and RNA structures and in protein synthesis. The role of trace elements in biology and medicine in connection with problems of health and disease associated with the geochemical environmental background (e. g., carcinoma, normal and pathological haematopoiesis, etc.) is discussed. The book gives a detailed description of the general aspects of trace element investigation including the most sensitive analytical methods, neutron activation analysis, X-ray, and optical emission spectroscopy, atomic absorption analysis, mass spectrometry, and the application of these methods to trace element analysis. Less attention is paid to optical spectroscopy on which trace element investigation is based. The book is complemented with a great number of tables and figures containing valuable data on the subject.

The book can be useful to all those who are just entering the field of trace element analysis and to those who are more deeply interested in these problems. The excellent book, written mainly from the physical viewpoint, gives general and special information on all the aspects of trace element analysis.

I. FÖLDES (Debrecen)

Immunobiology of trophoblast

Ed. EDWARDS, R. G., HOWE, C. W. S., JOHNSON, M. H.
Cambridge University Press, London (1975), pp. 284. £ 6.—.

The trophoblast at a very early stage in the development of an embryo is highly specialized for interaction with the mother. Being antigenically neutral and acting as a selective immunological filter between mother and foetus, it is of central importance in maintaining the survival of the foetus, antigenically foreign to the mother. The properties of these cells, their response to the endocrine and immunological components of pregnancy, are the central concern of this book. It is the first of a series of meetings in which specific topics in immunoreproduction will be debated. This volume is mainly based on the material of a conference held in 1974 in the Physiological Laboratory of the Cambridge University.

Antigenic topography and interactions of antibodies and cells with surface antigens are discussed by ALAN MUNRO on the basis of the fluid mosaic model of membrane structure (proposed by SINGER and NICHOLSON). After general and technical considerations he shows by electron microscopy of rabbit blastocysts that trophoblastic cells have all the features associated with secretory epithelial cells and suggests that the antigenic determinants expressed on the outside of the trophoblast differ from the determinants expressed on the inside. R. GARDNER writes about the pre-implantation origins, properties and early post-implantation development of trophoblast cells as revealed by recent experiments. BILLINGTON summarises the placental organization, ultrastructure and histochemistry in the most extensively investigated species: mouse and man, stressing that as the evolution of the viviparous state and the immunological system has occurred side by side all other placental forms must have been biologically successful in their development.

Separate chapters deal with the peri-implantation trophoblast, the post-implantation placentae, and the antigens shared by tumor cells, and foetal or gonadal cells.

Very interesing is HOWE's contribution about lymphocyte physiology during pregnancy. The use of *in vitro* tests for studying lymphoid cell–trophoblast interaction is reviewed and suggestions for further study are outlined. A preliminary experiment in which the transfer of mouse spleen cells sensitized to HCG causes loss of developing embryos is described.

A study of BORAND and co-workers reflects trophoblast endocrinology and immune privilege from another viewpoint: the treatment of trophoblastic cells with neuraminidase results in the loss of their immunological feature, but subsequent treatment of the cells with HCG restores protection to the trophoblast cells.

Discussing the immunogenicity of the placenta and trophoblast, BAGSHAWE and SYLVIA LAWLER also think that evidence for the presence of alloantigens on the trophoblast of laboratory animals remain controversial. In man, both ABO and HLA antigens are probably represented on trophoblast and in the case of HLA antigens may even be immunogenic.

ADINOLFI reviews the human placenta as a filter for cells and plasma proteins. Finally, by ANNE MCLAREN, evidence is reviewed bearing on the hypothesis (CLARKE and KIRBY, 1966) that antigenic disparity between mother and embryo is beneficial to the development of the embryo, and this effect contributes to the maintenance of histocompatibility polymorphism. It is concluded that the hypothesis is no longer tenable, as the experimental foundations on which the hypothesis was originally based are no longer secure.

Thus, from electron microscopy to histocompatibility polymorphism, all the existing body of knowledge and theories are questioned and discussed in this book.

GY. PETRÁNYI (Budapest)

Antibody structure and molecular immunology

Proceedings of the Ninth FEBS Meeting, Budapest 1974, Vol. 36.
Eds J. GERGELY and G. A. MEDGYESI, Akadémiai Kiadó, Budapest (1975, 170 pages), 60 figures.
Price: Forint 100.—.

The present volume contains the proceedings of the invited lectures delivered on immunochemistry at the Ninth FEBS Meeting held in Budapest, August 25—30, 1974. The subject of the book is divided into two parts.

The lectures of the first part deal with the problems of antibody structure and function. All the 12 lecturers are outstanding experts of this new topic of biochemistry and immunochemistry.

The first paper presented by J. D. CAPRA was concerned with the structure of heavy variable regions. New data on hypervariable regions, the combining site and idiotype specificity were presented. The topic of evolution of immunoglobulin V region genes was covered by A. C. WANG. J. C. JATON suggested some allotypic specificities in hypervariable regions.

F. M. POULSEN studied the small conformational changes of IgG molecule by hydrogen-tritium exchange technique. J. R. CLAMP found three types of oligosaccharide units in immunoglobulins. D. BEALE studied the structure of porcine IgM.

F. FRANEK attempted to study the antibody function with respect to multivalence of antibody and antigen, as well as the influence of macromolecules on the interaction. J. GERGELY and G. A. MEDGYESI reported on results concerning the preferential reassociation of autologous and heterologous chains of immunoglobulins. The localization of rat allotypic specificities were summarized by R. S. NEZLIN. O. V. ROKHLIN stated that allelic genes are not equally active during antibody formation. Rat monoclonal immunoglobulins were studied by H. BAZIN. The high incidence of IgE secreting tumours allowed the study of catabolism of this immunoglobulin. The complement binding activity of rat monoclonal immunoglobulins was presented by G. A. MEDGYESI.

The second part of the book deals with some problems of lymphocyte receptors and antibody synthesis. The five reports give an outline of the topic without covering all the current trends. The outstanding paper on control of antibody formation written by A. R. WILLIAMSON summarizes the different levels of control mechanisms regulating the biochemical events in immunoglobulin synthesizing cells. T. H. RABBITS approached the problems of V and C gene interaction with molecular hybridization experiments of messenger RNA.

The following papers report on the lymphocyte membrane receptors. R. M. E. PARKHOUSE found a T lymphocyte specific protein without immunoglobulin characteristics. The membrane associated IgM was found to be in correlation with immunocompetence of pig foetuses (L. JAROSKAVA). The localization and function of Fc receptors of B lymphocytes and macrophages were summarized by G. SÁRMAI.

At the end of each lecture references are presented.

The very interesting and valuable volume discloses many new aspects of molecular immunology and gives the most recent data on the correlation between structure and function of antibodies. The book may be of great interest not only for immunochemists but also for biochemists and molecular biologists involved in modern immunology. Without being a complete review of the field the volume gives rise to numerous thoughts and attracts attention to the trends of future immunochemistry.

S. R. HOLLÁN (Budapest)

Human genetics. Vol. 1

A. A. NICHIPOROVICH. Hall et Co., Boston (1975), pp. 120, US $ 22.— + 10%.

This book deals with those topics of human genetics, where the greatest progress has been made during the last few years. It has four chapters. The first chapter was written by BULANOV and GRINBERG about the linkage of human genes and about the mapping of the human chromosome. The different methods of mapping are described: the geneological analysis with mathematical procedures, the cell hybridization, the gene localization by the analysis of chromosome variants and of the cases with chromosome aberration (duplication, deletion), further, the *in situ* hybridization. The description of gene linkages and — if it is known — their localization can also be found in this chapter. The second part of the book was written by A. P. ZAKHAROV about new methods of chromosome examination: the examination of the different spiralization of the chromosomes, the fluorescent bands, Giemsa banding, the *in situ* hybridization and the supposed mechanism of these reactions. The author of the third chapter is K. D. KRASNOPOLSKAYA. This chapter deals with the investigation of the inborn errors of metabolism in cell cultures and gives a description about the general pathophysiology of this group of diseases. A well-constructed table gives a good survey about the large collection of data. In the last chapter, written by A. D. TAMARKINA, the biochemical aberrations of chromosome anomalies, mostly of trisomy 21, are described. At the end of each chapter a short summary and a good collection of references can be found.

This book gives a good survey and list of references for the scientist who is interested in the above-mentioned topics of human genetics. The results and the references of the last three years are not included in this volume, the first edition of which appeared in Russian in 1973.

D. SCHULER (Budapest)

Human gene mapping, 2

Rotterdam Conference, 1974.

Ed. D. BERGSMA. S. Karger, Basel—München—Paris—London—New York—Sydney (1975), pp. 310, SFr. 79.—

The mapping of the human chromosomes is one of the most fascinating and rapidly developing branch of human genetics. Recent results on human gene mapping presented at the 2nd International Conference are collected in this book. The first part of the volume contains the reports of the committees dealing with the map of the 1st and 2nd chromosome, with the chromosome constitution of the other autosomes and of the X chromosomes. The gene linkages, detected by family studies but with yet unknown localization, are also described. The chapter about the gene markers used in the human gene mapping (enzyme, protein, antigen and other markers) has a practical value for the interested scientists. In the second part of the book, short publications can be found concerning methods of gene mapping (e.g. *in situ* hybridization, gene mapping by exclusion in some chromosome aberrations, linkage analysis, etc.), about the gene localization of several markers and about gene linkages. Some of them represent new data, others confirm earlier, yet unproved, publications. The excellent collection of new data on human gene mapping and the high number of references make this volume very useful both for research workers of this topic, and for scientists interested in this new field of human genetics.

D. SCHULER (Budapest)

TIMOFEEFF-RESSOVSKY, N. V., VORONCOV, N. N., JABLOKOV, A. N.: **Kurzer Gundriss der Evolutionstheorie**

VEB Gustav Fischer Verlag, Jena (1975), pp. 285, figs 132, M 36.

No doubt that there is a great need of a modern, comprehensive book on the theory of evolution by which the experts of many different scientific branches can profit a great deal. The necessity to learn as much about the evolution as possible seems to be inevitable for every biologist in a broader sense, regarding everybody dealing with living things, as a biologist. This is why Professor STUBBE's (editor) contribution in preparing the German edition of TIMOFEEFF-RESSOVSKY's book is warmly welcome.

The original Russian edition was published in 1969, and the present German version is not merely a translation; also the structure of the book has been altered.

Writing a book by three different authors, recognized researchers in their subject, involves some advantages and, also, some disadvantages. This holds true to this book as well, especially because the authors are living far from one another (Moscow—Obninsk—Vladivostok) and belong to different generations of scientists. This means that different views have been gathered to form an entity, but it also means that some problems reappear several times throughout the text.

This book consists of three main parts, viz., I. Life and Evolution, II. The Process of Microevolution, III. Present Interpretation of the Basic Facts of Evolution. It deals with problems like the development of life on earth, Darwin's theory and the ideas of evolution, the elementary factors and material of the evolution, the main steps and directions of evolution, the micro- and macrophylogenesis, the progress, pace and forms of evolution, as well as the role of environment and adaptation including the problems of the biosphere. The book can readily be used for all those who wish to get acquainted with the most important problems of the evolution.

The ample list of bibliography, more than 900 references, gives a great support for those who wish to deal intensely with the problem.

G. SZEMERE (Szeged).

The use of genetics in insect control

Ed. R. PAL, M. J. WHITTEN. Elsevier/North-Holland, Amsterdam (1974), pp. 241.

This book consist of 11 papers, selected from the matters of the 14th International Congress of Entomology, held in Canberra, Australia, in August, 1972. As it is usual in such cases, certain unbalancedness is almost inescapable, particulary if the subject covered is so new and controversial. In my opinion, the best editorial policy was chosen: difficulties are not avoided, but uncovered.

Research programmes devoted to development of new genetic control methods are anything but not cheap and these methods are hardly to be useful if applied alone. Moreover, each insect species must be treated as a separate problem. On the other hand, it is also true that results achieved in a particular case — if they are adapted properly — might be helpful in solving other problems. Papers are selected in accordance with this consideration overlappings are successfully reduced. In spite of the essentially practical goals of these studies more academic aspects of genetics are inevitably enriched by them. To illustrate only (hoping that I will not be accused to be unfair either by authors of this book or by the reader), in this context I should like to mention the paper on incompatibility in *Culex pipiens*. But in every other paper one can find valuable additions to genetics, environmental sciences and other disciplines of biology. For these, and for elimination of false expectations and misbeliefs shared by specialists and non-specialists, this volume deserves everyone's attention interested in related problems.

T. PÁTKAI (Budapest)

HILL, D.: **Agricultural insect pests of the tropics and their control**
Cambridge University Press, London (1975), pp. XVI + 516, £ 12.

Professor DENNIS HILL's book is an excellent example how this kind of work should be written.

The heart of the book, the chapter on 250 major tropical insect pests, is organized along taxonomic lines. Discussion of every species follows the same pattern, including scientific name, common name, family, hosts, damage, pest status, life history, distribution (with maps!), control, further reading — this list speaks for itself. Additionally, good and clear line-drawings of imagos and, if it is necessary, pictures of both sexes together with developmental stages as well as figures showing characteristic forms of damage are given for each species. Obviously, in spite of the title, due to the world-wide distribution of many species, even this apparently special chapter is definitely useful for scientists working outside the tropics.

The same holds true for chapters on principles and methods of pest control, chemical control of pests, not to mention the glossary and the sizable bibliography. Indeed, while didactical and practical aspects are pursued down to the level of use of short drinks for transitional preservation of insect specimens under tropical conditions, this does not diminish at all the scientific correctness required. Consequently, altough the book is primarily intended for use as textbook for undergraduates and postgraduates, the result surpasses this intention considering not only the content but, I am afraid, the price too. It is well worth a place on the bookshelf of professional practitioners of identification and control of insect pests, moreover, in all agricultural or biology libraries. Nevertheless, being produced according to the well-known, always delightful standards of Cambridge University Press, it may be rather expensive for individuals other than the specialist.

T. PÁTKAI (Budapest)

DE BACH, P.: **Biological control by natural enemies**
Cambridge University Press, London (1974), pp. XI + 323, £ 5.50.

It is not difficult to appreciate the harmful effects of pesticides used increasingly in world's agriculture. This is stressed by the author, who shows many not yet fully exploited possibilities offered by biological control. According to his persuasively presented opinion, carefully designed, integrated use of biological control, other non-chemical methods and pesticides harmless to human environment would cause a substantial reduction in the amount of pesticides used. In this way, besides prevention of health and environmental problems, costs of pest control might well be reduced.

Owing to its marked clarity or even didactic exposition of the subject and the laudable intention of the author to cover almost all possible aspects in some detail, this volume is useful for everyone interested in these problems. For research workers, the vast amount of data, especially historical ones embedded in enthusiastic stories, are interesting.

It is somewhat disappointing that apparently little attention was paid to the illustrations. The darkness of several old, historically important, photographs is somehow explainable, but it is much more difficult to understand why there are two separate photos signed as Fig. 12 or why Fig. 20e — depicting an ordinary shipboard — is inserted upside down.

T. PÁTKAI (Budapest)

HOPE, A. A., WALKER, N. A.: **The physiology of giant algal cells**
Cambridge University Press, London (1975), pp. 201, £ 8.50—.

Microscopic algae have been excellent subjects of physiological experiments for a long time. Particularly, unicellular algae are suitable for solving physiological problems, as they are easy to influence and divide rapidly. Thus, results are available in a few hours.

The authors of this booklet are physicists, who discuss the biophysical physiology of giant algal cells. The habitude of unicellulars is independent, while in multicellular organisms vital processes proceed at a higher level and are much more complicated. Thus, the giant algal cells have proved very useful for experimental purposes.

The book has several great merits: (a) the subject has been excellently summarized and many valuable literary references have been listed; (b) mathematical formulas are applied whenever possible; (c) the precise presentation of the subject is exemplary; (d) new apparatuses and methods are excellently described; (e) the ion transport is thoroughly discussed.

Authors present a historical approach to the subject; after a retrospect, present knowledge is recorded, and the theoretical and practical perspectives are discussed.

The volume consists of 12 chapters, a subject index, preface and a list of symbols and abbreviations. Giant algal cells as experimental subjects (Charophyta, Chlorophyta: Hydrodictyon, Phaeophyta, Rhodophyta), The 200 year history of physiology, Water relations, Permeability to nonelectrolytes, Compartments and ionic concentrations, Ionic fluxes and kinetic models, Electrical properties of membranes, Action potentials in Charophyte cells, Studies of active transport-cations, Studies of active transport-anions, The transport and use of carbon dioxide and bicarbonate ions, Protoplasmic streaming are the subjects discussed in the chapters. Each chapter is divided in subchapters.

The book is completed by an Appendix. The titles are as follows: Notes on equations and their derivation, Rates of energy conversion in Chara et Griffithsia.

The list of references is very detailed; 363 titles and an index terminate the book.

The beautiful electron micrographs, the tables, graphs and diagrams are worth special mentioning.

The volume will be of interest to physiologists, biophysicists, university teachers and students. A Hungarian translation of the book is warmly recommended by the reviewer

T. HORTOBÁGYI (Budapest)

COOPER, L. P.: **Photosynthesis and productivity in different environments**
Cambridge University Press, London (1975), pp. 715, £ 22—.

This valuable manual presents the research work performed by the numerous plant physiologists and ecologists who have collaborated in the International Biological Programme for human welfare. The very divaricating papers and many data are synthesized in eight main chapters and numerous subchapters. Firstly, the utilization of light energy in the biosphere, viz., in woods, in agriculture, in the tundra and deserts is comparatively discussed. Hereupon the water ecosystems are dealt with, reflecting primary production.

Within the large ecosystems, authors consider the capability of photosynthesis of the single organs of various plants. In addition, the effects of life phenomena like respiration, internal transport, growth, mineral nutrition and those of environmental factors on plant productivity are examined.

At last, the possibilities of increasing the productivity of photosynthesis are discussed.

The volume substitutes many original sources by presenting a precise list of the origin of data given in this volume.

This excellent comprehensive manual is valuable for ecologists and plant physiologists as well as for students and teachers in training.

V. FRENYÓ (Budapest)

CUSHING D. H.: **Marine ecology and fisheries**
Cambridge University Press, London (1975), pp. 278, figs 68, £ 3.90—.

The author is particularly concerned with the application of quantitative methods of measurements for fish stocks. His newly published book has been focussed to fish production problems in the sea. A very useful glossary is found at the fore-part of the book containing the symbols and abbreviations being used in the text. The book covers some special fields

of marine ichthyology with the following 12 chapters: Production cycles in space and time; Production cycles in upwelling areas; The part played by nutrients in the sea; Models of production in the sea; The circuits of migration and the unity of fish stocks; Growth and death in the sea; The population dynamics of fishes; Fish stocks and the production cycles; Food webs in the sea; Temporal changes in the sea; Climatic changes in life in the sea; The regulation of fish populations by nature and by man.

The book is arranged in three main parts. The first four chapters describe the mechanisms of production in the sea, the basic parameters and the models that have been developed. The second group of four chapters deals with the biology of fish stocks, their population dynamics depending on the variability of the production cycles. The third and fourth group of chapters describes more general themes, the use of food-chain studies to fisheries biologists, the nature of climatic change and the match in time of larval production and that of their food.

The illustration is of high-quality standard, the literature cited comprises every essential work. The volume can be recommended to all those teachers, research workers and students who are interested in population and production ecology.

P. Biró (Tihany)

E. T. Burtt: **The senses of animals**

The Wykeham Science Series, WykehamPublications Ltd, London and Winchester (1974). pp. 160, £ 2.50.

The aim of this book to provide, in a short and accessible form, the most important information about the senses of animals. The volume is based on the author's lectures given to second year students. It consists of eight chapters complemented with an index and a short list of advised literature. Chapter 1 deals with the general principles of the structure and function of sense organs. The following chapters are devoted to the chemical senses (taste, olfaction and chemoreception in water and soil), mechanoreceptors and hearing, including echolocation. Two chapters cover the problems of vision in vertebrates and in arthropods and cephalopods separately. In the last two chapters the electric sense of fishes, the pain, temperature, humidity and magnetic field reception are described. In all chapters the discussion of physiological data is preceded by a description of the general morphology and ultrastructure of the given receptor and the basic physical principles of its function, which greatly facilitates the reading of the text. The volume is well illustrated and has a good technical presentation. It is written primarily for undergraduate students, but may be recommended for all those wishing to have a survey about the present state of knowledge of animal senses.

J. Kovács (Budapest)

Development and regeneration in the nervous system

British Medical Bulletin, Vol. 30, No. 2, May, 1974.

In the British Medical Bulletin as usual the actual problems of a rapidly developing branch of science are discussed. Biologists and physicians are widely interested in these subjects.

The 15 papers in the issue under review touch all the main problems being in the centre of the interest of neurological research.

In the Introduction J. Z. Jüng emphasizes that the total of the papers presents an up-to-date picture of the interrelations between the regulatory functions of the nervous system, inheritance, and the defence mechanisms of the organism.

The first three papers, "Axon and cell numbers" (M. C. Prestige), "Cellular responses to axotomy and related procedures" (W. E. Watson) and "Neural specificity" (R. M. Gaze), present valuable data on the cellular phenomena in the nervous system. R. F. Mark's paper, "Selective innervation of the muscle", presents an interesting review on the neuromuscular junction and its regeneration. R. Balázs in his paper "Influence of metabolic factors on brain development" discusses the influence of extraneural factors on the development of the brain. M. Berry and A. C. Richers ("Immunology and neural regeneration") present a new hypothesis to explain the errors in neuroaxon regeneration in the CNS of mammals and the errors in the immunoactive status and in the related protein synthesis. The regeneration is the subject of the last paper (W. J. McDonald: "Regeneration in relation to clinical

lesions of the central nervous system") as well. In a paper entitled "Plasticity of synapses" B. G. CRAGG analyses the numerical and intensity changes of synapses on a morphological and electrophysiological basis. M. J. KEATING ("The role of visual function in the patterning of binocular visual connections"), comparing the vision of amphibians and mammals, concludes that the visual function of amphibians plays an important role in the evolution and modification of binocular vision. In the paper "Causation of neural tube effects: clues from epidermiology" some important causal factors of CNS malformations are discussed by J. LECK. J. DOBBING and J. L. SMART ("Vulnerability of developing brain and behaviour") attempt to clarify the relationship between the changing physical and chemical conditions of the brain and the development of human behaviour. J. TIZZARD ("Early malnutrition, growth and mental development in man") examines the structure and the function of the brain in relation to the development of the child's intellect. Similar subjects are discussed by T. G. BOWER ("Development of infant behaviour") and by A. B. D. CLARKE and ANN M. CLARK ("Mental retardation and behavioural change").

There is no doubt that this issue deserves much interest.

I. TÖRŐ (Budapest)

Recent studies of hypothalamic function

Proceedings of the International Symposium on Recent Studies of Hypothalamic Function Calgary, 1973.
Ed. LEDERIS, K., COOPER, K. E. S. Karger, Basel (1974), pp. 434, SFr. 135—.

An increasing body of evidence indicates that the diverse functions of the hypothalamus are interrelated and that there is an overlap of divergent functions in hypothalamic areas. This suggests the possibility of a non-specificity of hypothalamic neurons. In the Symposium the leading workers interested in various aspects of the hypothalamus presented their recent findings and discussed the highly integrated structural and functional organization of the hypothalamus. Three hypothalamic functions were discussed in detail. (1) Hypothalamus and hormones. Sixteen papers were devoted to this subject. Among others, the control of anterior pituitary hormones (hypothalamic releasing hormones, neurotransmitters, hormonal feedback), the hypothalamo-neurohypophyseal system and the functional identification of hypothalamic neurons have been dealt with. (2) Metabolic and behavioural aspects of hypothalamic function. Contributions discussed effects of alcohol, angiotensin and essential amino acids on the lateral hypothalamus, role of the hypothalamus in the control of cardiovascular functions and of ingestive behaviours and hypothalamic changes in hibernators. (3) Hypothalamus in thermoregulation, dealing with fever, prostaglandins in fever, hypothalamic control of thermoregulatory behaviour, ionic concepts of the set-point for body temperature, hypothalamic blood flow and a neuronal model of temperature regulation. At the end of the Proceedings there is a summary of the final sessions in which general aspects of endocrine and non-endocrine functions of the hypothalamus were considered.

The book provides useful information to all those who are interested in the hypothalamus, i. e., to neuromorphologists, physiologists, neuroendocrinologists and clinicians.

B. HALÁSZ (Budapest)

MYERS, R. B., CANTINO, E. C.: **The gamma particle**

A study of cell-organelle interactions in the development of the water mold Blastocladiella emersonii
Monographs in Developmental Biology, Vol. 8. S. Karger, Basel—München—Paris—London—New York—Sydney (1974), pp. 117, SF. 67.

This monograph provides a comprehensive review on gamma particles of the zoospore of fungus *Blastocladiella emersonii*. The gamma particle is an unusual DNA-containing cytoplasmic organelle, which was discovered by one of the authors some 20 years ago. Later on, its structure, genesis and biochemistry were investigated in detail, and in this volume the most important results accumulated during this time are brought together.

The book consists of 12 chapters complemented with a list of references fully covering the literature on the subject. Following the historical introduction, the authors deal with the

ultrastructure, genesis and decay of the gamma particles. Separate chapters are devoted to their staining properties, isolation and chemical composition. It is shown that the particles contain DNA, RNA and high chitin synthetase activity. The effect of visible light on gamma particles is described in detail. In the last chapter the function of the gamma particles, their involvement in cell-wall synthesis, the problems of their autonomy, continuity and possible occurrence in other aquatic fungi are analysed.

The book is well written and contains a large number of illustrations. It may be most useful for mycologists, microbiologists and for all those interested in cell organelles carrying extranuclear DNA.

J. Kovács (Budapest)

Basic biology course

Tribe, M. A., Eraut, M. R., Snook, R. K.:
Book 1. Light microscopy, pp. 108, £ 2.75. *Book 2. Electron-microscopy and cell structure*, pp. 117, £ 2.75. *Book 4. Ecological principles.* pp. 160, £ 2.75. *Book 6. Photosynthesis*, pp. 77, £ 2.40. Cambridge University Press, London (1975).

The gigantic cumulation of knowledge calls for further development of teaching technics. The Sussex Inter University Teaching Project Team has attempted the writing-up of fundamentals in biology in this respect. The four volumes reviewed below are members of this series and display an excellent example of scheduled teaching. Each volume is excellently illustrated, both the schematic drawings and the question–answer method facilitate self-control during learning and thus profound knowledge can be acquired.

In the first book the various kinds of microscopic picture, the types of microscopes and the physics of microscopy are described, among others. The stereo- and phase contrast microscopes are discussed in such a clear way that the reader can learn to recognize at first sight the various sorts of objectives and their application in practice. Size analysis in microscopy and the technics of preparation are also discussed. Moreover, the rough structure of cells and chromosomes and the structure of *Drosophila* and certain cytological and genetical fundamentals are presented.

In the second volume an excellent atlas of cytology is presented in the form of questions and answers, and very good drawings help understanding. As a completion, modern methods applied in cell biology, for example, ultracentrifugation, replica method, freeze-etching and scanning electron microscopy are reviewed. A very useful dictionary is found at the end of the volume. References in the text help the application of the recording tape and film slides enclosed in the supplement.

In the fourth book sparkling diagrams displaying experiments guide the reader from the ecological problems of lakes to the closed ecological system of space-ships. A separate chapter deals with man and ecosystem, stressing problems due to demographic explosion and environmental pollution.

Although all volumes of the series are excellent, Volume VI seems to be the most didactic. Authors explain with the help of diagrams, chemical deductions and electron micrographs the extremely complicated process of photosynthesis. The most modern theories and experiments are reviewed, and the reader is shown the pathway leading to these; thus, a full picture is presented of the problems related to photosynthesis: the question of binding of light energy, its transformation and, finally, the course of fat and carbohydrate strorage are discussed. The volume is completed with a series of slides and a recording tape.

Scheduled teaching often seems forced. However, in these volumes it seems quite natural — and this proves the great experience of the authors in teaching. Could the teaching of all subjects be solved in this mode, it would mean a great facility to students and everybody interested in the subject.

G. Csaba (Budapest)

Progress in allergy, 18

Ed. Kallós, P., Waksman, B. H., de Weck, A. S. Karger, Basel—München—Paris—London —New York—Sydney (1975), pp. 477, figs 46, SFr. 187—.

The first volume of the present series appeared under the editorship of Kallós et al. with the aim to mate in a sequence of continuing reviews the clinical aspects of problems caused by allergic diseases and the perspectives opened by new serological and immunological proce-

dures. It inaugurated a popular trend: the serial publication of detailed review articles. This new volume contains reviews on actual topics of basic and clinical immunology by some of the most competent research workers in the field.

The antigen recognition by antigen-sensitive T and B cells is reviewed by DIENER and LANGUEAN from the University of Alberta. They expose most of the current views on the nature of cell receptors, on modes of antigen recognition, on immune induction and on the mechanism of triggering of antigen-sensitive cells. They show in a lucid manner how immunological research is undergoing a transition from parameters of antigen-induced lymphocyte population dynamics to the search of triggering mechanisms contingent on antigen receptors, the cell membrane as a dynamic structure which provides anchorage for antigenic receptors and the cytoplasm which translates the specific signals of antigen–receptor interaction into biochemical event.

The review on immunological tolerance by HOWARD and MITCHISON (London) gives a timely account of the current conceptions. Among the main mechanisms leading to an absence of immune response, the selective elimination of lymphocyte clones, the enhancement phenomenon produced by antibodes interfering with the access of antigen to lymphocytes, the reversible tolerance by blocking of lymphocyte receptors by antigen or antigen–antibody complexes and the identification of suppressor lymphocyte populations are thoroughly discussed. The most important advances in this subject, according to the reviewers, are the analysis of distinct tolerance events in T and B cells, a growing understanding of the binding to B cell receptors which leads to tolerance and the delineation and partial analysis of several active suppression systems.

The present state of affairs as well as their personal experience on antigenic competition are extensively presented by LIACOPOULOS (Hôpital Broussais, Paris) and BEN EFRAIM (University of Tel Aviv). Three main mechanisms are proposed: obliteration of the handling of antigens by macrophages, inhibition of antigen-specific T or B cells by other cells through release of inhibitory factors, finally the still controversial issue of competition for a limited number of potential immunocompetent cells.

The interest of molecular immunology is increasingly focused on the structural elements and dynamic interaction taking place on the lymphocyte membrane. The review by LADOULIS, GILL, CHEN and MISRA from the Pittsburgh School of Medicine presents not only an evaluation of a relatively crude knowledge but gives a perspective for research within the next years. Membranologists and biologists on one hand and classical immunologists on the other had little to say in common. The scene is now rapidly changing: the techniques of physical chemistry and membrane chemistry have developed to the point where many tools are now available for studying the structure and interactions of cell membranes and the progress in immunobiology has defined many critical phenomena in the immune response. The time has now come for combining these two approaches.

The review by HOLMGREN (Gothenberg) and SMITH (University of Texas) deals with a clinical problem: the immonological aspects of urinary tract infections. The antigenic composition and biological properties of germs and the persistence of antigens seem to relate directly to the severity and chronicity of infection. Whereas extensive data have accumulated on the presence and possible role of serum antibodies, another important aspect is discussed here: the possible role of local and cellular immune response in the infection and in the development of chronic inflammatory lesions.

Antigens involved in the production of experimental encephalomyelitis are reviewed by NIEDIECK (University of Hamburg). As shown in a review of her own work lipid antigens should not be neglected in the immunological evaluation of demyelinating diseases.

Cross reactions between antigens from mammalian tissues and bacteria are reviewed by LYAMPERT and DANILOVA (Gamaleya Institute, Moscow). This important topic gives a clue to understand autoimmune diseases and lesions consecutive to immunization against infectious diseases, as well as insufficient response to immunization. Our knowledge on cross-reacting antigens is far from complete: native proteins and polysaccharides contain hidden determinants which may be uncovered only by treatment with appropriate enzymes.

There is a continuosly increasing amount of knowledge in this dynamically developing field of medicine. The seven chapters of the present volume are based on about 1 900 references. In our world getting from day to day narrower personal contacts and verbal information reconquered their former role in certain fields of medicine which are in the centre of interest, as immunology and allergology: printed material as it is published will be outdated. Therefore, it is correctly stated by the contributors of the first chapter: since this manuscript was submitted for publication in February 1973 some of the contents may not accurately reflect the current state of knowledge. This statement stresses the fact that the emergency of immunology and allergy as one of the most active fields of medical and biological research makes a general

survey very difficult. One has to agree, however, with the introductory remark of DE WECK in this context that the publication of review articles on topics of basic and clinical immunology will remain an important and rewarding endeavour. This is the reason why this volume should be of special interest to nephrologists, allergologists and microbiologists as well.

K. KIRÁLY (Budapest)

Progress in allergy, 19

Ed. KALLÓS, P., WAKSMAN, B. H., DE WECK, A. S. Karger, Basel—München—Paris—London—New York—Sydney (1975), pp. 312, figs 19, SFr. 128—.

This new volume contains as usual reviews on topics of current interest in fundamental immunology. Particular emphasis is placed on the diverse populations of lymphocytes, on the problem of lymphocyte triggering, on the role of cyclic nucleotides as second messengers in immune response and on the mechanism of the macrophage's participation in the inflammatory process.

The immunologically competent cell capable of undertaking an immunological response is the small lymphocyte. The two-component concept of the lymphoid system: T cells, which are concerned with the delayed (cellular) allergic response, and B cells, which are the precursors of the antibody-secreting plasma cells, have been generally accepted. Lymphocytes are not sessile cells. FORD, from the Department of Pathology of the University of Manchester, gives a detailed account of the migration of lymphocytes and shows that lymphocytes are nomadic cells which have recently come from the blood and which return to the blood within a period of hours. A large pool of lymphocytes is shuffled continuously between the spleen, the lymph nodes, Peyer's patches and, on a much smaller scale, into many non-lymphoid tissues. Uncommitted and committed activated lymphocytes have their own migratory properties. The differential migration of these cells depends, firstly, on their selective affinities for the endothelium of small vessels in lymphoid tissue and inflamed tissue and, secondly, on the ability to segregate after entering the lymphoid tissue. The lymphocyte membrane with its display of different structures and receptors is the decisive factor, however, the molecular basis of these processes has remained obscure.

The presence of reaginic antibody in the serum of allergic patients was first demonstrated by PRAUSNITZ, who had passively sensitized his forearm by intracutaneous injection of serum from his allergic colleague, KÜSTNER. Identification of reaginic antibody as IgE was based on progress in structure and classification of immunoglobulins. In the review of T. and K. ISHIKAZA from the Johns Hopkins University, on the biology of immunoglobulin E, a comprehensive review is given on the molecular basis of reaginic hypersensitivity. The most important biological property of IgE is its capability to bind through its Fc portion with the specific receptors to the surface of basophilic granulocytes and mast cells of the homologous species. On contact with the specific allergen, basophils and mast cells with surface-bound IgE release histamine, slow-reacting substance and eosinotactic factor, which are responsible for clinical signs and symptoms of the immediate allergic reaction. The prerequisite of the releasing process is probably bridging of cell-bound IgE molecules by either the antigen or anti-IgE and not the "capping": the polar patch-like redistribution of cell surface IgE. Bridging of cell-bound IgE molecules may bring IgE receptors to close proximity causing changes in cell membrane structure, which initiates enzymatic sequences leading to the release of chemical mediators.

TADA, from the Chiba University, Japan, deals with the regulation of reaginic antibody formation in animals, a topic to which the author rendered important experimental contribution. The importance of the study lies in the identifications which cause the elevated reaginic antibody formation leading to human reaginic diseases; on the other hand, knowledge about the regulatory mechanism in reagin production would give a clue to the therapeutic application in these diseases. Besides extrinsic factors (antigen, its dose, route of administration) the crucial factors lie in the cellular events of the host, among them the inductive and inhibitory interactions between T and B cells, which lead to the enhancement and suppression of reagin response. These interactions, either antigen-specific or non-specific, are mediated by soluble factors whose chemical and biological properties are now in the process of being elucidated. Evidences on animals indicate that IgE B cells are highly T-cell dependent and more susceptible to the T cells' regulatory influences than other B cell types. IgE-producing cells seem to have a high-affinity receptor for antigen and thus their response is easily influenced by the

dose and form of the antigen. Different approaches to the suppression of reaginic antibody formation are discussed. The first is the induction of immunological tolerance in IgE-producing cells by deletion of certain B cell clones. Another promising approach is the induction of immunological tolerance in T cells by large doses of non-allergenic carrier achieving an antigen-independent tolerance. A further possibility is the antigen-specific suppression by suppressor T cells (a possible explanation for the long-term hyposensitization therapy). This can be antigen-independent as it is seen with some adjuvants, T cell mitogens which switch off reagin response. Serum IgE antibody (blocking antibody) can suppress pre-established formation. Further studies are, however, necessary before such an immunotherapy can be applied to human reaginic diseases which are hampered by the overt differences in the regulatory mechanisms between animal species.

The last review in the present volume, by HIRSCHFELD from the State Institute for Blood Group Serology, Stockholm, challenges the central dogma of immunology: the non-specificity of antibodies from the conceptual point of view. It is now generally accepted that not only the immunoglobulin population constituting an immune serum is highly specific for the eliciting antigen, but also the individual immunoglobulins constituting this population. However, the exceptions from the general validity of the dogma, i.e. the cross reactions between antigens which have, beclouded, from the early days of serology, the specificity of agglutinations, hamper their use for unequivocal diagnosis. HIRSCHFELD transformed serologic reactions into verbal codes and analyzed them. He states that individual antibodies are complex: cross reacting and multispecific. This view is in congruence with recent experimental results, viz., that antibody-combining regions contain subsites at which structurally diverse antigenic determinants bind. The merit of HIRSCHFELD's review is the analysis of the conceptual framework itself what he calls "metaserology" in contrast to conventional serology, which is the description of observations. According to HIRSCHFELD's idea, "metaserology" analyses the rules and structures of our own thinking which decide how knowledge is obtained and organized in serology and how data are thereby distorted. By the examples of the Rh and ABO blood group systems, it has been shown that the same observations may be explained on the basis of the different mapping surfaces of antibodies as well as on the basis of diversity of antigens.

The selective suppression of immune response, a therapeutically promising area since the successful prevention of erythroblastosis fetalis by passive sensitization with anti-Rh sera, is extensively reviewed by FITCH (Department of Pathology, University of Chicago). Among the different mechanisms mentioned (masking of antigenic determinants by antibody molecule, alteration of antigen catabolism, feed-back regulation) the suppressor effect of anti-idiotypic antibodies which react with receptors of specifically reactive lymphocytes is emphasized as an especially interesting and promising means. There are, however, tremendous difficulties in applying this approach to man.

The review by BIGAZZI and ROSE on spontaneous autoimmune thyroiditis in animals gives a model to understand the Hashimoto thyroiditis in man. The review summarizes the observations and investigations of the last decade on autoimmune thyroiditis, initiated first by WITEBSKY and extended and continued efficiently by the Buffalo group to hypothyroid obese chickens and later on other animal species (rats, dogs, monkeys) in which spontaneous autoimmune thyroiditis resembling Hashimoto thyroiditis of humans occur. The prerequisite of the development of this spontaneous disease is a genetic predisposition which seems to follow the pattern of polygenic inheritance. A viral infection of the thyroid in genetically susceptible individuals seems to be the primary event.

This new volume with its excellent and comprehensive reviews, each with up-to-date references, should be of special interest to allergists, experimental pathologists and microbiologists, as well as to basic and clinical immunologists.

K. KIRÁLY (Budapest)

DELLING, G.: **Endokrine Osteopathien (Endocrine bone diseases)**
G. Fischer Verlag, Stuttgart (1975), pp. 111, figs 32, DM 74—.

This booklet, the 98th in the series "Veröffentlichungen aus der Pathologie", reviews morphological subjects. Modern quantitative morphological and functional methods applied in connection with bone biopsy are discussed. The precise diagnosis of bone diseases can be only partially established by pathophysiological methods. The book draws attention to the fact that the classical X-ray pictures are rather unsuitable for the unambiguous determination of pathological courses in various bone diseases.

After a Preface and Introduction, bone preparation methods not involving decalcification are demonstrated, and their great advantage is stressed. This chapter involves microradiography and quantitative morphometry, and the criticism of these methods. The next chapter describes the normal bone structure as revealed by modern methods. The fourth chapter summarizes the newest literature of parathormone, calcitonin and vitamin D. The review of vitamin D and its metabolites and the demonstration of their hormonal properties are particularly modern. The subjects of the last main chapter are parathormone, calcitonin, "D hormone", growth hormone, thyroid hormone, glycocorticoids and the sex hormones. Based on the demonstration of the dynamic morphology of bone tissue, the author displays the clinical consequences of increased or reduced production of the above substances.

The extensive up-to-date list of references and the excellent illustrationo need special mentioning. The colour photos, beautiful in themselves, provide a great help to readers of little experience in morphological methods, in interpreting pathological pictures.

The methods presented in the booklet are a great aid to both the clinician and the research worker interested in the metabolic disorders of the bone.

We hope that this short review will draw attention of Hungarian experts to the theoretical and practical significance of up-to-date methods in bone morphology and will stimulate introduction of these methods.

I. HOLLÓ (Budapest)

Proceedings of the Third Conference on Origin of Life
Planetary Astronomy. Ed. MARGULIS, L. Springer Verlag, Berlin—Heidelberg—New York (1973), pp. 268, figs 28.

The volume presents the topics of debate of the conference organized by the "Smithsonian Institute" in the course of its Interdiscipline Communication draft programme. The Conference was held between 27 February and 1 March, 1970, in Santa Ynez (California). This was the third in a series of Conferences on the origin of life.

Nineteen famous representatives of biology, biochemistry, geology, earth and space research, exobiology and borderline sciences participated in the conference. Scientific knowledge on the Moon and Mars were analysed and discussed from the aspect of the origin of life.

The discussions were equally divided between the Moon and the Mars. The data concerning the Moon were more actual, while informations on the Mars were often based purely on speculation.

After an analysis of data concerning the Moon surface, Moon formations, craters, the so-called regoliths, the problems of Moon glass were discussed. The age of the Moon was discussed in detail, further on, the theories of the possible origins of the Moon. Finally, the results of the organic-chemical analysis of Moon samples and their microbiology were presented.

Knowledge concerning the Mars was obtained in the first place by observations made by the Mariner 6 and 7. The main subject was the composition of the Mars atmosphere. The "atmospherical pressure", aqueous vapor, CO_2 and N_2 were central subjects of the debate. Data obtained up to the present on the Mars surface, the craters, the "weather" conditions are in their majority disputable or speculative.

The extremely interesting discussions — sometimes only consisting of remarks and half sentences — are exciting for the reader, but represent a difficult task for the reviewer. Besides the readiness, the sparkling of the discussions, the often very friendly form, it deserves mentioning that an immense scope of knowledge, enlightened from several aspects, was presented at this conference.

The volume is a real mine of information for biologists, astronomers, chemists, biochemists, geologists and for workers of many other fields of science.

GY. KISZELY (Szeged)

COWAN, S. T., STEEL, C. C.: **Manual for the identification of medical bacteria**
Cambridge University Press, London (1974), pp. 238, US $ 14.95.

The second edition of the internationally famous volume consists of nine chapters, viz., Classification and nomenclature; Media: constituents and sterilisation, Principles of isolation; Characters and characterisation; Theory and practice of bacterial identification;

Characters of Gram-positive bacteria; Characters of Gram-negative bacteria; Identification by cards; Taxonomy in theory and practice. In an Appendix, preparation of culture media, staining of bacteria, classification, international nomenclature and abbreviations and other questions of practical importance are dealt with. In a separate chapter the most important chemicals and substances and their manufacturers are listed.

This volume is a great practical aid for routine bacteriological laboratories. The so-called mini-definitions, the large number of detailed diagnostic tables, the clear style and conciseness deserve special mentioning.

This book has not been written for use in the library, its real place is on the shelf of the bacteriological laboratory.

The beautiful getting-up of the volume credits the Cambridge University Press.

MÁRIA M. HERBAI (Budapest)

Current topics in microbiology and immunology, Vol. 65

Springer Verlag, Berlin—Heidelberg—New York (1974), pp. 160, figs 26, DM 66.—, US $ 27.—

The 65th volume of this well-known series reviews the newest results in three interesting subjects, namely, (1) the molecular biology of adenoassociated viruses (K. I. BERNS), (2) the most recent knowledge of the commitment of B lymphocytes (J. LEFKOVITS) and (3) the DNA-dependent *in vitro* enzyme synthesis (M. SCHWEIGER and P. HERRLICH). All three reviews are well illustrated and present highly valuable references.

Although the book was planned on the first place for experts in the given subjects, it will be of great expedience to physicians, biologists and university students.

The prompt presentation of the newest data and publication of the opinion of authorities on the subject both credit the Publishers. As usual the volume is of adequate size and of best quality.

MÁRIA M. HERBAI (Budapest)

KISS, F. A.: **Vascularization and tissue differentiation**

Studia Biologica Hungarica, 14.
Akadémiai Kiadó, Budapest (1975), pp. 168.

During many decades of investigation on the problems of bone formation and in general of bradytrophic tissues, KROMPECHER's school has recognized the significance of vascularization. This volume contains work performed on one of the side-paths of these investigations, examining and proving the stimulating effect of various tissue extracts, especially adrenal extracts, on vascularization. The following experimental model was applied by the author: (a) in controls: various organs and tissues were transplanted onto the chorioallantoic membrane of the chicken embryo, the ingrowth of blood vessels was investigated; (b) the degree of growth was determined in such cases when the transplants were immersed in an adrenal extract before transplantation. The experiments unambiguously proved that the vascularization of bradytrophic tissues (Wharton's jelly, immature cartilage, embryonic brain, keloid) needed a previous immersion in the adrenal extract. Mature cartilage was not vascularized in the same circumstances. The experiments are important from the point of developmental biology: they prove the necessity of a vascularization factor during tissue differentiation. From the practical point of view, the vascularization factor(s) may be involved in callus formation in corneal vascularization or even in the reparation of myocardial infarctions.

The morphobiological effectuation of the experiments is impressive, the documentation is convincing. Only one group of disturbing questions has remained open: what substance induces vascularization? further on, of what origin is this substance? — cortico- or medullo-adrenal origin or whether the effect is the result of a joint effect of both and if so, then why?

The getting-up of the book is beautiful, the illustrations and tables are demonstrative. This volume, summarizing the work of an eminent school, in which the author himself has played a prominent role, will be valuable for biologists, surgeons, and workers of borderline sciences.

G. CSABA (Budapest)

Printed in Hungary

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STUDIES ON CARTILAGE FORMATION XIX. OXYGEN AND GLUCOSE SUPPLY OF THE REGENERATING ARTICULAR SURFACE

Cs. Hadházy and S. Varga

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(Received 1975—02—05)

Abstract

Complete removal of the articular cartilage in dogs is followed by regeneration of the articular surface. At the site of the bone wound, granulation tissue develops, which later differentiates into cartilage. The O_2 and glucose supply of the regenerating articular surface is ensured by the synovial fluid, by the large exposed surface of the medullary cavity, and by the capillary network of the granulation tissue. Oxygen and glucose supply of the articular surface in different stages of differentiation has been statistically analyzed. It is suggested that in the early stage of regeneration O_2 supply comes predominantly from the capillaries of the granulation tissue. Later on, as capillarization regresses, the oxygen supply originating from the synovia and medullary cavity, assumes a more important role. In the stage of cartilage regeneration an oxygen-deficient state can be supposed in the entire articular surface, but areas differing in oxygen supply may be formed owing to local differences (due mainly to the extent of vascularization and degree of regeneration of the subchondral bone layer). At the site of chondrogenesis, conditions allowing aerobic metabolism of cells with reduced O_2 requirement seem to be ensured. Glucose supply deriving from the above-mentioned sources satisfies the highest glucose requirements of the cells in the regenerating articular surface.

Introduction

During bone repair we usually find cartilage in the environment exposed to mechanical forces. Formation and accumulation of cartilage is usually preceded by a marked reduction in the capillary network of the granulation tissue. The joint occurrence of vascular pauperization and chondrogenesis [21] has led to the supposition that a causal relation may exist between these phenomena [11, 21, 22]. In this conception which attributes a formative or predisposing effect to decreased circulation, the question of O_2 and glucose supply is of essential importance.

The experimental material consisted of the regenerating articular surface of dogs [11]. Before investigating this material as regards its O_2 and glucose supply, we had studied the fluid circulation of the intact articular cartilage which comes from the synovial fluid, from the arcade-like capillary network reaching the margin of the articular cartilage (circulus articuli vasculosus) and from the so-called subchondral capillaries underlying the articular surface

[7, 9, 29]. Schematic illustration of the blood and fluid supply of the articulation is shown in Fig. 1.

There is a striking difference in capillarization between regenerating and intact cartilages. The articular surface (granulation tissue) is characterized by a marked capillary network deriving from the subchondral vessels, which had been cut through at the operation but soon regenerated, and to a small extent from the circulus articuli vasculosus. As it has been demonstrated by different methods [12] the extent of capillarization regresses as regenera-

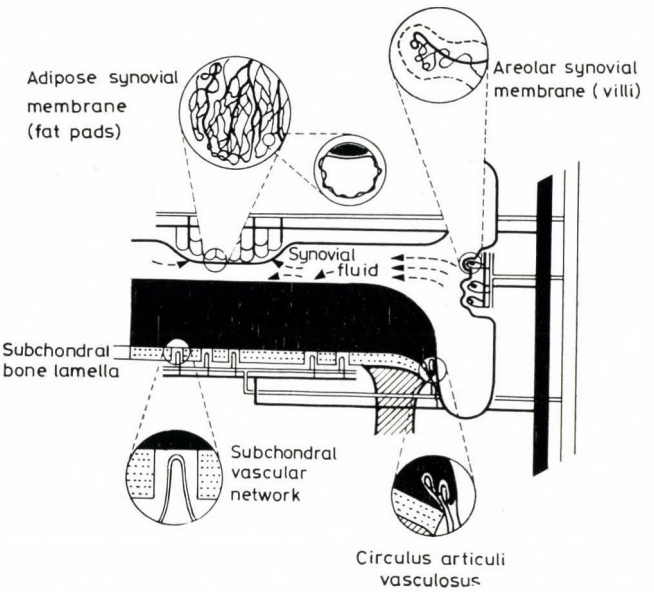

Fig. 1. Scheme of nutritive supply of articular cartilage after OTTE [29], modified by the authors. Fluid supply originating from the villi (their capillaries) of the areolar synovial membrane. The fluid is absorbed through the portions of the adipose membrane by means of capillaries with a special structure. Further supplying source: the *subchondral capillary network*, from the medullary cavity. The capillary loops of this network are in part, found in the small communications of the subchondral bone layer. The peripheral cartilage portions are partly supplied by the *circulus articuli vasculosus*

tion progresses. Another characteristic feature is that the medullary cavity is extensively open in the direction of the "articular surface". Though owing to the regeneration of the subchondral bone layer, the open boundary surface between the granulation tissue and spongiosa decreases, it also serves as a source of supply. The synovial fluid of the operated joint is likewise important in this respect.

On the basis of known principles, we have made attempts to study the different components of this supplying system (synovial fluid, medullary cav-

ity and vascular network of the granulation tissue) to get information regarding the extent of oxygen and glucose supply of the regenerating articular surface.

Material and method

The data analyzed in the present study derive from experiments on 146 dogs. Some of the experiments had been carried out earlier. The animals were operated on their left knee joints. After opening the joint, the articular cartilage of the distal part of the femur, together with a few millimeters of the underlying spongiosa, was removed, a new articular surface was formed from the spongiosa, and the wound was closed (for details, see [11]). Animals were killed 7, 20, 33 and 70 days after operation.

From each age group the material of 10 dogs was used in the experiment. Granulation tissue pieces (3 × 3 mm) with a bony layer were excized from the condyles of the regenerating articular surface and from the intercondylar fossa. The pieces were fixed in Susa's mixture and embedded in paraffin. The sections (10 μm) were stained with haematoxylin-eosin. In each animal 3 different places of the articular surface were examined, on 10 sections each. The thickness of the covering cartilage was measured and the numbers of the cross-sections of capillaries seen in the restricted visual fields of an eye-piece were counted. From the extent of the area examined and the thickness of the section we have calculated the length (L) of the capillary as described by OPITZ and SCHNEIDER [28]. This value and the data of the thickness of the cartilage were corrected to fresh material by taking in consideration the shrinking effect of the histotechnical procedures. In the section, the closed part (spongious trabecula) and the open part (spongious cavity) of the borderline between the granulation tissue and spongiosa have also been determined and their ratios calculated.

Oxygen consumption was measured by Warburg's manometric method [27] in material taken from 48 dogs (12 dogs in each age group). In addition, in material derived from another 48 dogs, anaerobic glycolysis was determined, likewise with Warburg's method [13]. From the quantities of CO_2 obtained on the basis of the formula given by WARBURG [34], according to which 1 ml CO_2 corresponds to 4 mg glucose utilized by the tissue, we have calculated the consumption of glucose.

In further 10 dogs the O_2 pressure in the blood of peripheral capillaries was examined with Clark's electrodes.

Results

Bleeding caused by the operation gives rise to a haematoma from which granulation tissue develops. On the 7th postoperative day (Fig. 2) a richly vascularized granulation tissue covered by fibrin is seen on the spongious bone. In the superficial layer of the spongiosa, bone formation is going on. The new bone trabeculae show an irregular pattern. After 20 days (Fig. 3), the tissue of the articular surface is more massive and less vascularized. There is a more active bone formation at the border of the bone and granulation tissue; the new bone trabeculae situated in the vicinity of the borderline show a parallel arrangement.

On the 33rd day (Fig. 4), small cartilage islets are present at the border between the spongiosa and granulation tissue. These islets and their environments are devoid of vessels. Formation of bone trabeculae, parallel with the boundary surface is continued. In the 70-day stage (Fig. 5), the so-called subchondral bone layer closing the main part of the spongious cavities, makes

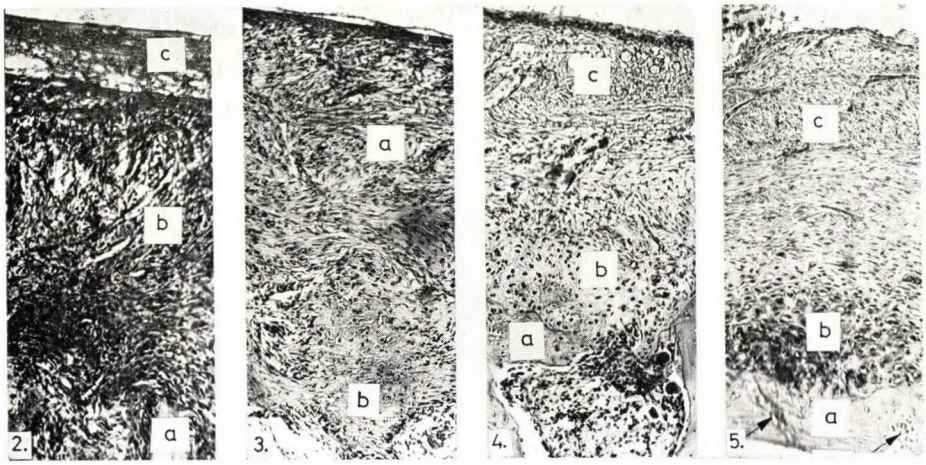

Fig. 2. Regenerating articular surface, *7-day stage*. The spongiosa stumps (a) are covered by richly vascularized granulation tissue (b), enveloped by fibrin (c). Haematoxylin-eosin, × 95

Fig. 3. Regenerating articular surface, *20-day stage*. Poorly vascularized granulation tissue (a). Below this layer bone formation (b) is going on at the granulation tissue–spongiosa border. Haematoxylin-eosin, × 95

Fig. 4. Regenerating articular surface, *33-day stage*. On the spongiosa granulation–tissue border, in the partly regenerated subchondral bone layer (a) a small cartilage islet (b) can be seen. More superficially vascularized connective tissue (c) is visible. Haematoxylin-eosin, × 95

Fig. 5. Regenerating articular surface, *70-day stage*. On the border of the spongiosa and granulation tissue the subchondral bone layer contains nutritive communications (a) ↗ and a moderately large cartilage islet (b). Its immediate environment is devoid of vessels; towards the surface a connective tissue layer containing narrowed and occluded vessels (c) can be seen. Haematoxylin-eosin, × 95

its appearance. Supported by this layer, large coalescing islets are present in the regenerating articular surface near the spongiosa. The rest of the "articular surface" is composed of a poorly vascularized fibrous tissue. Occasionally, capillaries of narrow lumina are seen.

In earlier investigations we studied the changes in the vascular pattern of the regenerating articular surface by different methods. Comparing the total area of capillary cross-sections with the cross-section of the articular surface we found a gradual marked decrease of vascularization. Taking the "articular surface" in the 7-day stage as 100% (at this time the total area of capillary cross-sections was 6.03% of the cross-section of the articular surface), this value was found to be 56%, 36%, and 22% on the 20th, 33rd and 70th postoperative day, respectively [12].

A similar decrease was noted in the haemoglobin content, the respective percentages being 100%, 63%, 48% and 23% [23].

The morphological features of the vascular pattern in the articular surface have been studied by filling up the vessels with India ink [12]. The results are summarized in Fig. 6.

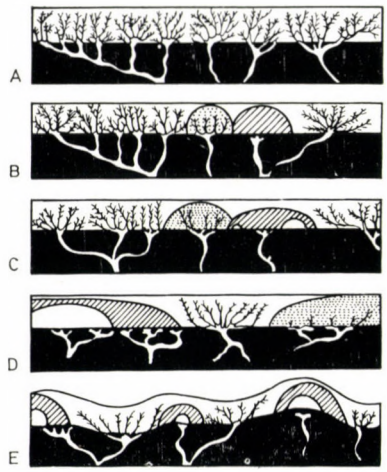

Fig. 6. Schematic illustration of the results obtained after injection with India ink [12], showing the conditions of vascularization of the regenerating articular surface 7 (*A*), 20 (*B*), 33 (*C*) and 70 (*D*) days after surgical intervention. The dotted areas correspond to poorly vascularized, the finely lined ones to avascular territories, and the white fields represent cartilage islets. *E* shows the unevenness of the articular surface, the vascular conditions of areas emerging from the surface or lying in deeper layers

The data regarding the thickness of the articular surface, the length of capillaries, the open part of the granulation tissue spongiosa border and the oxygen and glucose consumption are shown in Table 1.

Table 1

Parameters of the regenerating articular surface used for the determination of its 0_2 and glucose supply

Postoperative day	Thickness of art. surface (μm)	Length of capillary L = cm/cm^3	Open part of spongiosa border (per cent)	O_2 consumption A = ml O_2/min/cm^3 tissue	Glucose consumption: Q = mg glucose/min/cm^3 tissue
7	958	4550	66.5	7.60×10^{-4}	19.56×10^{-4}
20	488	3230	45.1	25.87×10^{-4}	45.16×10^{-3}
33	629	2270	31.2	27.07×10^{-4}	37.32×10^{-3}
70	810	1240	25.0	15.76×10^{-4}	36.12×10^{-3}

The oxygen supply of the cell is determined by the partial pressure (pO_2) of the diffusible O_2 present in its immediate environment. Continuous diffusion

needs an oxygen source and continuous consumption. Constant (non-rhythmical and non-periodical) oxygen source together with a constant consumption leads to a "steady state". In such cases there is a characteristic pO_2 in given sites of the tissue and this may be mathematically approximated [10, 14, 19, 20, 28, 33, 34].

The thickness (d) of a tissue with an oxygen consumption "A" through which O_2 of a given pressure can still diffuse was termed "aerobic boundary layer" by WARBURG [34]. In the case of smooth-surfaced tissue open on one side (as regards synovial fluid supply, these conditions are given) the thickness of this layer was determined by the following equation:

$$d_{(cm)} = \sqrt{\frac{2Dp_0}{760A}} = 2.08 \times 10^{-4} \sqrt{\frac{p_0}{A}} \qquad (1)$$

where D is the diffusion constant of O_2, p_0 is the pO_2 mmHg present on the supplying surface, A is the O_2 consumption expressed in ml/cm^3 tissue/min. Though according to WARBURG, D is dependent on tissue structure, he always used the value of 1.64×10^{-5} cm^2/min at 37 °C in his calculations. (In the equations the O_2 pressure and not the molar concentration of O_2 is given. According to KROGH this is correct as the dissolution constant is included in the diffusion coefficient [see 10]). The D value, originally introduced by KROGH for muscle, is virtually identical for muscle and connective tissue [19]. The same value was found for spherical protozoa [32]. Since we could not determine the D for granulation tissue, we have used this value. The A value is indicated in Table 1. For p_0 — if the value measured in human joint [8] is accepted — we may take 38 mmHg. On the basis of the above values Warburg's equation can be solved. As a result we obtain the thickness of the aerobic boundary layer. By modifying this equation, OPITZ and SCHNEIDER [28] calculated the thickness of the "aerobic threshold layer" (d') as follows:

$$d'_{(cm)} = K_1 \sqrt{\frac{p_0 - p_{crit.}}{A}} = 2.08 \times 10^{-4} \sqrt{\frac{p_0 - p_{crit.}}{A}} \qquad (2)$$

This value represents the distance from the supplying surface to the critical oxygen pressure (pO_2 *crit.*). The pO_2 *crit.* is the O_2 threshold value required for the maintenance of aerobic processes, in other words, the O_2 pressure level below which tissue respiration decreases owing to lack of oxygen. In warm-blooded animals this value is 2—4 mmHg [28] at body temperature. This has been verified in cell cultures as well [5, 6, 30]. K_1 is a constant dependent on tissue structure which, in agreement with OPITZ and SCHNEIDER [28] is equal to Krogh's constant.

Obviously, the thickness of the threshold and boundary layers in oxygen-consuming tissues is not characteristic of normal cell life. As we study regeneration and differentiation, the optimum O_2 supply should be considered. In human subcutaneous connective tissue — *in vivo* — 15.23 mmHg [30], and 30 mmHg [3] pO_2 have been measured. These data reflecting the conditions present at the moment of measurement, cannot be considered general values. The O_2 supply of certain cells was found to be optimal in a pO_2 domain characterized by a minimum (pO_2 *opt. min.*) and a maximum (pO_2 *opt. max.*) value. For mammalian fibroblasts this interval, *in vitro*, ranges from 20 to 200 mmHg [4], for mouse sarcome I cells from 30 to 60 mmHg [17], and for LS mouse cells from 40 to 100 mmHg [18]. On the basis of these findings the pO_2 *opt. min.* of mammalian fibroblasts, i.e., the lower limit of optimal O_2 supply is approximately 20 mmHg. As there are no data available on our material and cells, we considered more than one values (10, 20, 30 mmHg) as the lower limit of optimum O_2 supply. *The thickness of the tissue layer with optimum oxygen supply* (d'') can be calculated by substitution of these data in equation (2):

$$d''_{(cm)} = K_1 \sqrt{\frac{p_0 - p_{opt.\,min.}}{A}} = 2.08 \times 10^{-4} \sqrt{\frac{p_0 - p_{opt.\,min.}}{A}} . \qquad (3)$$

The thickness of the layer optimally supplied with synovial fluid: d_s^+, d_s^{++}, d_s^{+++} (calculated for 10, 20 and 30 mmHg minimum pO_2) and those of the so-called threshold (d'_s) and boundary layers (d_s) are shown in Table 2.

Removal of the subchondral layer and the exposure of the spongious cavity render the diffusion of O_2 from the bone marrow's capillaries possible. The p_0 of the subchondral oxygen supply is not identical with the p_0 of the synovial fluid. Being unknown, it was characterized by the mean oxygen pressure of peripheral (capillary) blood. In dogs, this was 73 mmHg. When examining subchondral oxygen supply, one should consider that regenerating subchondral bone layer causes a reduction in size of the openings of the spongiosa cavities. Consequently, the diffusion boundary surface becomes smaller and smaller. For the estimation of this reduction the closed and open parts of a unit borderline were measured and their ratios calculated. From this — supposing that there are identical conditions in the direction perpendicular to the borderline (plane of section) — we obtained the closed and open parts of the boundary surface. From p_0, the data mentioned above, and equation (3), we obtained the thickness of the aerobic boundary (d_m) threshold (d'_m) and optimally supplied layers (d_m^+, d_m^{++}, d_m^{+++}), related to the entirely open surface of subchondral O_2 diffusion, calculated for the pO_2 values 10, 20 and 30 mmHg. These data were reduced in proportion to the actually open part of the boundary surface (Table 1). The results are shown in Table 2.

Table 2

Results of calculations concerning O_2 and glucose supply of the regenerating articular surface

	Marks	Postoperative day			
		7	20	33	70
Radius of the "anatomical cylinder" (μm)	R_A	74	88	105	142
Oxygen supply:					
Aerobic boundary layer, capillary of the art. surface $\Delta p = 73$ mmHg	R_A	325	71	70	87
Aerobic boundary layer, synovia $\Delta p = 38$ mmHg	d_s	465	252	253	323
Aerobic boundary layer, medullary cavity $\Delta p = 73$ mmHg, corr.	d_m	427	158	106	112
Aerobic threshold layer, capillary of the art. surface $\Delta p = 73-4$ mmHg	R'	318	69	68	85
Aerobic threshold layer, synovia, $\Delta p = 38-4$ mmHg	d_s'	440	239	233	305
Aerobic threshold layer, medullary cavity $\Delta p = 73-4$ mmHg corr.	d_m'	417	153	104	109
Boundary layer of opt. oxygen supply, capill. of art. surface $\Delta p = 73-10$ mmHg	R^+	305	67	66	82
Boundary layer of opt. oxygen supply, synovia $\Delta p = 38-10$ mmHg	d_s^+	399	216	211	277
Boundary layer of opt. oxygen supply, medullary cavity $\Delta p = 73-10$ mmHg corr.	d_m^+	398	146	99	104
Boundary layer of opt. oxygen supply, capill. of art. surface $\Delta p = 73-20$ mmHg	R^{++}	283	62	61	76
Boundary layer of opt. oxygen supply, synovia $\Delta p = 38-20$ mmHg	d_s^{++}	278	174	170	222
Boundary layer of opt. oxygen supply, medullary cavity $\Delta p = 73-20$ mmHg corr.	d_m^{++}	366	134	91	95
Boundary layer of opt. oxygen supply, capill. of art. surface $\Delta p = 73-30$ mmHg	R^{+++}	257	57	56	70
Boundary layer of opt. oxygen supply, synovia $\Delta p = 38-30$ mmHg	d_s^{+++}	213	116	113	148
Boundary layer of opt. oxygen supply, medull. cavity $\Delta p = 73-30$ mmHg corr.	d_m^{+++}	329	121	82	86
Glucose supply:					
Boundary layer of glucose supply, synovia $C_0 = 110$ mg%	d_s^G	1077	710	781	794
Boundary layer of glucose supply, medullary cavity $C_0 = 110$ mg%, corr.	d_m^G	716	320	244	199

The values stand for μms.
Correction: data calculated with the value of the open part of the boundary surface

The third oxygen source of the regenerating articular surface is the blood flowing in its capillaries. In this case the conditions of diffusion can be evaluated on the basis of Krogh's concept. Accordingly, the tissue may be divided into cylinders (Krogh's tissue cylinder, see Fig. 7) each of which is supplied by a

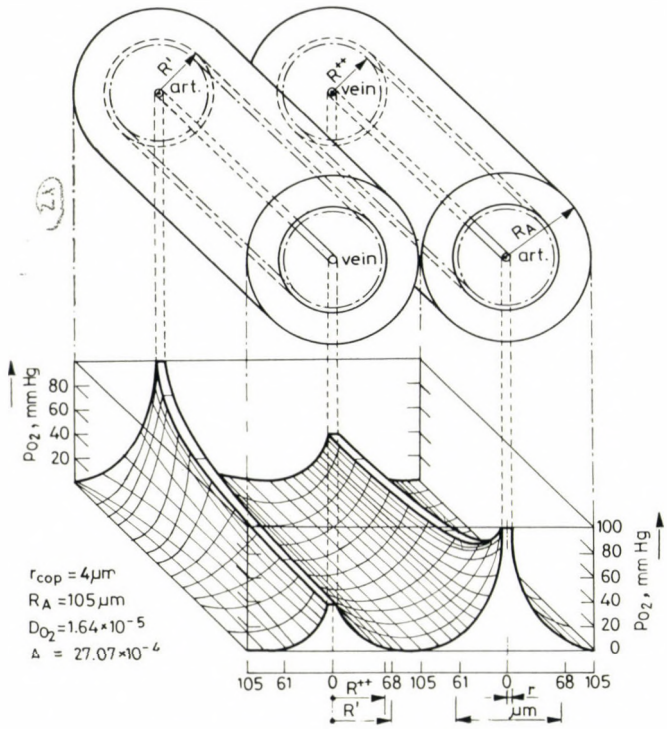

Fig. 7. Krogh's "tissue cylinder" of two "neighbouring" capillaries in 33-day granulation tissue of the articular surface. The radii of these cylinders (R_A, R', R^{++}) were obtained from calculations supposing uniform vascularization of the tissue. R_A = the radius of the anatomical tissue cylinder, i. e. the half of the distance between two capillaries. R' = the radius of the tissue cylinder representing the aerobic boundary layer supplied by the capillary. R^{++} = the radius of optimally supplied tissue cylinder (if $pO_{2\ \text{opt. min.}} = 20$ mmHg). The direction of the capillary circulations is illustrated according to LÜBBERS' concept. For the calculation of R' and R^{++} the mean O_2 pressure ($pO_2 = 73$ mmHg) of the capillary blood was considered. In the lower part of the Figure, note the conditions of O_2 pressure in the tissue cylinders belonging to 2 capillaries (at the arterial end $pO_2 = 95$ mmHg; at the venous end $pO_2 = 38$ mmHg).

capillary localized in its axis. In conformity with Fick's law, oxygen — corresponding to its fall in tension — diffuses in the tissue perpendicularly to the axis of the capillary. Oxygen pressure at a given site of the tissue is determined by the pO_2 of the capillary blood and the fall in pressure occurring in the tissue (Δp). Δp depends on the O_2 consumption by the tissue (A), on the

diffusion surface (capillary radius: r), on the length of diffusion's path (radius of the supplied cylinder: R), and on the diffusion constant (D). With Krogh's formula:

$$\Delta p = \frac{760}{4D} \cdot A \left\{ R^2 \left(4.6 \log \frac{R}{r} - 1 \right) + r^2 \right\} \tag{4}$$

D and A as in equation (1), r = the radius of the capillary of average calibre, i.e. 4 μm [25]. Instead of R, on the basis of the relation

$$R_{cm} = \sqrt{\frac{1}{L}} \tag{4a}$$

the capillary length L (cm/cm^3) may also be used [28]. The R obtained by the solution of (4a) is the half distance between two capillaries, i.e., the radius R_A of the so-called "anatomical cylinder".

$\Delta p = p_0 - p_R$. In agreement with KROGH's view, OPITZ and SCHNEIDER [28] suggested that p_0 is equal to pO_2 at the venous end of the capillary, i.e., to the O_2 pressure in the venous blood. This view was disputed by BENZINGER [1], who claimed that the mean O_2 tension of the capillary blood (physically dissolved O_2) should be considered. LÜBBERS [23] agrees with this concept, starting from the fact the R is greater at the arterial end than at the venous one, and that the blood in the neighbouring capillaries is not necessarily flowing in the same direction. In common with LÜBBERS, we have considered 73 mmHg as the mean O_2 pressure of the capillary blood (Fig. 7). From equation (4), by substituting the determined Δp-s, the R-s characteristic of them can be calculated. We have proceeded as follows: for p_R values we have given various O_2 tensions (0, 4, 10, 20, and 30 mmHg). The results (R, R', R^+, R^{++}, R^{+++}) are shown in Table 2.

Summing up the O_2 supply coming from three sources into the layers of the regenerating articular surface, the conditions shown in Fig. 8 are obtained.

The question of glucose supply of the regenerating articular surface can also be approached mathematically. It may be supposed that under physiological conditions the cells — independently of the extracellular concentration — consume a quantity of glucose characteristic of them and can fully utilize the amount of glucose present in their environment. In this case the concentration of glucose decreases departing from the supplying surface until zero, i.e., when reaching the depth where a complete equilibrium exists between its diffusion and consumption. This depth, the *thickness of the boundary surface* of glucose supply (d^G), can be calculated by the integrated form of Fick's law [2, 24]:

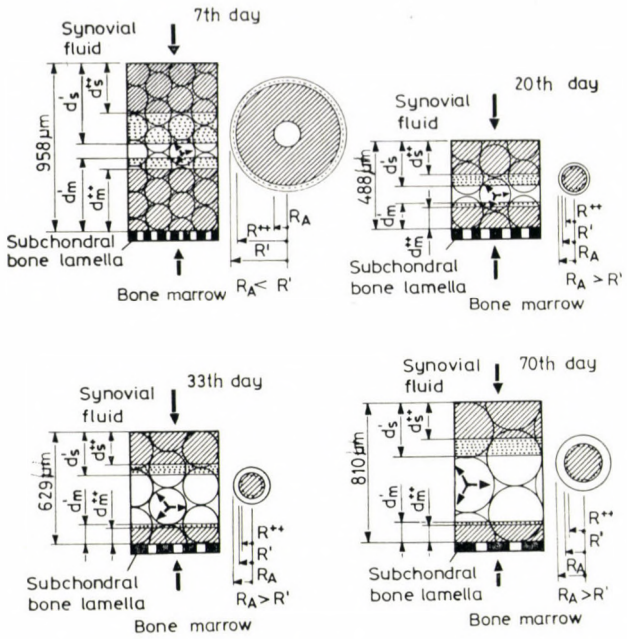

Fig. 8. Changes of O_2 supply in the tissue of the articular surface in the course of regeneration. Scheme supposing uniform capillarization which considers the diffusion originating from the possible oxygen sources (capillaries of the granulation tissue, synovial fluid, medullary cavity). The thickness of the aerobic boundary layer of the synovial and medullary supply (d'_s, a'_m) and the thickness (d_s^{++}, d_m^{++}) of the optimally supplied layer ($pO_{2\ \text{opt. min.}} =$ 20 mmHg) was related to the whole thickness of the articular surface. Naturally, when illustrating the complete supply, diffusion from the capillaries should also be considered. The R' and R^{++} are indicated outside the "articular surface" because the scheme representing the articular surface only the cross-section of the "anatomical cylinder" could be illustrated.

As it appears from the Figure, in the course of regeneration a considerable modification occurs in the thickness of the layers supplied by capillary diffusion. E. g., on the 7th day $R \gg R_A$, i. e. the capillary ensures an abundant O_2 supply for its own tissue cylinder. On the other hand, on the 33rd day $R' < R_A$, i.e., O_2 deficiency may be present in the peripheral part of the tissue cylinder (see also Fig. 7). Legend to marks is given in Table 2.

$$d^G_{(\text{cm})} = \frac{2\,p\,C_0}{Q} \tag{5}$$

where p = the permeability coefficient (cm²/sec) for glucose; C_0 is the glucose concentration (g/cm³) at the supplying surface; Q is the glucose consumption by the tissue (g/cm³/sec), p was taken as equal to 1.725×10^{-6} cm²/sec according to MAROUDAS and co-workers [24], who obtained a value of 2.9×10^{-6} cm²/sec in the articular cartilage at body temperature when the glucose solution on the boundary surface was mixed (functioning joint). Without mixing (resting joint) a value of 0.55×10^{-6} cm²/sec was obtained. We have used the mean of these two values. BYWATERS [2] established the value 5.4×10^{-5} cm²/min in his investigations on *one* slice of articular cartilage. We know

from experience that the glucose concentration in the synovial fluid is about the same as in the blood. On the basis of the measurements of FALCHUK and co-workers [8] we have considered C_0 as equal to 110 mg/100 ml. Q was taken to correspond to the maximum (anaerobic) glucose consumption of the cells of the regenerating articular surface (see Table 1). The boundary layer of glucose supply from the synovial fluid and subchondral space (d_s^G and d_m^G) was calculated, the latter corrected as described for the determination of O_2 supply. The results are shown in Table 2.

Discussion

The conditions of oxygen and glucose supply in the regenerating articular surface are rather intricate. (Entering into the particulars involved more and more problems which we attempted to solve on the basis of the data obtained from our material and from other tissues and in part by some hypotheses.) Owing to structural inhomogeneity, our results do not reflect the actual conditions. Furthermore, lack of diffusion constant characteristic of the tissue, neglect of the longitudinal diffusion in the tissue cylinder, considering oblique sections as cross-sections, regarding d_m as a layer of uniform thickness, etc. all have interfered with correct evaluation. For these reasons, the results of calculations are considered as a model which may only lead to approximative conclusions. As regards glucose supply the questions seem to be simpler to solve.

We have shown that the oxygen supply of the regenerating articular surface is highly dependent on the extent of the capillary network present in the tissue. In a densely and uniformly capillarized tissue (e.g. in the 7-day material) the oxygen pressure present at the boundary surface of the anatomical tissue cylinder (R_A) — because of the lack of slight degree of pressure gradient — may inhibit synovial diffusion and may limit considerably the diffusion from the subchondral space. In this tissue the latter effect seems to be of on importance.

Reduction of the capillary network involves an increase in the intercapillary distance (and in the R_A). If the latter exceeds the thickness of the layer supplied with oxygen, i.e., $R_A > R$ or R', oxygen deficiency will develop in the peripheral part of the anatomical tissue cylinder, and the tension gradient developing in such cases, permits both synovial and subchondral diffusion. These may be regarded as additional oxygen sources, the area supplied by them (d_s', and d_m', respectively) is restricted to a portion of the articular surface. At 20, 33 and 70 days following operation similar conditions may arise and, considering the total articular surface, the presence of an O_2 deficiency may be supposed (see Fig. 8).

The capillaries of the regenerating articular surface are, however, unevenly distributed: there is a much higher number of capillaries in the areas in the proximity of the articular cavity than in the environment of the spongiosa. Consequently, the local O_2 supply is not uniform, the densely vascularized areas may be regarded as better supplied with oxygen (complemented with synovial O_2 diffusion), as compared to the area nearer to the spongiosa. In the latter area, however, owing to its poor capillarization (consequently, to higher pressure gradient), we have to reckon with subchondral O_2 diffusion as well. In this period, i.e. at the time of formation and growth of cartilage islets, the spongiosa-granulation tissue boundary surface is, in part, open. Thus, the tissue layer in the vicinity of the spongiosa cannot be regarded as deficiently supplied. At certain sites (where cartilage formation is going on) the conditions of metabolic processes requiring oxygen seem to be ensured. Local differences (varying thickness of the articular surface or size of the gaps in the regenerating subchondral layer, concentration of capillaries, etc.) may result in further differences in the local O_2 supply (Fig. 9).

The literature on the O_2 tension in wounds at different stages of healing and in granulation tissues is poor. Low O_2 pressure (about the ciritical pO_2) was found in fluid aspirated from wounds [35]. As healing progresses the O_2 pressure changes between 5 and 28 mmHg [15].

In the fluid taken from the peripheral part of the granulation tissue developed in a metal sieve cylinder, the following tensions were measured at different intervals after implantation: 12 mmHg on the 5th day [16], 9—13 mmHg on days 15 to 18, and 14 mmHg on days 25 to 38 [35]. In the centre of 8-day old muscle wounds, vascularized in 86% or less of their area, 4.4 mmHg was measured, while at the periphery the pO_2 was 26 mmHg. In fully vascularized wounds — in the centre and peripheral parts alike — the oxygen pressure was identical with that of the neighbouring healthy tissue [31]. The latter examinations have shown that a decrease in the pressure difference needs a large part of the wound to be vascularized. In our cases the matter in question is not vascularization but the inverse process of it, the decrease in vascular supply. Nevertheless, the data given above are instructive: the consequence of decreased vascularization in this respect is the same whether it is due to insufficient vascularization of the tissue, or to capillary occlusion.

According to our calculations, the synovial fluid alone is able to ensure adequate glucose supply and, consequently, the loss due to decreased capillarization can be compensated to a certain extent by subchondral glucose diffusion. In BYWATERS' [2] opinion, the synovial fluid is capable of ensuring an adequate glucose supply for a 3 mm thick cartilage layer. This observation has been confirmed by MAROUDAS and co-workers [24]. These authors, however, claim that in an immobilized joint this distance is only 1.5 mm.

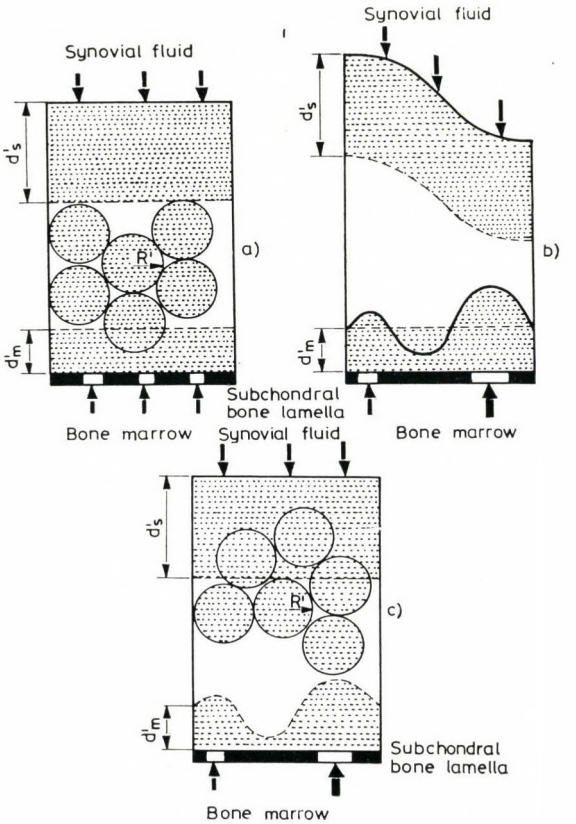

Fig. 9. O_2 supply minimally required for oxidative processes in the 70-day regenerating articular surface, under different boundary conditions. In the representative part chosen as unit of the articular "surface" (including 6 anatomical tissue cylinders) the aerobic boundary layers (d'_S and d'_M), and cross-sections of the aerobic tissue cylinder of the capillaries are indicated. *A* = Under "ideal" conditions (the thickness of the articular surface portion chosen as unit is constant, the openings of the subchondral bone layer are about the same size and uniformly distributed; the capillaries are localized in the middle layer of the "articular surface" (the supplied area (d'_S, d'_M) the cross-sections of the aerobic tissue cylinders of the capillaries) is smaller than the unit articular surface. This indicates that the minimum O_2 supply required for oxidative processes is not ensured everywhere in the tissue, even under such "ideal" conditions. *B* = Here the effect of the irregularities of the thickness of the "articular surface" portion chosen as a unit, and that of the uneven size and distribution of the subchondral bone layer are shown. The changes — even independently of the capillaries — may cause local differences in oxygen supply (areas with better or worse O_2 supply). *C* = Under boundary conditions approximating the real ones (locally smooth synovial surface, the openings of the subchondral bone layer showing irregular size and distribution, capillary network converging towards the synovial surface) local differences in supply may also arise, but in the vicinity of the synovial and medullary boundary surface the O_2 supply seems to be satisfactory

Since in the period examined even this thickness is not attained by the regenerating articular surface, insufficiency of glucose supply can be excluded.

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HISTOCHEMICAL STUDIES ON RESERVE SUBSTANCES AND ENZYMES IN FEMALE GAMETOPHYTE OF ZEA MAYS

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Abstract

Cytochemical changes during the early development of maize caryopsis are reported. Changes in the localization of different reserve substances (e.g., polysaccharides, proteins, nucleic acids and lipids) and enzymes (acid phosphatase, esterase, lipase, phosphorylase, succinate dehydrogenase, cytochrome oxidase and peroxidase) have been studied in unfertilized and fertilized ovules. Before pollination very feeble enzyme activity (acid phosphatase, succinate dehydrogenase, cytochrome oxidase and peroxidase) was observed. Reserve substances were present in low amounts before pollination. Pollination stimulated the accumulation of several reserve substances and enzymes in the tip of the nucellus, micropylar zone. Just prior to, during and after fertilization, the cells in the micropylar zone had strong reaction for several enzymes indicating temporary enhancement of metabolic activity in the micropylar zone. The role of antipodals in the storage of reserve food products and nutrition of embryo and early stages of endosperm development is discussed. The pattern of enzymatic changes within the embryo sac reflected the biochemical changes operative during quiescent and active stages. The nucellus of *Zea mays* contains many enzymes required for hydrolysis of reserved food substances. A role of acid phosphatase in autolysis of nucellar cells, after fertilization is suggested. Post-fertilization increase in the activity of enzymes and accumulation of reserve materials is interpreted as reflecting a presumed increase in the metabolic rate relative to growth and differentiation.

Introduction

The embryo sac occupies an important position in the life cycle of plants. Its organization and detailed developmental pathways differ considerably among species [13]. Recently electron-microscopic studies have demonstrated that the differences extend to the sub-cellular level [9, 10]. The female gametophyte of *Zea mays* has been studied ultrastructurally [6], but the work concerning the distribution of different substances and enzymes during its development has not attracted much attention. The present communication describes the changes in the distributional pattern of several reserve substances and enzymes before and following pollination. The histochemical data presented in this paper are compared with those on embryological details.

Material and method

The materials used for studying the development of caryopsis resulted from self-pollinating of plants of the variety Vijay grown during 1974 in the Punjab Agricultural University Botanical Gardens. Pollinations were made at 7 a. m. Collection of developing caryopsis was made at five-day intervals.

In all the enzyme localization studies, fresh unfixed tissue was used. The mature ovules were sectioned with Weswox hand and table microtome, without embedding. The ovules were sectioned 10—20 μm thick and transferred to buffers of a particular pH value depending upon the enzyme to be localized. The section were then lifted with a brush and were put in the desired reaction mixture and incubated for a specific time.

The procedures of enzyme localization were those described by JENSEN [9] and CHAYEN and co-workers [5]. For the localization of polysaccharides, nucleic acids and proteins, mature ovules after fertilization were fixed in Nawashin fixative, Craf I for 0—5 day caryopsis and Craf III for all other collections. The material was dehydrated in an ethyl alcohol—TBA dehydration series and embedded in paraffin. The blocks were sectioned in a Weswox Junior rotary microtome at 8—10 μm.

For conveniences of description and precise assessment regarding relative concentrations, as observed under light microscope, the visible reactions were scored. Feeble or negligible (—), faint (+), normal (++), rich (+++), intense (++++) and strong (+++++) categories were distinguished.

Results

Polysaccharides

Polysaccharides were localized by the alcian blue—periodic acid—Schiff (AB—PAS) series as recommended by MOWRY [16] and DIBOLL and LARSON [6]. They occurred in the form of cell wall, cytoplasmic polysaccharides and storage starch. The nucellar cell walls and the integument cell walls were rich in acid mucopolysaccharides (Fig. 11). The gametophyte wall was AB-negative

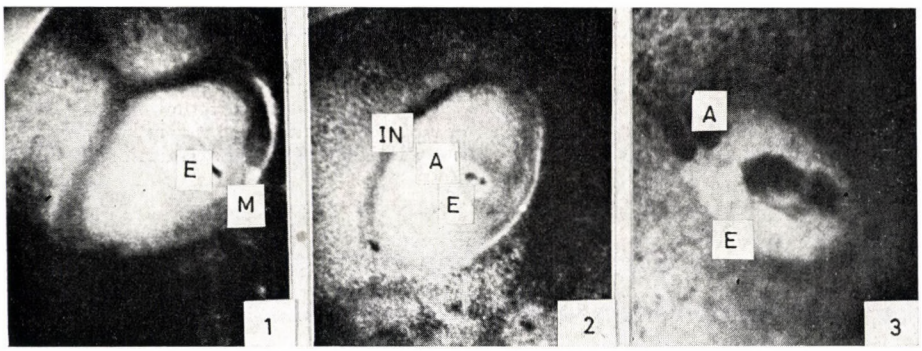
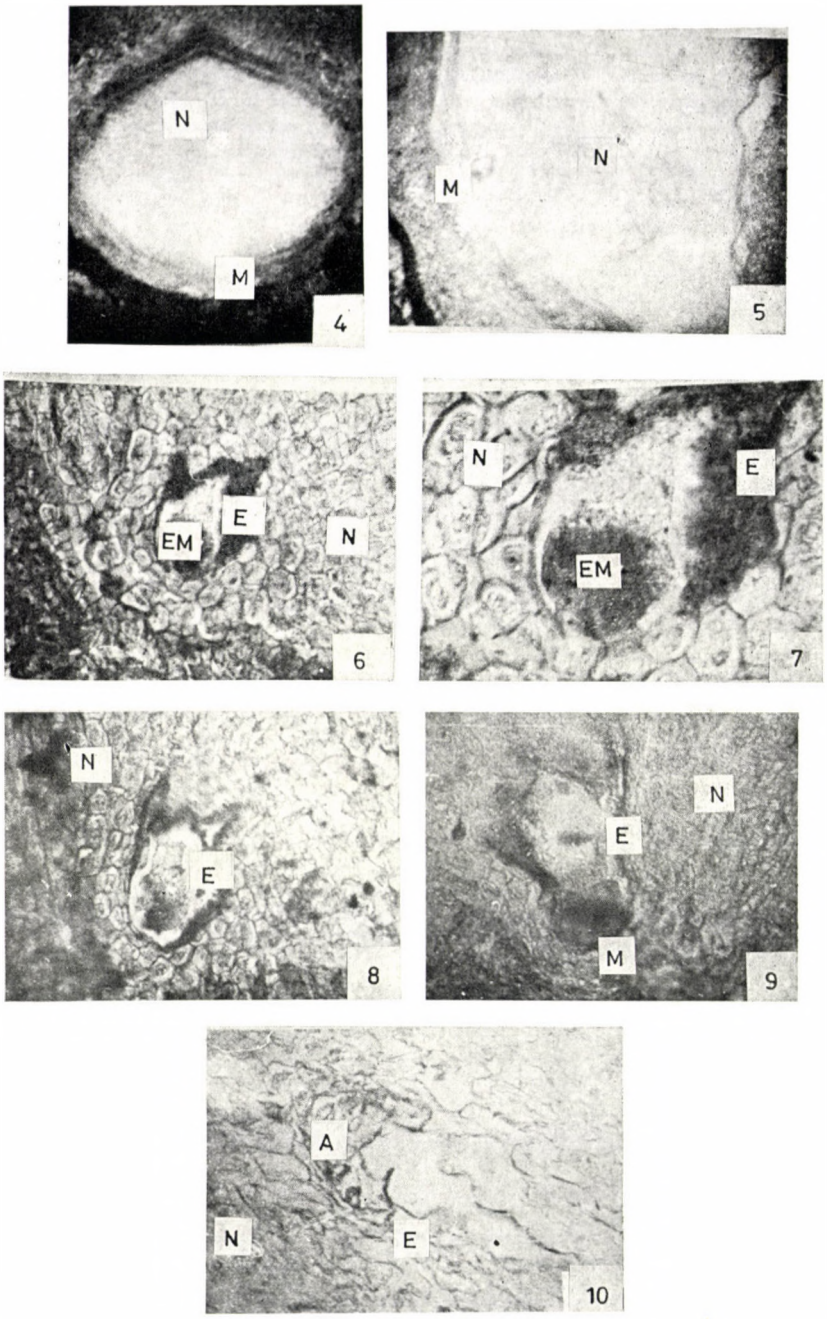


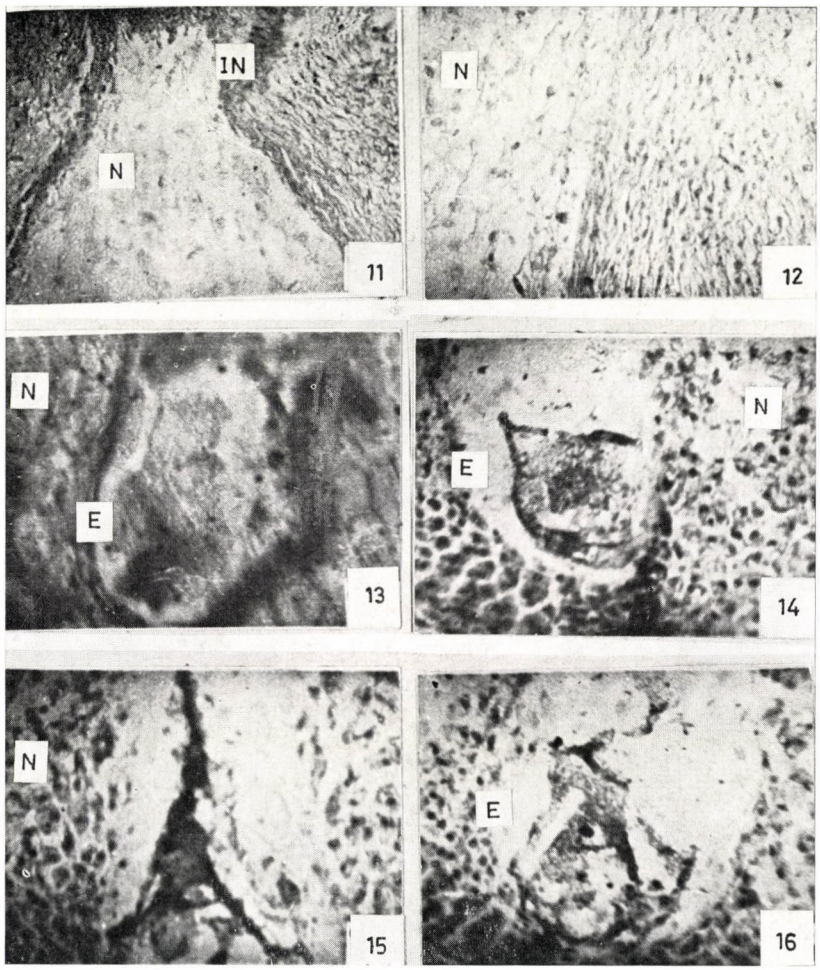
Fig. 1. Proteins. Ovule showing large amounts of protein in integument and embryo
N = nucellus, EM = embryo, A = antipodals, En = endosperm, E = embryo sac, M = micropyle, IN = integument

Figs 2—3. Lipids. *2* — Ovule with embryo, antipodals, and integuments rich in lipids; *3* — Enlarged view of embryo sac showing antipodals, embryo and nucellar cells adjoining embryo sac having great amount of lipids

and PAS-positive. In the developing embryo sac, an intense cytoplasmic PAS reaction was observed (Fig. 5). Nucellus cells also contained storage starch grains and this was confirmed by the IKI reaction. Nucellus cells showed yellow while the outer integument and chalaza showed deep blue starch grains. Within the embryo sac, the egg cell in its cytoplasm and the synergids showed faint PAS-positive tinge. The zygote was AB-negative and



Figs 4—10. PAS-positive polysaccharides. *4* — Ovule with strong PAS reaction in integuments; *5* — Ovule section showing strong PAS reaction in embryo sac wall and globular embryo; *6* — Embryo sac showing thick PAS-positive wall and an intense reaction in the basal tier of the globular embryo; *7* — Enlarged view of Fig. 6.; *8* — Embryo sac with thick PAS-positive wall and an intense cytoplasmic reaction in nucellar tip cells; *9* — Micropyle end of embryo sac showing intense PAS reaction; *10* — Chalazal end of embryo sac with antipodals depleted of cytoplasmic polysaccharides



Figs 11—12. Acid mucopolysaccharides. *11* — Portion of ovule with intense AB reaction in cell walls of integuments; *12* — Enlarged portion of Fig. 11.

Fig. 13. A portion of embryo sac with thick PAS-positive wall rich in neutral polysaccharides in the basal tier of the embryo

Figs 14—16. Nucleic acids. *14* — A portion of embryo sac showing nucellar cells in the micropylar zone rich in DNA and RNA. Note the intense RNA reaction in the tunica layer of the globular embryo; *15* — Portion of embryo sac with intense RNA reaction in the developing endosperm; *16* — A portion of embryo sac with nucellar tip cells rich in RNA

showed acid polysaccharides. In the globular embryo an intense PAS reaction was noticed near the basal end. The upper part of the globular embryo had small starch grains. The central cell contained traces of polysaccharides along its peripheral cytoplasm. The wall of the embryo sac was very thick, with strong polysaccharide deposition (Figs 6, 8). However, near the polar ends, where the eggs and the antipodals are situated, it was thin and stained less intensely

(Figs 9, 10). The wall of the embryo sac near the micropyle was strongly positive (Fig. 9). At this stage, nucellus cells contained storage starch. The reaction was highly intense in the outer, and less intense in the inner integument. The tip of the inner integument had large amounts of polysaccharides. Fig. 9 shows the polysaccharide distribution in the micropylar region of the ovule. Fig. 9 shows disintegrated antipodals with depletion of their polysaccharide contents.

Proteins

Protein was distributed in all the tissues of the maize ovule, in the developing embryo sac, antipodals and integuments (Fig. 1). The nucellus cells, especially those adjacent to the embryo sac, were also rich in proteins. After fertilization, the intensity of the reaction considerably increased, especially in the chalazal end of the nucellus.

Lipids

All the components of the embryo sac, except the central cell, contained acid lipids.

The most intense reaction was observed in the antipodals (Figs 2 and 3). Integuments also showed rich accumulations of lipids. High amounts were localized in the embryo. In sharp contrast to other tissues, nucellar cells showed faint lipid reaction.

Nucleic acids

Figs 14, 15 and 16 show the distribution of nucleic acids.

Enzymes

Oxidases

Peroxidase. Peroxidase activity was demonstrated by the benzidine reaction. Jensen's technique was followed. Before fertilization, there was considerable activity near the micropylar end. However, most part of the nucellus showed feeble enzyme activity. When fertilization was proceeding, part of nucellus surrounding the embryo sac was intensely positive. It appears that the activity of peroxidase was stimulated by pollination. About the time of fertilization, the micropylar end and the chalazal area showed strong activity.

Cytochrome oxidase. The site of the enzyme activity was indicated by formation of indophenol-blue granules. The distribution was comparable to that of succinate dehydrogenase. At the time of fertilization, the tip of the nucellus was strongly positive and the reaction intensified severalfold in the nucellar cells adjacent to the embryo sac. The nucellus tissue near the chalazal zone showed strong staining. Similarly, in the globular embryo, an intense activity was visible.

Transferases

Phosphorylase. To localize phosphorylase activity we studied the presence of newly formed red and old blue starch following the action of the enzyme on glucose-1-phosphate. The distribution of this enyzme is demonstrated in Fig. 7. The nucellus showed a very strong reaction in the integuments and in the tip. The antipodals also stained strongly and so did the chalazal zone and the nucellar tip and outer integument. The tip of the inner integument was also intensely positive. Phosphorylase simulated starch in distribution.

Hydrolases

Acid phosphatase: For the localization of acid phosphatase, Gomori's technique was employed.

Unfertilized ovules had intensely positive antipodals. However, nucellar cells were feebly stained. Integuments show an intensely positive reaction. The micropylar end of the nucellus was also intensely stained.

After fertilization, the activity considerably increased in the nucellar cells adjacent to the embryo sac. The micropyle also showed intense activity. Within the embryo sac, the globular embryo had strong activity and the same was true of the disintegrating antipodal cells.

Lipase: Lipase was observed in all the tissue of the nature ovule approaching fertilization. Integuments showed intense reaction. The nucellar cells near the nucellus tip showed an intense activity.

Dehydrogenases

Succinate dehydrogenase: This enzyme had a more pronounced activity than the hydrolases. The reaction in the integuments and in the nucellus cells was moderate while the micropylar and chalazal cells of the nucellus end were strongly positive. The reaction is very strong in the tip of the nucellus.

Following pollination and subsequent fertilization, there was a general increase in the enzyme activity, especially in the micropylar region, the nucellus and the chalazal end. Seemingly, the activity was concentrated in the active growth regions including the zygote.

Discussion

Histochemical studies on the development of caryopsis in *Zea mays* indicated the presence of diverse types of enzymes and variety of reserve products. Extensive changes occurred in different enzymes during the development of the ovule.

It should be noted that, in maize, very few enzymes have been reported in the ovules and the developing fruit, compared to pollen. Possibly, the ovule and the seed, being closed systems, are difficult to be examined bio-

chemically. Our studies clearly indicate that the female gametophyte in general resembles pollen grains, viz., similar enzymes are present and the megagametophyte and the surrounding tissues are physiologically highly competent and efficient in both.

Ovules, however, lacked enzymes, especially in the egg apparatus, which was usually associated with green plastids. Similarly, in the pollen grains, isoenzymes, especially those of peroxidase, esterase and some phosphatases have been reported [2]. However, for the embryo sac such data are lacking. One possible reason could be the structure of ovule and the size of the embryo sac itself. It is commonly observed that only a single megaspore differentiates, and expands into the embryo sac. Probably, the functional megaspore is considerably specialized. It would be rewarding to study the changes in the levels of enzymes and growth hormones in the functioning megaspore and embryo sac as well as in the surrounding tissue, during different stages of morphogenesis. The mechanism of differentiation is reverse to that of stomata, the megaspore-embryo sac undergoes differentiation and expansion whereas the nucellus cells remain unchanged in structure. In the epidermal cells where stomata differentiate and expand the epidermal cells undergo considerable enlargement but no differentiation. The embryo sac has a great array of enzymes and several physiologically active substances which lend this structure unique physiological and/or biochemical characteristics. It is pertinent to mention that three hydrolases (acid phosphatase, acetylesterase and lipase) which occurred within the developing ovule were not freely and quickly diffusible. Such phenomena helped in delimiting further growth and differentiation of the embryo sac. Prior to fertilization, activity of succinate dehydrogenase, cytochrome oxidase and peroxidase increased severalfold, especially in the micropylar region and cells adjacent to this zone and in zones where the pollen tube entered the ovules.

Intense activities of succinate dehydrogenase and cytochrome oxidase indicated high levels of respiration in the micropylar region. The rate of high metabolism in this region was also evidenced by the localization of large amounts of lipids, polysaccharides and starch. ZINGER and PODDUBNAYA-ARNOLDI [22] and VERMANI [21] also observed an increase in the activity of peroxidase enzyme just before fertilization. Seemingly, increased respiratory activity provided to sustain an increased rate of tube elongation.

Association of peroxidase with secretory-glandular organs was suggested by ZINGER and PODDUBNAYA-ARNOLDI [22] and HESLOP-HARRISON [8] compared the tip of integuments in some cases to anther tapetum. DIBOLL and LARSON [6] suggested that synergids and the tip of integuments were secretory in function and produced chemotropic substances.

Furthermore, such secretions helped in the development of pollen tube and were potential sources of basic material contributory to pollen wall biogen-

esis. Recently LOWEUS and LABARCA [12] have proposed a pathway by which polysaccharide fragments would be transferred from the styler canal into the pollen tube cytoplasm, where they would be utilized. It is suggested that the micropylar zone is a second nutritional source which contributes significantly to the wall material of the growing pollen tube.

The nuclear division within a functional megaspore seems to indicate initiation of embryo sac "germination". During this stage, high amounts of peroxidase and acid phosphatase were present. Several workers have suggested the involvement of these two enzymes in cell division and cell wall formation as well as in the general process of differentiation [14, 20]. The nuclear division within the megaspore was accompanied by low levels of cytochrome oxidase or succinate dehydrogenase. Obviously, during nuclear division, the respiratory activity was low. BROWN and DYER [4] have already indicated that the level of O_2 uptake is higher during the interphase than in other stages. Therefore, the possibility of involvement of respiratory activity and/or its level in regulating the growth and differentiation of embryo sac is evident. The question of how final division of nuclei is not accompanied by wall formation remains unanswered. It is easy to conceive that within the embryo sac there is some selectivity of operational investment of wall. However, the mechanism of the selectivity is not clear. DIBOLL and LARSON [6] have studied the development of the megagametophyte in *Zea mays*.

DIBOLL and LARSON [6] observed that the cell wall of synergids was the thickest near the micropyle but it is membranous adjacent to the polar nuclei. The distribution of polysaccharides as observed by us, was consistent with their findings. However, with alcian blue, the coloration was very poor.

DIBOLL and LARSON [6] observed abundant plastids and lipid bodies, but no starch. We observed a faint PAS reaction in the cytoplasm, but granular insoluble polysaccharides were not seen. The filiform apparatus was strongly PAS-positive. In other words, a rich PAS-positive filiform apparatus dominated the micropylar region of synergids. DIBOLL and LARSON [6] published a similar pattern, viz., a contact between the filiform apparatus and nucellus cells. JENSEN [10] made similar observations in cotton. We propose that nucellus was the chief source of metabolites which were passed on to the filiform apparatus. The latter served as a pathway of entry for these metabolites. Richness in polysaccharides of the filiform apparatus may be an additional source of carbohydrate exudate available to the growing pollen tubes. However, the extent of fragmentation of these polysaccharides before their utilization in the biogenesis of wall polysaccharides in the pollen tube is not clear. LOWEUS and LABARCA [12] suggested the MIO pathway through which the hexoses would be converted into the units of cell wall polysaccharides.

Antipodals were rich in cytoplasmic polysaccharides, reserve starch, proteins and lipids. In addition, several enzymes, e.g., peroxidase, succinate

dehydrogenase, cytochrome oxidase and lipase were localized there. During the later stages, antipodals contributed metabolites to the embryo sac and the endosperm. The same was suggested by VERMANI [21] who studied *Zephyranthes*. An excessive amount of DNA in antipodals also pointed towards their secretory function. Intense enzyme activities and high amounts of lipids and proteins suggested a high metabolic rate in these cells. Antipodals have multiple roles, viz., absorption, storage and secretion [8]. The presence of large amounts of lipids in the antipodals agrees with the electron microscopic studies of DIBOLL and LARSON [6]. DIBOLL observed that in the unfertilized megagametophyte mitochondria in antipodal cells were compact, but became hypertrophied after fertilization. Presumably, the mitochondrial swelling resulted from an increased biochemical activity. Incidently, the localization of succinate dehydrogenase and cytochrome oxidase in our material supported the electron microscopic observations of DIBOLL. Antipodals are therefore a zone of special physiological interest.

Antipodals also control and/or regulate and initiate, the nuclear division of the primary endosperm nucleus in the initial stages and during the further development of endosperm. In cotton, during maturation of the megagametophyte, JENSEN [9] found degenerated functionless antipodals. He also proposed that synergids possibly take over the activities of the antipodals. DIBOLL failed to observe similar changes in maize. Histochemical studies in maize antipodals indicated large amounts of reserve products in the form of starch, lipids and proteins, which thus seem to play an important nutritive role. The histochemical differences between antipodals and synergids suggest that synergids do not take over the role of antipodals.

The nucellar tissue showed intense enzyme activities and accumulated lipids, polysaccharides, starch, etc., especially in the chalazal region. It appears that the nucellus was a pathway through which metabolites passed into the embryo sac, and the chalazal region was a possible site of entry. Both the nucellus and antipodal cells are rich in starch, polysaccharides and lipids. Some authors suggest that initial contribution to the embryo sac nutrition is made by the nucellus. In the later stages the nucellus cells disintegrated and as mentioned above these cells were full of reserve materials. HESLOP-HARRISON [8] compared this region with the anther tapetum. It seems, therefore, that there are several tissues in the ovule, including antipodals, nucellus and integument, which are secretory in function.

The nutritional role of different structures is indicated below:

Embryo sac Endosperm in initial development	Nucellus
Endosperm Zygote in initial development	Antipodals

Embryo sac Zygote	Integument
Zygote	Endosperm, suspensor of embryo

A comparison of unfertilized and fertilized ovules revealed that the former had feeble oxido-reductase activity and was extremely poor in reserve substances; similarly, the hydrolase and transferase activities were low. However, a short time before fertilization, there was a gradual, but considerable increase in these enzyme activities. After fertilization, the increase was heghtened. This was possibly linked with reserve substances. In the phase approaching seed and/or caryopsis formation the metabolic activity considerably decreased. Hydration may be a possible factor.

Mature embryo sac contained little amounts of polysaccharides and showed low metabolic activity. Most of the enzymes were also feebly localized. Our histochemical investigations support the electron microscopic studies of DIBOLL, viz., in the mature unfertilized megagametophyte, the rate of membrane synthesis was very low.

It is presumed that like the ripe pollen the embryo sac was in a state of quiesence. This could be attributed to the non-availability of metabolites from the adjacent tissue. Nearing fertilization, most of the metabolites increased considerably. Presumably, pollen tubes conveyed some triggers which activated the mobility, accumulation and availability of physiologically active substances. Following this, most of the enzyme activities also increased.

There was an amplification of the mitochondrial enzymes cytochrome oxidase and succinate dehydrogenase, immediately after fertilization. The structural changes resulting from fertilization, observed by DIBOLL and by us, are interpreted as reflecting the requirements of rapid growth after fertilization when zygote and endosperm nuclei are dividing. DIBOLL observed hypertrophy of mitochondria following fertilization. BROWN and DYER [4] reported that the dividing cells had lower respiratory activity than the growing ones. NARAIN [17] demonstrated that cytokinin-induced growth, during active cell division, displayed lower respiration rates. The zygote did not divide while the endosperm nucleus did. This may be attributed to the cytokinin and/or the respiratory level. DIBOLL and LARSON [6] suggested that large polymorphic mitochondria, as observed in egg, may not necessarily mean a high rate of respiration. Indeed, we did observe low succinate dehydrogenase and cytochrome oxidase activities, which might be associated with post-fertilization metabolic activity.

Developing maize ovules stained with AB – PAS revealed acid or neutral polysaccharides in various cell walls. These existed in several forms, viz., soluble sugars, insoluble cell-wall polysaccharides (cellulose, pectin, etc.) and even storage starch. Storage polysaccharides contribute to the cellular energet-

ics and to the production of energy-rich triphosphates. The presence of storage starch indicated potential energy which could be utilized during different growth and differentiation processes in plants. Therefore, the presence of starch and its utilization in the organization of various tissues is very significant. Our results indicate that insoluble polysaccharides play a significant role in the differentiation of the embryo sac.

The occurrence of starch grains in the cytoplasm reflects a high rate of polysaccharide metabolism, which was contributory to the organization and differentiation of the megagametophyte.

The magenta colour of the embryo sac wall in PAS preparations indicates the presence of hydroxyl groups while the presence of carboxyl groups in the nucellar cell wall is indicated by alcian blue staining [6]. The significance of altered cell wall chemistry or the structural absence of plasmodesmata in relation to intercellular circulation is not clearly understood. However, ISRAEL [23] described the disapperance of plasmodesmata from MMC in *Dendrobium*. He postulated that this loss of cytoplasm continuity might alter the obligatory flow of metabolites between the nucellus and spore mother cells and thereby triggered the initiation of megasporogenesis by establishing a peculiar environment within the spore mother cells. DIBOLL observed a similar appearance of plasmodesmata from the wall of the developing maize megaspore mother cell. Perpetuation of this condition apparently prevailed throughout the embryo sac development and might be important to that development. These observations were interpreted as strengthening the concept that the female gametophyte of seed plants was a plant generation, developmentally distinct from the sporophyte, but one which had evolved physiological dependence upon the sporophyte.

The thick embryo sac wall acted as a "molecular filter" which allowed specific substances to pass from the nucellus into the embryo sac and contributed to its differentiation. Nucellus, integuments especially near the chalazal, showed high accumulation of insoluble polysaccharides, but after the process of fertilization these were gradually depleted from the nucellus. Similarly, before the process of embryogenesis, the antipodal cells were rich in starch, which was presumbly consumed during the formation of the endosperm. This suggests that starch is a basic metabolite required during the early differentiation of the endosperm or even of the embryo. In the globular embryo of maize the terminal tiers revealed absence of starch and a marked loss of cytoplasmic PAS reaction. On the other hand, the basal tiers had large amounts of stored starch and cytoplasmic polysaccharides. Tissue differentiation begins in the old globular embryo with the organization of growth centres — one at the tip of the terminal tier and another in the middle tier, laterally contributing to the scutellum, a storage tissue. The shoot root axis showed less cytoplasmic polysaccharide.

The localization of acid phosphatase was mostly observed in the cytoplasm as well as in the cell wall of the mature embryo sac. The activity was also found in the antipodals as well as near the micropyle of female gametophyte during fertilization. The zygote also contained high amounts of this enzyme. In the cytoplasm of the megagametophyte, DIBOLL identified lysosomes growing in amount after fertilization. We suggest that acid phosphatase is linked with the differentiation and the maturity of the embryo sac. Thus, our studies are in agreement with those of MIA and PATHAK [15] and VERMANI [21]. GAHAN and MCLEAN [7] postulated that acid phosphatase was associated with sugar transfer across the sieve plate. Since the enzyme was very active near the embryo sac or adjacent to the nucellus, we suggest that the activity of this enzyme may be physiologically concerned with the exchange of materials.

Following fertilization, the enzyme activity increased not only in the zygote but also in the cytoplasm of the embryo sac. Therefore, the possibility that it assisted in the absorption of metabolites from surrounding nucellus cells seems logical. Probably, this enzyme is also concerned with cell wall formation as well as liberation of energy by general process of hydrolysis. In this connection, we may recall that levels of acid phosphatase increased considerably near the micropylar end just about the process of fertilization. Occasionally, both succinate dehydrogenase and cytochrome oxidase also exhibited intensified reaction prior to, during and after the process of fertilization. The enzyme activity also increased considerably on the margin of the nucellus and the integument. Similar situations have been reported in *Zephyranthes* by VERMANI [21] viz., both the enzymes were associated with mitochondria and were involved in transfer of electrons to oxygen. Both the enzymes thus found simultaneously in the same tissue integrated into the process of cellular respiration.

Phosphorylase activity was observed in the embryo sac, outer boundary, integuments and the ovary wall, but was not in the micropyle and in the nucellus tissue. The occurrence of starch simulated the localization of phosphorylase. Furthermore, ovule also had other enzymes, esterase and lipase among them, which were concerned with hydrolysis of other food material. Presumably, these enzymes also increased in amount following the process of fertilization. Considering that, following the process of fertilization, the nucellus undergoes a gradual autolysis, the role of acid phosphatase-positive granules, i.e. lysosomes, in such a process has become more lucid.

Moreover, during this process another hydrolase, 5-nucleotidase, was also possibly involved. The presence of lipids and lipase in the tissues of ovule, nucellus, antipodals, etc., suggests that these were the source of metabolites which made available energy during megasporogenesis and development. Thus, lipase acted on lipids and resulted in the formation of free fatty acids

The latter were converted into sucrose following the action of intermediary enzymes or fatty acids released energy through the β-oxidation cycle.

Peroxidase was observed in ovary wall and in the embryo sac and its adjacent areas, except the nucellus proper. Near the process of fertilization, the amount of peroxidase increased in the micropyle, in the antipodals and in the egg apparatus zone. However, the central cell was completely devoid of peroxidase activity. It is of special interest that this enzyme was intensely present during the differentiation of the embryo sac. Presumably, it was closely associated with the process of differentiation. VAN FLEET [20] observed the occurrence of this enzyme in the differentiating tissue of root, as well as in the developing nucellus and embryo sac. MALIK and VERMANI [14] demonstrated a considerable increase in the peroxidase level during the embryo sac differentiation. In a recent study the activity of this enzyme is correlated with the differentiation of stomata and trichomes. Peroxidase was therefore involved in the cellular differentiation. Furthermore, meristemoids showed high accumulation of this enzyme and there was good amount of expansion and differentiation. As regards the distribution of AA VERMANI [21] found a positive correlation between peroxidase and ascorbic acid in *Zephyranthes* sp. Furthermore, the differential distribution and extent of flavonoids in regulating the process of differentiation of the embryo sac was also discussed. We do not have any information on the occurrence of ascorbic acid. However, SUTHER, BHATT and SHAH [19] observed localization of ascorbic acid during various developmental phases of maize. The synergids and antipodals were rich in ascorbic acid. Similarly, supply of ascorbic acid to zygote was through the synergids and the antipodals. In our studies, the localization of peroxidase agreed well with histochemical localization of ascorbic acid. Thus, we may suggest that peroxidase was definitely concerned with the growth centres, locales of zygote and primary endosperm nucellus. However, with the development and maturity of the fruit, the amount of peroxidase decreased. The maize ovule contains many enzymes which are needed for the hydrolysis of food reserves, e.g., lipase, acetyl esterase, acid phosphatase, etc. SIMOLA and SOPANEN [18] suggested that the function of α-glycero-phosphatase may be connected with lipid synthesis as well. All the enzymes exhibited essentially similar distribution. BROUILLARD and OUELLET [3] made similar observations in wheat. These authors found that some phosphatase enzymes possessed esterase activity. We may explain our observations on similar grounds. The developing aleurones and scutellum were also rich in hydrolases. However, epithelium had low amount of them. Possibily these layers secreted hormones and factors which stimulated enzyme activity in the aleurone. According to JONES [11] , GA_3 enhanced the production of at least six hydrolytic enzymes in the aleurone cells of barley. The occurrence of these enzymes in relation to seed germination may be visualized. Seed coat is a very important barrier to

water absorption. Testa, however, lacked the activity of these enzymes but had fat deposits and proteins. It is questionable whether such activities had any significance for the maturity of the seed.

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PHOSPHATASES IN THE ACCESSORY RESPIRATORY ORGANS OF TWO FRESH-WATER FISHES

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Abstract

The distribution of enzymes, viz., alkaline phosphatase, acid phosphatase, adenosine monophosphatase and adenosine triphosphatase was studied by histochemical methods in the accessory respiratory organs of two fresh-water fishes *(Clarius batrachus* and *Heteropneustes fossilis)*. Enzymes have been used as markers to differentiate between functional and non-functional cells of the dendritic organ of *Clarius* and of the air chamber of *Heteropneustes*. The variations in the enzyme activities have been correlated with the functional capacity of each respiratory organ. It is attempted to understand the physiological role of these enzymes in the process of aerial breathing.

Introductior

The accessory respiratory organs of fishes provide an excellent example of adaptation by modification. These organs are variable in structure in different fishes. For example, in *Clarius batrachus*, the accessory respiratory organ consists of a (1) suprabranchial chamber, (2) gill plates, (3) a dendritic organ or respiratory tree and (4) a respiratory membrane, whereas in another fresh-water fish, *Heteropneustes fossilis*, it is made up of (1) a respiratory sac or air chamber, (2) extended gill plates and (3) a respiratory membrane. The structure and functioning of these organs have been studied thoroughly [3, 6, 7, 9, 10, 11, 12], but their enzymes have not been analysed so far, and their role in the process of aerial breathing is unknown.

The present work was carried out with two species of fishes, viz., *Clarius batrachus* (commonly called Mangur) and *Heteropneustes fossilis* (commonly called Singhi). Both inhabit fresh-water of Muzaffarnagar. An attempt was made to localize a few enzymes, viz. alkaline phosphatase, acid phosphatase, adenosine monophosphatase (AMPase) and adenosine triphosphatase (ATPase) in the respiratory tree of *Clarius batrachus* and the air chamber of *Heteropneustes fossilis*, employing histochemical parameters. Whether these enzymes play any role in the aerial breathing of fishes forms the main part of the present study.

Material and method

Living fishes, *Clarius batrachus* and *Heteropneustes fossilis* were collected from the native river Kalinadi and brought to the laboratory. Respiratory tree from *Clarius* and air chamber from *Heteropneustes* were removed intact from the living fishes and as a whole fixed in chilled absolute acetone, and 10% buffered neutral formalin in the refrigerator. Fixed frozen, fresh frozen, and paraffin-embedded sections thus prepared were processed for the following enzymes, employing the indicated specific histochemical methods.

Alkaline phosphatase	—	Calcium cobalt method [5].
		Azo dye method [2].
Acid phosphatase	—	Lead nitrate method [5].
Adenosine monophosphatase	—	Calcium method [15]
Adenosine triphosphatase	—	[14].

Haematoxylin and eosin stained sections were also prepared to show the position of various cell types. Suitable controls for enzymes were run simultaneously.

Results

Morphology

Clarius batrachus possesses two respiratory trees or dendritic organs on each side. The first one, borne by the second gill arch, is smaller. The second respiratory tree is better developed and is borne by the 4th epibranchial of the fourth arch. The whole of the dendritic organ is supported by an internal cartilaginous skeleton (Fig. 1).

Respiratory sac or air chamber of *Heteropneustes fossilis* is formed by the backward extension of suprabranchial chamber and remains embedded

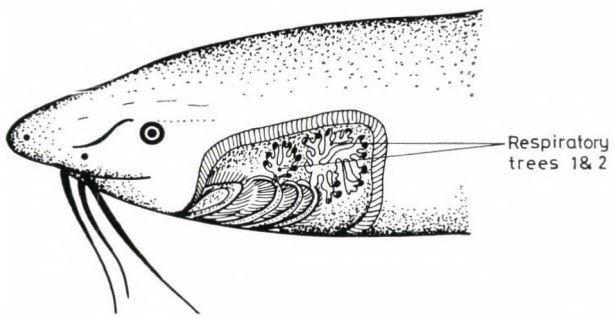

Fig. 1. The first dendritic organ in *Clarius batrachus* is borne by the second gill arch and seems to be smaller in size. The second respiratory tree is better developed and borne by the fourth epibranchial of the fourth arch. (Natural size)

in the trunk myotomes. In a full-grown specimen it was found reaching up to the middle of the tail region. The anterior part of the air sac is wider than the posterior part. The inhalent aperture, which is formed by the approximation of the second and third fans, opens in the bottom of the anterior chamber, which is continuous backwardly and forms the tubular sac (Fig. 2).

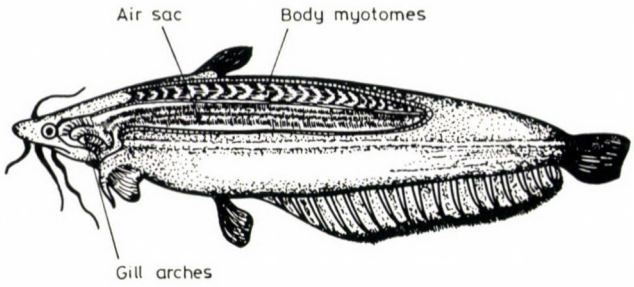

Fig. 2. The air chamber in *Heteropneustes fossilis* is formed by the backward extension of suprabranchial chamber. It reaches up to middle of the tail region remaining embedded in the trunk myotomes. (Half of the natural size)

General histology

Clarius batrachus. The terminal parts of the respiratory tree end in blunt knobs. The knobs in transverse section seem to consist of a core of cartilage in the centre. This is surrounded by an inner uniform connective tissue and an outer epithelial layer. The latter forms the outermost covering of the knobs. The complete epithelial layer is further characterized by 8 folds. These are separated from one another by a U shaped depression representing a non-vascular area. Equal sets of blood vessels are associated with each fold. A single fold of a typical knob may very well be compared with a primary gill lamella (Fig. 3).

Heteropneustes fossilis. The respiratory sac of *Heteropneustes* seems to be totally different from the respiratory tree of *Clarius*. The cavity of the air chamber is thrown in folds which are more prominent in the anterior region than elsewhere. Only one fold is more prominent than the others. Each fold is covered by the muscular coat, followed by connective tissue fibres and by the fine layer of the respiratory epithelium. The whole air sac is covered by muscular coat. The main blood supply is clearly seen in the region of connective tissue fibres (Fig. 4).

Enzyme histochemistry

Alkaline phosphatase. In *Clarius* the epithelial layer of the knobs reacted strongly for alkaline phosphatase while the cartilaginous skeleton and connective tissue fibres showed complete absence of the enzyme. The muscle lining of the epithelium showed a weak positive reaction. The restricted activity in the 8 folds showed that the enzyme marks its presence in the vascularized areas of the respiratory tree (Fig. 5).

In the air chamber of another specimens the muscular coat exhibited a normal reaction while the inner lining of the respiratory epithelium of folds reacted strongly. The activity in the respiratory epithelium was found to be discontinuous, indicating the importance of cells rich in alkaline phosphatase in the process of aerial breathing (Fig. 6).

Acid phosphatase. Acid phosphatase activity in *Clarius* resembles that of alkaline phosphatase. The epithelial covering showed strong positive reaction. U-shaped spaces between the folds are clearly seen. Adjoining muscles

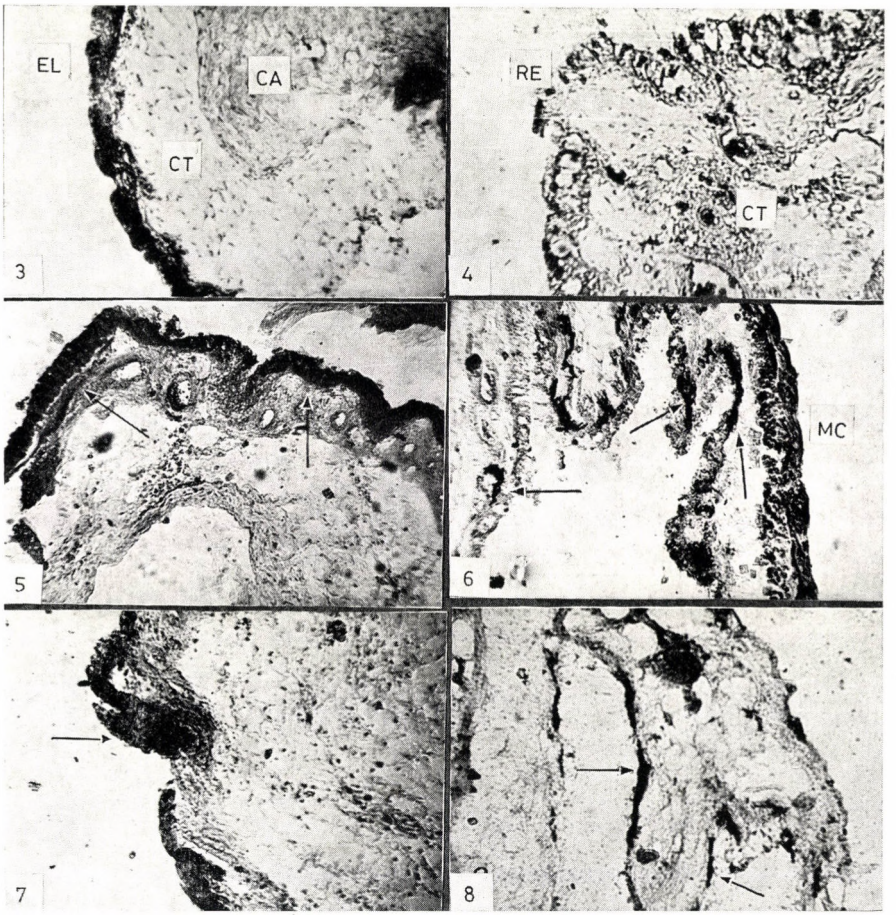

Fig. 3. T. s. of a single knob of respiratory tree shows the outer epithelial covering tissue that is supported by a central core of cartilage. U-shaped regions representing the nonvascular area are also seen. × 125

Fig. 4. T. s. of the air chamber of *Heteropneustes.* Note one of the folds in the cavity of air chamber seems to be more prominent. The tubular air chamber is covered by a muscular coat, followed by connective tissue fibres and the layer of respiratory (RE) epithelium. Haematoxylin and eosin, × 125

Fig. 5. The respiratory tree of *Clarius.* In each knob strong positive reaction for alkaline phosphatase is shown by the epithelium alone. The muscular layer shows a weak positive reaction. × 80

Fig. 6. T. s. of air chamber shows a discontinuous pattern of alkaline phosphatase reaction in the inner lining of the respiratory epithelium of each fold. × 80

Fig. 7. Positive acid phosphatase reaction in the epithelium covering knobs. Note the U-shaped spaces between folds. × 80

Fig. 8. Outer layer of respiratory epithelium. Positive acid phosphatase reaction. Just the opposite of the alkaline phosphatase. × 80

exhibited a weak reaction while the connective tissue and the cartilage were totally negative (Fig. 7).

In *Heteropneustes* the localization of alkaline phosphatase is totally different. The inner lining of the folds was negative while the outer layer showed strong positive reaction. The muscular coat did not give any reaction (Fig. 8).

Adenosine monophosphatase (5-nucleotidase). The presence of AMPase could be shown in the epithelial covering alone. This lining is perforated at regular intervals. The perforations did not show any activity but the bar-like structures lying between perforations were strongly positive. The so-called bars seem to be connected with each other by horizontal small AMPase-positive rods just like those of gill lamellae (Fig. 9).

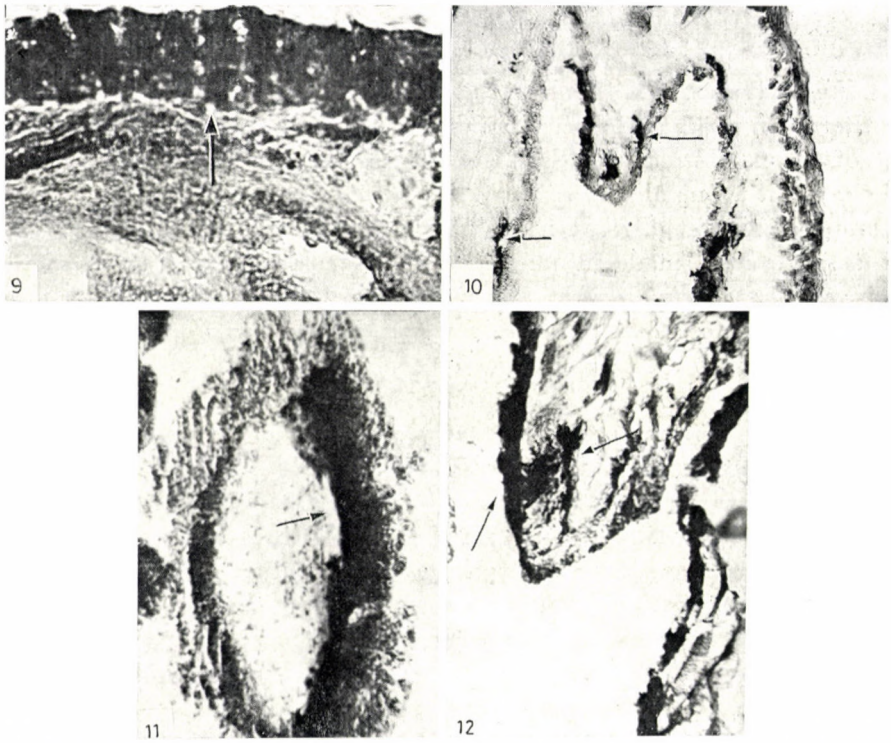

Fig. 9. Section stained for AMPase. Outer lining seems to be perforated at regular intervals. The lining of these perforations and the horizontal bars are strongly positive. × 320

Fig. 10. Heteropneustes. Few median cells of the prominent fold exhibit positive AMPase reaction while outer as well as inner linings appear negative. × 80

Fig. 11. In fixed frozen sections of the knobs, good AMPase activity is observed in the epithelium alone. × 125

Fig. 12. In *Heteropneustes*, outer and inner linings of epithelium are strongly positive for ATPase while the connective tissue and the muscular coat show no reaction. Fixed frozen section, × 80

In *Heteropneustes* the picture is totally different. Neither the inner nor the outer layer of the folds and sac gave positive reaction but the median cells showed enzyme activity. The pattern of distribution was found to be discontinuous again (Fig. 10).

Adenosine triphosphatase. In *Clarius*, good activity was noted for ATPase. The epithelium exhibited a strong positive reaction while no other parts showed any reaction (Fig. 11).

In the respiratory sac of *Heteropneustes*, the folds showed a well-defined enzyme reaction, which was the most pronounced in the middle prominent fold. The outer and inner linings were strongly positive while the middle portion of cells and the muscular coat showed no reaction (Fig. 12).

Discussion

From the anatomical and histological evidences presented here and by other workers [3, 7, 11] it is proved that the dendritic organ or respiratory tree of *Clarius batrachus* is derived from the primary and secondary gill lamellae. Each terminal end of this tree, the so-called knob or bulb seems to be composed of eight gill filaments that have fused and formed this new structure. The same studies made in another fish *Heteropneustes fossilis* show that respiratory sac or air chamber is important in air breathing and works much after the fashion of a fountain pen. However, DAS [3], who described the structure of respiratory epithelium, concluded as follows: "It has a thin epithelial wall underlain by loose connective tissue, it becomes richly vascular, deriving its blood supply from the afferent and efferent blood vessels of the fourth branchial arch that gives rise to vascular tongues." Later on MARLIER [7] described the same vascular tongues as papillae in *Saccobranchus*. However, it can be said that the tongues, papillae or folds are synonyms. The air first enters the anterior chamber through the inhalent slit and then goes into the posterior tubular part. The same air returns into the opercular chamber through the narrow slit between the third and the fourth fans. Thus, morphological studies support the respiratory function of the respiratory tree in *Clarius* and of the air sac in *Heteropneustes*.

Enzymological evidences presented here further prove the above statement. Four different phosphatases were traced in the epithelial regions of both fishes. How these enzymes are useful in the process of respiration? The basic idea that none of the enzymes is present in a non-vascular region, e.g. in the connective tissue and cartilage is an answer in itself. Histological studies show that in *Clarius* the epithelium is the outer lining while in *Heteropneustes* epithelium lines the internal folds, indicating that vascularized regions are differently displaced in both the fishes.

The presence of alkaline phosphatase in the complete epithelium covering of respiratory bulb suggests that enzyme facilitates the oxygen transfer to the internal structures. In the other fish the same function may be assigned to alkaline phosphatase on the basis of its localization. Acid phosphatase seems to be related with absorption though in *Clarius* it was found in the same regions as alkaline phosphatase. It is worth mentioning here that both these enzymes though present in the same region are responsible for their specific functions. In the other fish, the outer epithelium of folds shows remarkable activity for acid phosphatase, indicating that enzyme positive areas first come in direct contact with air. The discontinuity in the enzyme distribution further suggests that gas exchange takes place only through these regions and not via the entire surface of folds.

Though AMPase and ATPase show strong positive reaction in the respiratory organs of both fishes, manifestation of their activity seems not to be directly related to the process of gas exchange. The absence of AMPase from the respiratory epithelium in *Heteropneustes* suggests this view. However, in *Clarius*, activity was noted in the form of bands revealing a new fact, viz. that the respiratory epithelium in *Clarius* bear perforations. Presumably the air enters the bulb through these pores, where we have noted a strong positive reaction for alkaline phosphatase and acid phosphatase.

ATPases have been found in muscles, in the mitochondrial, ribosomal and cytomembranous fractions. Amongst the suggested contributions of ATPases to life of a cell are their direct metabolic functions, coupling oxidation and phosphorylation [13]. The same functions may very well be asigned to ATPase in the respiratory organs of these fishes.

Functional and non-functional cells could not be differentiated by routine histological stains. But by applying phosphatase(s) technique(s) it became clear that the respiratory tree or air chamber is not respiratory *in toto* but only their epithelial lining plays a vital role in the function. The conclusion that phosphatases are integrally concerned with the aerial breathing of fishes is thus inescapable. The study on the functional role of these enzymes in the accessory respiratory organs of the amphibious vertebrates will be of great academic interest.

Acknowledgements

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THE DEVELOPMENT OF THE IPSILATERAL RETINOTHALAMIC PROJECTIONS IN THE XENOPUS TOAD

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Abstract

The appearance of ipsilateral terminal degeneration in the thalamic visual centers is studied with the aid of the Fink—Heimer II technique in *Xenopus* larvae and postmetamorphic toadlets following unilateral eye removal. The youngest age at which degeneration can reliably be shown in the lateral geniculate complex (nucleus of Bellonci and corpus geniculatum thalami) was at stage 62, that is, 10—12 days before metamorphic climax. The whole compliment of the ipsilateral retinothalamic projection develops only in postmetamorphic toadlets, and it is much more abundant in *Xenopus* than in *Rana* species. The first appearance of the ipsilateral degeneration in the lateral geniculate complex coincides in time with the first ipsilateral visuotectal responses described by others. The possibility that the two phenomena may have a closer relationship than the mere time coincidence, is discussed.

Introduction

The primary optic pathway consists of contralateral and ipsilateral retinothalamic projections, and only of contralateral retinotectal and retinotegmental projections in the anuran brain [11, 15]. Physiological experiments have also revealed the existence of an ipsilateral retinotectal projection which emerges from the binocular visual field [5]. This projection is mediated indirectly by a polysynaptic pathway which originates in the contralateral tectum and passes through the diencephalon [7]. Responses evoked with visual stimuli can be recorded from the contralateral optic tectum at very early larval stages [4]; ipsilateral visuotectal responses, however, do not appear before the metamorphic climax, when the eyes in *Xenopus laevis*, gradually assume a frontal position and adopt a binocular visual field [1, 6].

The direct retinothalamic projections, which terminate in the nucleus of Bellonci, the corpus geniculatum thalamicum and in the pretectal region, originate in the temporal part of the retina [9, 14], which is involved in binocular vision. In view of the fact that these thalamic areas give rise to abundant

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thalamo-tectal connections [10, 16], the probable role of the direct ipsilateral retinothalamic connections in the establishment of the indirect retinotectal projection may be supposed. In the present work, the question whether there is a temporal coincidence between the development of the ipsilateral retinothalamic connections and the appearence of the ipsilateral visuotectal responses was investigated. While this work was in progress, CURRIE and COWAN [2] gave an account of the delayed development of the ipsilateral retinothalamic connections which were first detectable, with the autoradiographic technique, during the metamorphic climax in *Rana pipiens*. The present results obtained from *Xenopus* material with the terminal degeneration technique, corroborate their observations.

Material and method

The experiments were performed on *Xenopus laevis*. Spawning was induced by hormone injection (Choriogonin, Richter) given to both male and female specimens. The developing larvae were reared on diluted baby food filtered through cotton wool and staged according to the normal table of development made by NIEUWKOOP and FABER [12]. Starting at stage 50 (about 15 days after egg laying) larvae were unilaterally enucleated at every second successive stage till the end of metamorphosis, which is stage 66 and is about two months after egg laying. In addition to larval operations, the eye of a few postmetamorphic toadlets of about 4 weeks of age was removed unilaterally. Following enucleation larvae and toadlets were kept alive for 5—7 days; then they were killed, and the brains carefully removed and fixed in a 10% formalin solution at 4 °C for a period of 6 days. The fixed brains were embedded in egg yolk, and 20 μm thick frozen sections were cut in the coronal and horizontal planes. Degenerated nerve terminals were shown with the Fink—Heimer II [3] technique.

Results

In general, the larval brain lends itself to degeneration studies much less readily than the adult brain. Degenerated terminals in the contralateral optic tectum could only seldom and fragmentarily be shown in our material, although the presence of optic fibers has been demonstrated with physiological methods [4] and with the autoradiographic technique [2] in younger larvae of *Xenopus* and *Rana*, respectively. For some reason unknown to us, the thalamic optic centers are more favourable places. Degeneration could be shown in the pretectal neuropil area better than in the tectum, and it appeared most frequently in the nucleus of Bellonci and in the corpus geniculatum thalamicum. The present description to illustrate the development of the ipsilateral retinodiencephalic projection is, therefore, confined to these two latter nuclei. In view of the fact that, especially in young larvae, the nucleus of Bellonci and the corpus geniculatum thalamicum cannot easily be distinguished from each other in impregnated specimens, they will be referred to as the lateral geniculate complex.

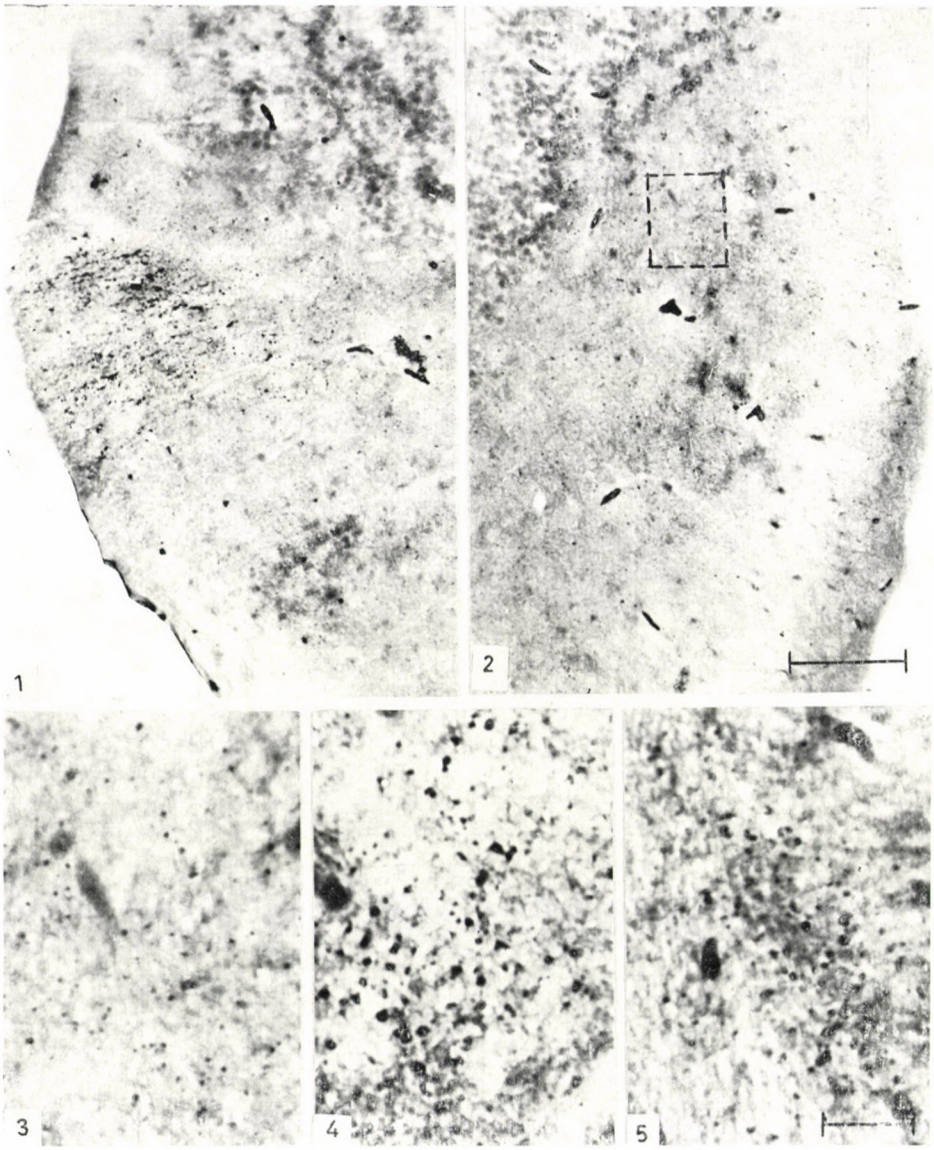

Fig. 1. Horizontal section of the dienchephalon contralateral to eye enucleation made at stage 60. Terminal degeneration outlines the lateral geniculate complex

Fig. 2. The ipsilateral diencephalon of the same brain. Broken line indicates the region of the lateral geniculate complex

Fig. 3. Enlarged photograph of the region enframed in Fig. 2, showing the first sign of ipsilateral degeneration in the ipsilateral geniculate complex following eye removal at stage 60

Figs 4—5. Degeneration in the lateral geniculate complex following ipsilateral eye removal at stage 64 and 66, respectively. Scale indicates 100 μm in Fig. 2, and Figs 1 and 2 are of the same magnification. The 20 μm scale in Fig. 5 applies to Figs 3 and 4 as well

The lateral geniculate complex, as it is outlined by degenerated terminals of the contralateral retinodiencephalic fibres, occupies a triangular area in horizontal sections (Fig. 1), and an oval area in coronal sections of the brain of young larvae. Silver particles indicating degenerated terminals tend to form smaller and larger clusters among which smaller silver grains can be seen. Although it cannot clearly be established from the present material, this clustering gives the impression as if the optic fibres were terminating in "puffs". With increasing age the density of silver particles increases and by the time of metamorphosis (stage 66) they are distributed evenly within the area of termination (Fig. 6). In the pretectal neuropil area degeneration, whenever it has been shown, appears in a form like in the lateral geniculate complex. It seems, however, that this area of termination becomes distinctly demarcated by degeneration later in the development (stage 64) than the geniculate complex.

In postmetamorphic toadlets the lateral geniculate complex is thickly filled with degenerated terminals (Fig. 8). At the appropriate level, the corpus geniculatum thalamicum can clearly be separated from the nucleus of Bellonci as it has been described for adult *Rana* species [11, 15].

The first sign of ipsilateral degeneration in the lateral geniculate complex appears at stage 60, which is about two weeks before metamorphosis (Figs 2 and 3). It must be clearly stated that this early degeneration gives a rather ambiguous picture. It consists of very fine dots which are barely larger than the well-known silver dust in unsuccessfully impregnated specimens. Nevertheless, their appearance is confined to the course of the optic tract, and they are more numerous in the area of the lateral geniculate complex (Fig. 3) than elsewhere.

At stage 62 larger silver particles characteristic of terminal degeneration can be detected in the lateral geniculate complex. They are small in number and, as far as it can be established from the incompletely impregnating series, form a small group at the oro-dorsal aspect of this area which corresponds, probably, to the nucleus of Bellonci. A few days later, at stage 64, this group of silver granules grows in size (Fig. 4), and new ones appear at the medial and caudal aspects of the geniculate complex. A tendency of clustering of the granules seen in the contralateral projection, can be observed on the ipsilateral

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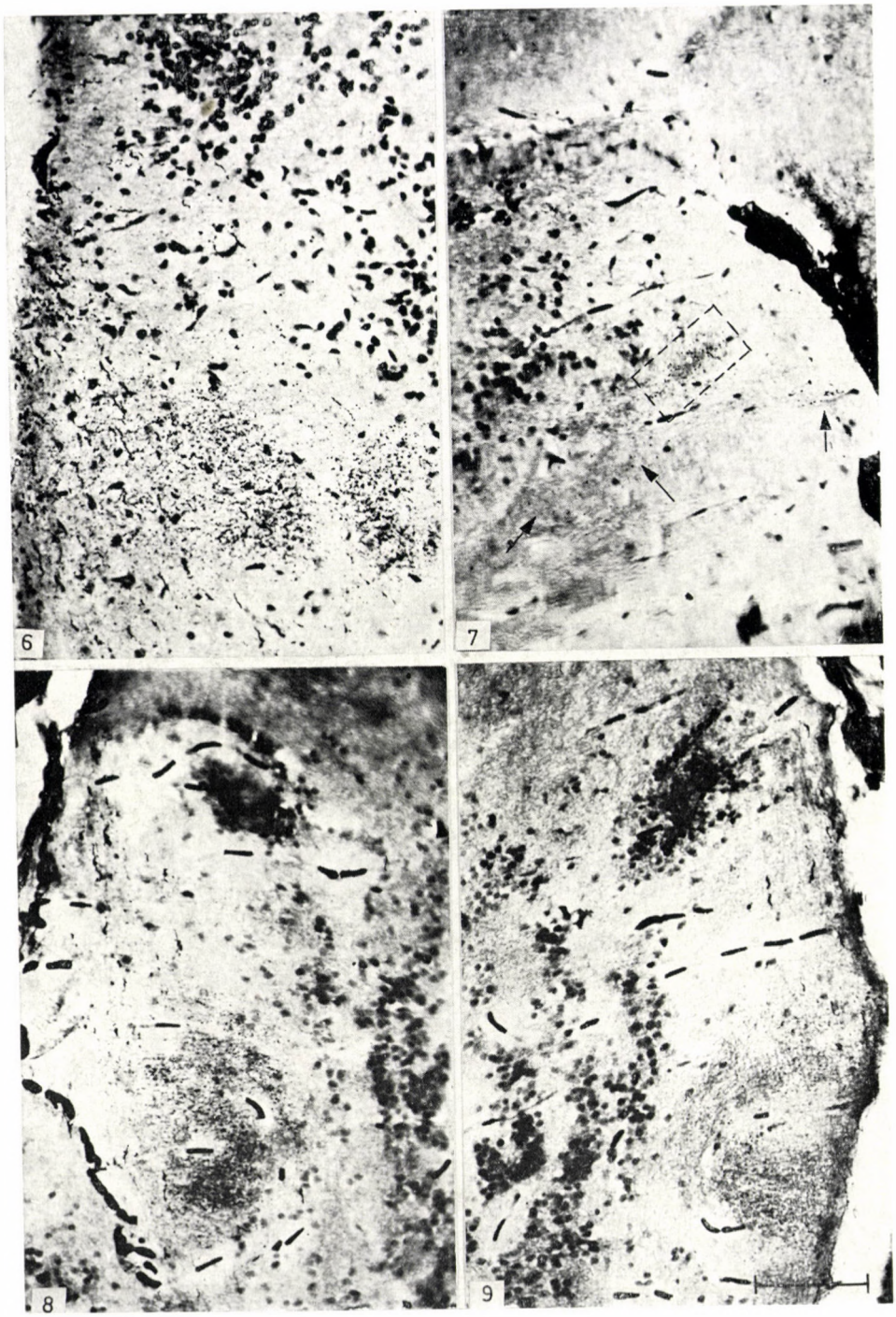
Fig. 6. Horizontal section of the diencephalon showing terminal degeneration in the contralateral lateral geniculate complex following eye removal at stage 66

Fig. 7. The same brain as in Fig. 6, ipsilateral to eye removal. Arrows point at patches of terminal degeneration in the lateral geniculate complex. The enframed region is enlarged in Fig. 5.

Figs 8—9. Horizontal sections of the diencephalon of a 4-week-old toadlet showing terminal degeneration in the contralateral and ipsilateral lateral geniculate complex, respectively. Scale in Fig. 9 indicates 100 μm, and applies to all photographs. Oral is upward in Figs 6 and 7, and downward in Figs 8 and 9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side as well. At the time of the metamorphic climax, at stage 66, a few granules are localized also at the outer surface of the lateral geniculate complex (Fig. 7). The amount of degeneration does not grow appreciably. The main differences between this and younger stages are that in the metamorphic toadlet the clustering tendency of silver granules is no more visible (Fig. 5) and, in contrast with younger stages, the ipsilateral projection can more consistently be found. However, in view of the difficulties in showing degeneration in the larval brain, it cannot be decided whether the less frequent occurrence of degeneration in younger larvae indicates the absence of the ipsilateral projection, or a technical failure in a larger proportion in larval brains than in metamorphic brains.

In postmetamorphic toadlets the entire area of the ipsilateral lateral geniculate complex is outlined by degenerating terminals (Fig. 9). Silver granules indicating degeneration are deposited almost as densely as in the contralateral side with the exception of a central core which is free of degeneration. This pattern of degeneration corresponds to that seen in the adult brain [10], and indicates that the ipsilateral retinodiencephalic projection is much more abundant in *Xenopus* toad than that found in *Rana* species by other authors [2, 11, 15].

An ipsilateral projection to the pretectal area cannot be demonstrated before stage 62 in the present material. It appears in the form of scattered degeneration in the caudal part of the thalamus, and seems to become gradually confined to the area proper during development. The incomplete impregnation of this part of the brain does not warrant a detailed description of the development of the ipsilateral pretectal projection.

Discussion

The first sign of degeneration in the diencephalon ipsilateral to eye removal appears at stage 60 with the Fink-Heimer technique. But in view of ambiguity of this early degeneration, it is safer to conclude that the first ipsilateral retino-diencephalic projection is detectable at the next stage investigated: at stage 62. CURRIE and COWAN [2] were able to show the first uncrossed retinodiencephalic connections at stage XX in *Rana pipiens*. According to a comparative table of anuran development [12], this stage of *Rana* tadpoles corresponds to a developmental stage between 61 and 62 of *Xenopus* tadpoles. The Fink-Heimer technique and autoradiography applied to two different anuran species give the same result in the study of the development of the uncrossed retinothalamic connections.

It is interesting to refer to the fact, discussed also by other authors [1, 2], that the development of the ipsilateral projection coincides in time with

the gradual reorientation of the eyes from a lateral into a frontosuperior position during metamorphosis. It is by this migration of the eyes that the postmetamorphic animal acquires the binocular visual field. As shown in other experiments, the uncrossed optic fibres originate in the temporal part of the retina [9, 14]. The temporal retina gives rise to a substantial contralateral projection as well, and this develops presumably together with other crossed retinal projections to the diencephalic visual centers. The problem of why the uncrossed part of the projection develops such a late time during development, and of whether the migration of the eyes has, or has not, any relationship to the appearance of the ipsilateral projection, is open to question. A few possible answers are discussed in CURRIE and COWAN's [2] paper.

There is another interesting time coincidence between the development of the ipsilateral retino-diencephalic projection and the appearance of visually evoked responses in the ipsilateral optic tectum [1, 6]. Using the same anuran species, the former authors were able to record the first ipsilateral tectal response from stage 62 onward. However, the ipsilateral responses were obtained only in a proportion of the animals investigated. This proportion tended to increase from stage 62 to stage 66, at which latter stage the ipsilateral responses were consistently present. This finding is similar to the present observation that the presence of degeneration in the ipsilateral diencephalic optic centre is more consistent when the eye was removed at stage 66 than at younger stages, though the absence of degeneration cannot be regarded as conclusive a result as the absence of responses in a physiological experiment.

In their work investigating the pathway for the ipsilateral tectal responses, KEATING and GAZE [7] suggest the involvement of an intertectal linkage through the postoptic commissural system. The ablation of one tectum gives rise to sporadic degeneration and only in the deeper layers of the tectum [8, 13], and this sparse amount of direct intertectal connections cannot be made responsible for the ipsilateral tectal responses. The assumed intertectal linkage must, therefore, be a polysynaptic pathway, and LÁZÁR's [8] observation on the abundance of tecto-thalamic projections on the diencephalic visual centres suggests that the relay sites may be in these same synaptic areas. These data and the several aspects of coincidence in the development of ipsilateral retino-diencephalic connections and in that of ipsilateral tectal responses, raise the possibility of a closer relationship between the two latter phenomena. Further experiments are necessary to investigate this possibility.

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MORPHOLOGY OF THE POST-OVULATORY FOLLICLE OF HOUSE SPARROW

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Abstract

The morphology of the post-ovulatory follicle (or corpus luteum) in the sparrow *(Passer domesticus)* ovary has been investigated with special reference to the origin of luteal cells which finally fill the follicular activity. The development and degeneration of luteal cell mass has been described in three phases. The luteal cell mass consists of hypertrophied granulosa luteal cells during the first phase and of both granulosa and thecal luteal cells during the second phase. During the second phase owing to their different staining reactions, both types of luteal cells can be differentiated. In the advanced stages of regression, i.e. during the third phase, the whole luteal cell mass consists of thecal luteal cells and connective tissue elements as the granulosa luteal cells had degenerated and disappeared by this stage.

Introduction

Two types of corpora lutea occur in the ovaries of vertebrates. One is formed by atresia of a mature or maturing oocyte and is known as the corpus luteum of atresia. The other type is derived from the empty follicle after ovulation; it is the corpus luteum of ovulation [4, 12]. Very divergent opinions have been expressed in regard to the origin and function of the vacuolated luteal cells in the corpus luteum of ovulation in birds. Their origin has been attributed either to the granulosa cells alone [17] or to the thecal cells [14] or to both [1, 8]. Keeping in view this controversy, the present study was undertaken to follow the origin of luteal cells as well as the other associated histological changes during development and degeneration of the post-ovulatory follicle in the house sparrow *(Passer domesticus)*.

Material and method

The ovary of the house sparrow was used for the present study. Female specimens were obtained by shooting them in the vicinity of Ludhiana during the breeding seasons (April to July and September to October). Recovery of the egg from the oviduct was the criterion for a recent ovulation. Some females were collected at night from the nests having eggs. After the usual dissection, the ovary was removed and fixed in alcoholic Bouin's fluid for 18—24 h and washed in 50% alcohol to remove excess of picric acid. Then the material

was dehydrated as usual, cleared in benzene and embedded in paraffin wax. Serial sections were cut at 5—7 μm and stained with haematoxylin and eosin. Ovarian material from some specimens was also fixed in formaldehyde-calcium, postchromed in dichromate-calcium and frozen sections were coloured with Sudan black B [2].

Results

Granulosa and thecal layers of the follicle after the discharge of egg undergo a series of morphological changes leading first to its development and subsequently to its degeneration. Only the major changes occurring in the thecal and granulosal layers will be described here.

Thecal layers

Immediately after ovulation, the thecal layers, consisting of theca interna and externa, are thickened to form well-developed layers of variable morphology and thickness around the granulosa cell mass (Fig. 1). This is the first phase of corpus luteum development. The theca externa is separated from the theca interna by a thin layer of deeply-stained connective tissue elements (Fig. 1). This layer disappears in later stages of corpus luteum development.

Theca externa. The theca externa is not of uniform thickness as at certain places it separates into three layers, i.e. theca compacta, theca spongiosa and outermost fibrous layer (Figs 1, 2) which show the same histological organization as that reported in the blackbird *Agelaius* [13]. With the involution of corpus luteum the distinction between these different theca externa layers disappears as a result of a progressive deterioration of their connective tissue elements, which starts from the theca compacta and gradually advances towards the outer layers (Figs 3, 4). This results in the formation of large intercellular spaces.

Theca interna. The theca interna forming the middle zone is more cellular and thinner than the theca externa (Figs 1, 2). Blood capillaries containing erythrocytes are present among the theca interna cells (Fig. 5). The transitional zone lying between the theca interna and granulosa luteal cells shows some eosinophilic material which extends in between the cellular elements of both layers (Figs 5, 6). Septa consisting of fibroblasts originate from the theca interna and then gradually invade the granulosa luteal cell mass.

The theca interna cells are arranged radially around the granulosa cell mass (Figs 5, 6). They stain more darkly than the granulosa cells and have

* Photomicrographs 1—6, 8, 9 and 11 have been prepared from paraffin sections of material fixed in alcoholic Bouin's fluid, stained with hameatoxylin and eosin. Photomicrographs 7, 10, 12 have been prepared from frozen sections coloured with Sudan black B. Figures 1—11 are prepared from the sparrow ovary and Fig. 12 is taken from the common mina ovary.

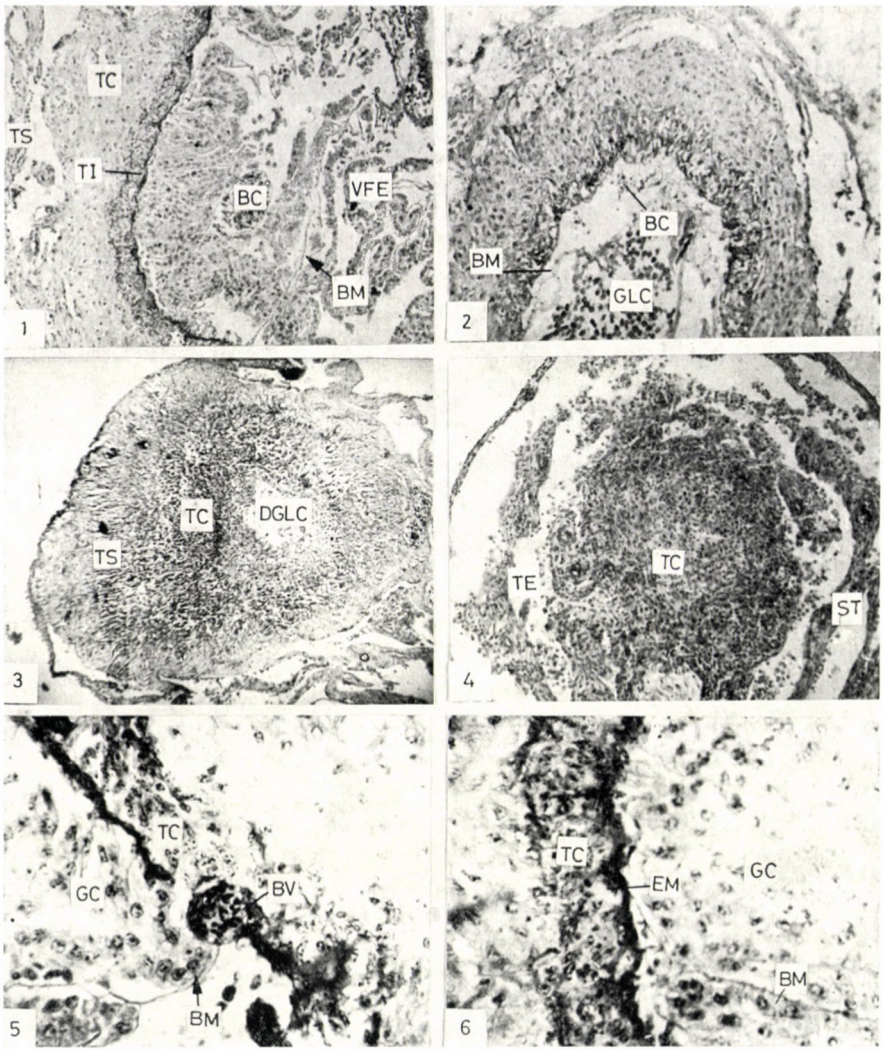

Fig. 1. Portion of newly ruptured follicle showing villus-like folds of follicular epithelium (VFE). Th blood cells (BC) are seen in between the folds formed by the basement membrane (BM) inside the theca interna (TI). Note the two layers of theca externa, i.e., theca compacta (TC) and theca spongiosa (TS). × 100

Fig. 2. Portion of the post-ovulatory follicle showing the hypertrophied granulosa luteal cells (GLC) which have detached from the basement membrane (BM). Note the blood cells (BC) among the granulosa cells. × 100

Fig. 3. The regressing post-ovulatory follicle showing the degeneration of granulosa luteal cells (DGLC) in the central region. The distinction between the thecal layers in disappearing. Note the thecal cells (TC) and thecal stromal cells (TS) among the granulosa cells. × 150

Fig. 4. Post-ovulatory follicle in its final stage of regression. The luteal cell mass at this stage is entirely formed by the thecal luteal cells (TC) and stromal tissue (ST). × 150

Fig. 5. Portion of newly-ruptured follicle showing the blood vessel (BV) in the theca interna cells (TC). Note the granulosa luteal cells (GC) which are closely attached to the basement membrane (BM). × 400

Fig. 6. Portion of post-ovulatory follicle showing accumulated eosinophilic material (EM) in the transitional zone of theca interna (TC) and granulosa cells (GC). Basement membrane (BM) is also seen. × 400

spherical to oval nuclei; their nucleoplasm contains one nucleolus and some distinct chromatin granules lying adjacent to the nuclear envelope as well as in the centre of the nucleus (Figs 5, 6). In addition to these histological differences, the theca interna cells show less lipids in Sudan black B preparations (Fig. 7.) In advanced stages of development of the corpus luteum, when the eosinophilic material of the transitional zone disappears, the thecal cells or theca luteal cells start invading the granulosa luteal cell mass (Fig. 8). This invasion forms the most characteristic feature of the sparrow corpus luteum. Ultimately, they form the small masses which lie among the granulosa luteal cells (Figs 9, 10). This can be designated as the second phase of corpus luteum formation during which both types of luteal cells fill the follicular cavity more or less completely.

Granulosa Luteal Cell Mass

The granulosa cell mass lies inside the thecal layers during the first phase. The cells form villus-like projections into the lumen of the newly-ruptured follicle (Fig. 1). The granulosa luteal cells form masses (Fig. 2). No mitosis is seen in the granulosa cells at any stage of the development of the corpus luteum.

After ovulation, the granulosa cells appear to develop more cytoplasm (Fig. 11). Vacuoles apparently formed by the removal of lipid droplets during dehydration and paraffin embedding are present in the cytoplasm. Theca interna cells continue to invade the granulosa luteal cell mass during various developmental stages till both types of cells are intermixed (Figs 9, 10).

The luteal cell mass is formed by the intermixing of cells of granulosal and thecal origin during the second phase (Figs. 9, 10). Its close examination shows that the granulosa and theca luteal cells form distinct groups or patches mixed with each other. However, they can be distinguished in frozen sections coloured with Sudan black B, in which the granulosa luteal cells show more sudanophilic lipid than the theca luteal cells (Figs 7, 10). The nuclei of thecal luteal cells are oval and contain large number of chromatin granules whereas the nuclei of granulosa luteal cells are pycnotic (Fig. 9). The cytoplasm of thecal luteal cells is also more eosinophilic than that of granulosa luteal cells. Similar structure of the corpus luteum has been observed in the common myna *Acridotheres tristis* (Fig. 12). Blood cells, which do not lie within vessels, are seen throughout the corpus luteum (Fig. 9).

The granulosa luteal cells start regressing earlier than the thecal luteal cells; their nuclei become shrunken and irregular in shape (Figs 3, 9). Their cytoplasm becomes more vacuolated. The outer boundaries of granulosa luteal cells simultaneously become indistinct (Fig. 9). During the third phase, the granulosa luteal cells disappear from view, leaving behind a nodule of thecal luteal cells (Fig. 4). Finally the thecal luteal cells also disappear from view.

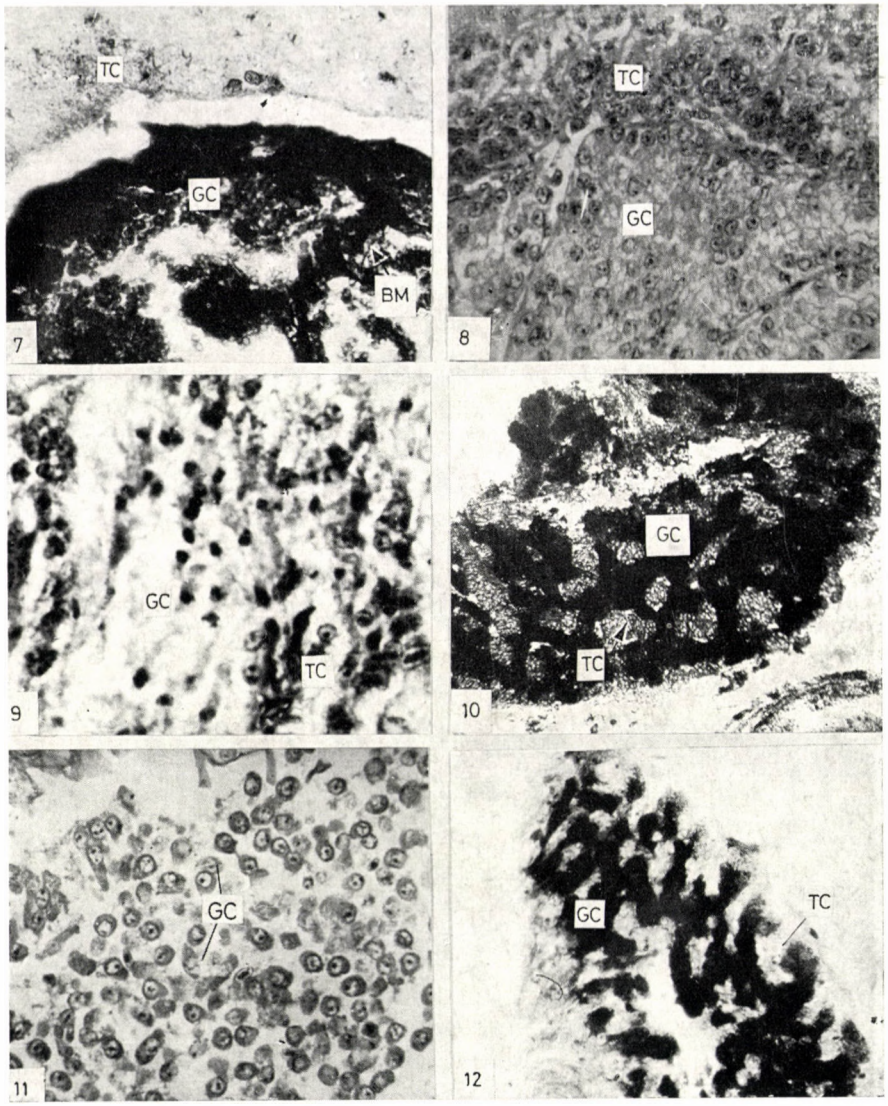

Fig. 7. Portion of newly-ruptured follicle showing the presence of lipids in theca interna cells (TC). The granulosa luteal cells (GC) are highly lipophilic. The theca interna cells (TC) contain very little sudanophilic lipids. Note the basement membrane (BM). × 100

Fig. 8. Portion of post-ovulatory follicle, showing the disappearance of eosinophilic material and subsequently leading to the movement of thecal cells towards the granulosa luteal cells (GC). Theca interna cells (TC) have oval nuclei and strain darkly, whereas the granulosa luteal cells (GC) have spherical nuclei and vacuolated cytoplasm. × 400

Fig. 9. Portion of the luteal cell mass in its second phase showing patches of theca luteal cells (TC) and granulosa luteal cells (GC). Note the pycnotic nuclei of granulosa luteal cells and scattered blood cells (BC). × 400

Fig. 10. Fully-developed corpus luteum showing patches of highly sudanophilic granulosa luteal cells (GC) lying among less sudanophilic patches of theca luteal cells (TC). × 100

Fig. 11. Highpower view of the post-ovulatory follicle shown in Fig. 2. The hypertrophied granulosa luteal cells (GC) show eosinophilic cytoplasm. × 750

Fig. 12. Portion of the post-ovulatory follicle from the ovary of myna showing intermixing of granulosa luteal cells (GC) and theca interna cells (TC). Thecal cells are still showing continuity with the outer layer. × 100

Discussion

The present studies on the post-ovulatory follicle of house sparrow have clearly shown that the luteal mass is constituted by granulosa cells during the first phase, by both granulosa and thecal luteal cells during the second phase, and only by the thecal luteal cells and connective tissue elements in the final regressional stages. The double origin of luteal cells is supported by the preliminary observations on the evolution of corpus luteum in the common myna (*Acridotheres tristis*), in which the theca luteal and granulosa luteal cells also form distinct cell types having the same morphological and cytological features as those described for the house sparrow.

Luteal cells of thecal origin have not been reported previously in the corpus luteum of the pigeon, blackbirds and other avian species [5, 6, 14]. Probably the cited workers failed to distinguish the thecal luteal cells from the granulosa luteal cells. However, in the post-ovulatory follicle of the fowl, the luteal cells multiply in the theca interna and subsequently migrate into the lumen to obliterate the follicular cavity [14]. They attributed the origin of the entire luteal cell mass to the thecal layers, thus neglecting the role of granulosa cells in this regard. Based on electron-microscopic studies some authors [7, 17] attributed the origin of luteal cells to the granulosa cells, paying no attention to the contribution of thecal layers. Further studies on the corpus luteum of fowl have shown that the luteal cell mass is derived from both epithelial and thecal cells [1, 8]. The later observations are in good agreement with the results of present study. From this discussion, it is quite clear that very divergent views have been expressed as regards to the origin of luteal cell mass even in the same bird species (e.g. fowl). These differences of opinions may be due to the fact that the post-ovulatory follicles investigated by previous workers may have been at different stages of their evolution and involution.

A large number of blood cells migrate into the lumen of the newly-ruptured follicle in the sparrow as also reported by other workers [8, 13]. In the origin of luteal cells, the corpus luteum of the house sparrow and common myna closely resembles that of some mammalian species [3, 9] as both the granulosa and thecal luteal cells have been described. It is of interest that the thecal layers do not make any luteal cell contribution in the post-ovulatory follicles of Amphibia and Reptilia [10, 11, 15, 16]. However, the post-ovulatory follicles in some species of elasmobranches and teleosts have been found to show double origin of luteal cells [12] like those described for the sparrow and myna. The luteal cells, whether derived entirely from the granulosa cells or both from the granulosa cells and theca cells, in the ovaries of non-mammalian vertebrates develop the cytological (including ultrastructural) and histochemical features of mammalian luteal cells [12]; the presence of steroidogenesis has also been demonstrated in the corpora lutea of some

non-mammalian vertabrate species in *in vitro* biochemical experiments. Further work should be continued with biochemical techniques to determine the nature, amount and function of steroid hormones of corpus luteum in non-mammalian vertebrates.

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ROLE OF ULTIMOBRANCHIAL BODY AND CORPUSCLE OF STANNIUS IN REGULATION OF THE PLASMA CALCIUM AND PHOSPHORUS LEVELS IN THE TELEOST *HETEROPNEUSTES FOSSILIS*

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Abstract

Effects of NaF, $CaCl_2$, NaCl and mammalian calcitonin on the histology of ultimobranchial (UTB) and corpuscle of Stannius (CS) and plasma levels of calcium and phosphorus in the teleost *Heteropneustes fossilis* are recorded. Administration of NaF, NaCl, and mammalian calcitonin resulted in varying degree of hypocalcaemia and hyperphosphataemia, whereas hypercalcaemia and hypophosphataemia developed during $CaCl_2$ treatment. These treatments also produced various histological changes in UTB and CS. It is suggested that CS and UTB are involved in metabolisms of Ca and P, and in osmoregulation. Moreover, it is an important hypocalcaemic mechanism in this fish in combating hypercalcaemia.

Introduction

Although fishes comprise the largest and most diverse group, until recently very little had been known about the endocrine control of their calcium and phosphate metabolism. Earlier studies regarding the endocrine control of these metabolisms in fishes suggest the presence of at least two functional systems, viz. the hypocalcaemic corpuscle of Stannius (CS), the ultimobranchial (UTB) and hypercalcaemic pituitary.

Parathyroid gland has generally been considered absent in fish [13, 20]. Rasquin and Rosenbloom [21] suggested that the UTB might have functions analogous to that of parathyroid gland of higher vertebrates. These findings were first to indicate that UTB might be related to calcium metabolism, but the gland had remained neglected until recently when calcitonin was extracted from fish UTB [3, 19]. The discovery of calcitonin as hypocalcaemia hormone in mammals [4] support expectations that this hormone might be important in fish calcium metabolism, too. Earlier reports concerning administration of this hormone in fishes indicated its failure to elicit hypercalcaemia in the killifish, catfish and Coho salmon [19]. Subsequently, partially purified mammalian calcitonin was shown to be effective in catfish [15] and eels [2]. Recently Pang [19] has suggested that calcitonin might be related

to osmoregulation, since eel calcitonin has been shown to decrease serum osmolarity [16].

Since the first identification by STANNIUS [22], CS have been mistaken for adrenal cortical tissue of higher vertebrates. It was demonstrated later that these bodies had no relation to the adrenal system in fish [9, 11]. FONTAINE [10] was the first to demonstrate that surgical removal of these tissues from European eel *Anguilla anguilla* produces hypercalcaemia. PANG [19] reviewed similar findings by various investigators working with eels and goldfish and reported the hypercalcaemic effects of stanniectomy in the killifish *Fundulus heteroclitus*. Various workers also indicated that this gland is involved in osmoregulation [21, 17, 6]. Thus, the role of UTB and CS in Ca and P metabolism in fishes is poorly understood even today and the literature is contradictory and confusing. The present report is an attempt to elucidate the role of UTB and CS in the catfish *Heteropneustes fossilis*.

Material and method

Several male specimens of *H. fossilis* were obtained from the local market during the months August to December, 1974 for various experiments. They were acclimatized in fresh water for 15 days at room temperature (25° to 27 °C). The specimens 45—60 g in weight and 8—10 inches in length were selected.

The fishes were divided in to various groups of twenty each for experiments. The groups were kept in separate aquaria each containing 20 litre of fresh water. The water was changed once a week.

The groups 1, 2 and 3 were kept in water containing sodium fluoride (60 mg/l), calcium chloride (150 mg/l) and sodium chloride (0.7%), respectively. Group 4 kept in fresh water, served as a control. Fishes from each group were killed after thirty days of treatment.

A daily injection of mammalian calcitonin (CIBA, 5 MRC units/kg/day) was given to fishes of the fifth group and were sacrificed after 7 days along with the control group which had received distilled water.

At the end of experiments fishes were anesthesized in 1% paraldehyde and blood was obtained for biochemical estimations by cutting the caudal region. Total serum calcium was estimated by flame photometer. For phosphorus the method of FISKE and SUBBAROW [12] was employed.

For histological observations UTB and CS were carefully removed and fixed in Bouin's fluid for 24 h. The paraffin sections were cut at 5 μm and stained with haematoxilyn-eosin.

Results

Biochemical observations. Fishes treated with NaF, NaCl and calcitonin showed hypocalcaemia and hyperphosphataemia. NaF treatment resulted in significant depletion of calcium and increase in blood phosphate level, whereas NaCl- and calcitonin-treated fishes showed slight depletion in the levels of Ca and P but these effects were not significant statistically. On the other hand,

Table 1

Effect of various treatments on the histology of UTB and CS and on the plasma calcium and phosphorus levels in H. fossilis

Treatment	Duration days	UTB		CS		Plasma (mg/100ml)	
		cell diameter μm	nuclear diameter μm	cell diameter μm	nuclear diameter μm	Ca	P
Control	30	7.12±0.52	2.96±0.12	6.85±0.40	5.52±0.98	8.20±0.15	5.0±0.25
NaF	30	5.40±0.52 P < 0.001	2.52±0.20 NS	6.36±1.93 NS	5.58±0.50 NS	5.58±0.12 P < 0.001	7.3±0.16 P < 0.001
$CaCl_2$	30	11.11±0.25 P<0.001	3.92±0.17 P<0.001	8.72±0.60 NS	6.96±1.60 NS	9.20±0.25 P<0.001	4.1±0.19 P<0.001
NaCl	30	8.42±0.23 NS	3.60±0.15 NS	9.40±0.72 P<0.001	6.72±0.40 NS	7.10±0.83 NS	6.80±0.13 NS
Calcitonin	7	3.58±0.40 P<0.001	1.85±0.17 NS	6.40±0.72 NS	4.72±0.40 NS	7.20±0.53 NS	6.30±0.10 NS

NS = Not significant
Values are mean ± SE (standard error).
Twenty animals were used in each experiment.

treatment with $CaCl_2$ produced significant hypercalcaemia and hypophosphataemia (Table 1).

Histological observations. Cellular and nuclear changes during various treatments in UTB and CS are summarized in Table 1.

UTB control. In *H. fossilis* the encapsulated UTB is oval and located in the transverse septum between the abdominal cavity and the sinus venosus, just ventral to the oesophagus. It is composed of polygonal cells (7.12±0.52 μm) with a centrally placed nucleus (Fig. 1).

Experimental. $CaCl_2$- and NaCl-treated fishes showed hypertrophy of cells and nuclei (Fig. 2), whereas during NaF treatment the nucleus and cell diameters were decreased. Mammalian calcitonin treatment in this fish resulted in reduction in cellular and nuclear diameter of UTB, indicating reduced activity of the gland (Fig. 3).

CS control. The corpuscles are flattened oval bodies embedded in mesonephron and enclosed in a fibrous capsule. They are composed of columnar cells possessing secretory granules in the cytoplasm. The average cell and nuclear diameters were 6.85±0.4 μm and 5.52±0.92 μm, respectively (Fig. 4).

Experimental. $CaCl_2$ and NaCl treatments produced hypertrophy of both cell and nucleus (Figs 5—6). On the other hand, there was slight reduction in the cellular and nuclear diameters during mammalian calcitonin treatment, but the values were not significant statistically.

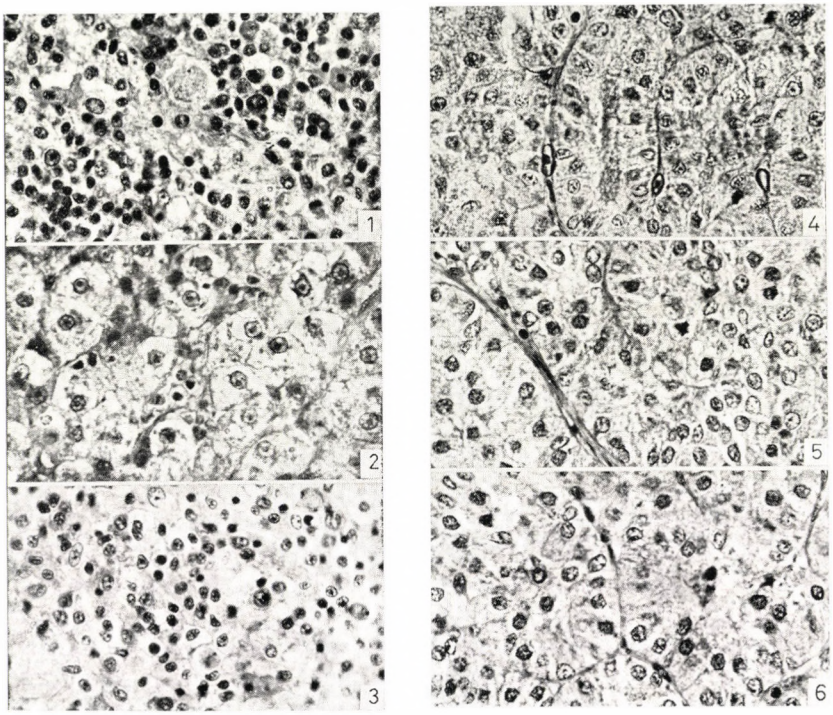

Fig. 1. Ultimobranchial gland of control fish; × 750

Fig. 2. Hypertrophied UTB from $CaCl_2$-treated fish; × 750

Fig. 3. UTB showing reduction in cell and nuclear diameter after calcitonin treatment; × 750

Fig. 4. CS from control fish; × 750

Fig. 5. CS showing nuclear and cellular hypertrophy following NaCl treatment; × 750

Fig. 6. Hypertrophid CS of $CaCl_2$-treated fish; × 750

Discussion

In the recent years considerable advancements were made in our understanding of hormonal control of calcium regulation in fish. Fish calcitonin was first demonstrated in UTB of dogfish, *Squalus suckleyi* [3]. Hypocalcaemic activities were subsequently observed in ultimobranchial extracts of the sharks *Prionace glauca* and *Heterodontus francisci* [23] and the teleost, *Oncorhynchus nerka* and *Gadus macrocephalus* [3]. Fish calcitonin showed longer lasting hypocalcaemic effects in killifish [3]. DAKE and co-workers [8] reported an increased plasma calcitonin in fishes injected with $CaCl_2$ solution. A similar increase in plasma calcitonin level of salmon was reported in fishes kept in $CaCl_2$-containing media [8]. The response of UTB in *H. fossilis* to hypercalcaemia ($CaCl_2$) and hypocalcaemia (NaF) was reflected as reduction in size of

cells and hypertrophy of cells, respectively, suggesting relationship of this gland with calcium metabolism.

Fresh-water-adopted *Anguilla anguilla* [7] and *Ictalurus melas* [15] exhibited hypocalcaemia and hyperphosphataemia when treated with mammalian calcitonin. On the other hand, the same treatments were ineffective in other piscine species. In the present study mammalian calcitonin failed to produce significant changes in Ca and P levels. However, there was reduction in the cellular and nuclear diameter of UTB, which may be due to a higher concentration of circulating calcitonin. The failure of response to mammalian calcitonin in some fishes may be due to species specificity of this hormone.

In the recent studies on hypocalcaemic effects of calcitonin in *F. heteroclitus* [19], UTB showed hypertrophy under both hyper- and hypocalcaemia. It was further suggested that the cellular activity of the UTB may be related to physiological processes other than calcium metabolism, viz., osmoregulation. In *H. fossilis* NaCl treatment for 30 days produced hypertrophy of the gland accompanied by hypocalcaemia and hyperphosphataemia. These findings indicate the possible role of UTB in the osmoregulation of this fish.

The effects of stanniectomy on serum calcium and other electrolyte levels in the eel *Anguilla anguilla* were first reported by FONTAINE [10]. Since then the hypercalcaemic effect of stanniectomy has been confirmed in eels [5, 6, 1], goldfish [17] and killifish [19]. One of the most consistent findings of these studies was the hypocalcaemic function of this tissue. There was almost 50% decrease in serum inorganic P in stanniectomized killifish [7, 19]. Decrease in phosphorus level was observed in the original studies of FONTAINE [10]. In *H. fossilis* altered cellular activity is seen during NaF and $CaCl_2$ treatments suggesting the possible involvement of this gland in regulation of serum Ca and P levels.

Histological features of CS under different environmental conditions have been studied by various workers [14, 17, 18, 21] in order to determine its physiological functions. These investigations indicated that this gland might be involved in osmoregulation. Hyperactivity of this gland during NaCl treatment in the present study gives further support to these observations. Further, it is suggested that in *H. fossilis* CS and UTB are involved in Ca and P metabolism and osmoregulation. It is an important hypocalcaemic mechanism in combating hypercalcaemia.

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DRY WEIGHT AND 5HT CONCENTRATION IN THE CEREBRAL GANGLIA OF ANODONTA (MOLLUSCA) DURING ACTIVITY

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Abstract

As shown by interferometry, the amount of protein in neuron bodies of *Anodonta cygnea* L. cerebral ganglia changed in direct relation to the duration of the active state of the mollusc. The amount of serotonin in cerebral ganglia changes independently of the amount of protein in nerve cells, although it varies consistently with the activity and rest of the animal.

Introduction

Stimulation of vertebrate neurons brings about changes in protein and RNA content in these cells [1, 3]. It is also known that alternation of activity and rest periods in *Anodonta* is controlled by cerebral and visceral ganglia. Periodic activity of animals coincides with the changes in the activity of the CNS [5]. This periodicity is manifested in changing of basophilia of ganglionic nerve cells [7]. The purpose of our study was to determine the dry weight as the total index of the number of macromolecules, mainly of proteins, in the neurons of the cerebral ganglion and to measure the amount of serotonin in the total cerebral ganglion of *Anodonta* at various times of the active and rest states of the animals. The activity of molluscs was objectively estimated by the position of their valves. The active state corresponds to the open position of valves, the rest state to their closed position [2].

Material and method

Adult molluscs with a small scatter in weight (200 $\pm$ 10 g) were chosen. The animals were kept in running-water pond aquariums while the position of the valves was constantly recorded with an actograph constructed at the Biological Research Institute, Tihany. Both cerebral ganglia were isolated at different periods of activity and rest. One of them was fixed, embedded in paraffin and cut in a microtome for determining the dry weight of neurons by interferometry. In another ganglion the amount of serotonin was determined by spectrophotofluorimetry. The latter method is based on butanol-heptane extraction and subsequent interaction of serotonin with ninhydrin [6]. The reaction product was estimated quantitatively.

To calculate the dry mass of each neuron $\left(m = \frac{\Delta\varrho \cdot L}{h}\right)$ the thickness of paraffin section (h) was determined. Then the concentration of the dry mass ($\Delta\varrho$) and the area of the

same cell ($L = d_1 \cdot d_2$) were measured in a hundred or more neurons of the cortical zone of each ganglion, where d_1 and d_2 were the diameters of the neuron body projection. To obtain commensurable values, we calculated the dry weight of neurons taking the section thickness as 1 μm [1].

Results and discussion

Fig. 1 presents histograms of the dry weight and dimensions of cerebral ganglionic nerve cells at different times after starting the active or the rest period. Both values, especially the dry weight, vary significantly. Distribu-

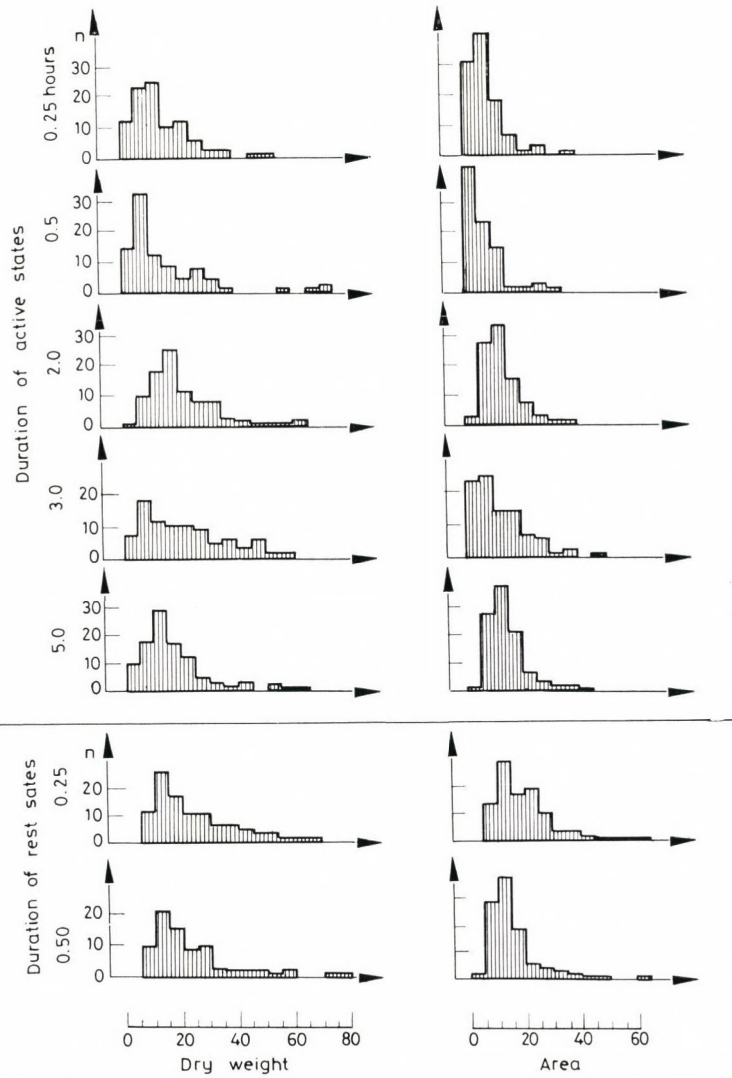

Fig. 1. Histograms of dry weight (left column) and area (right column) of neurons of *Anodonta* cerebral ganglia at different duration of activity and rest states. Abscissa: dry weight and area of neurons in arbitrary units; ordinate: the number of cells (n)

tions of values are asymmetrical; as a rule cells of large dimensions and weight are more pronounced. Owing to an asymmetrical character of the histograms, the calculation of mean values and other parameters of normal distribution seems inexpedient. In further calculations, the neurons studied were divided into three conditional groups: light (weight: 5 – 20 units), moderate (21—40 units) and heavy (41—75 units). The reproducible results of interferometry

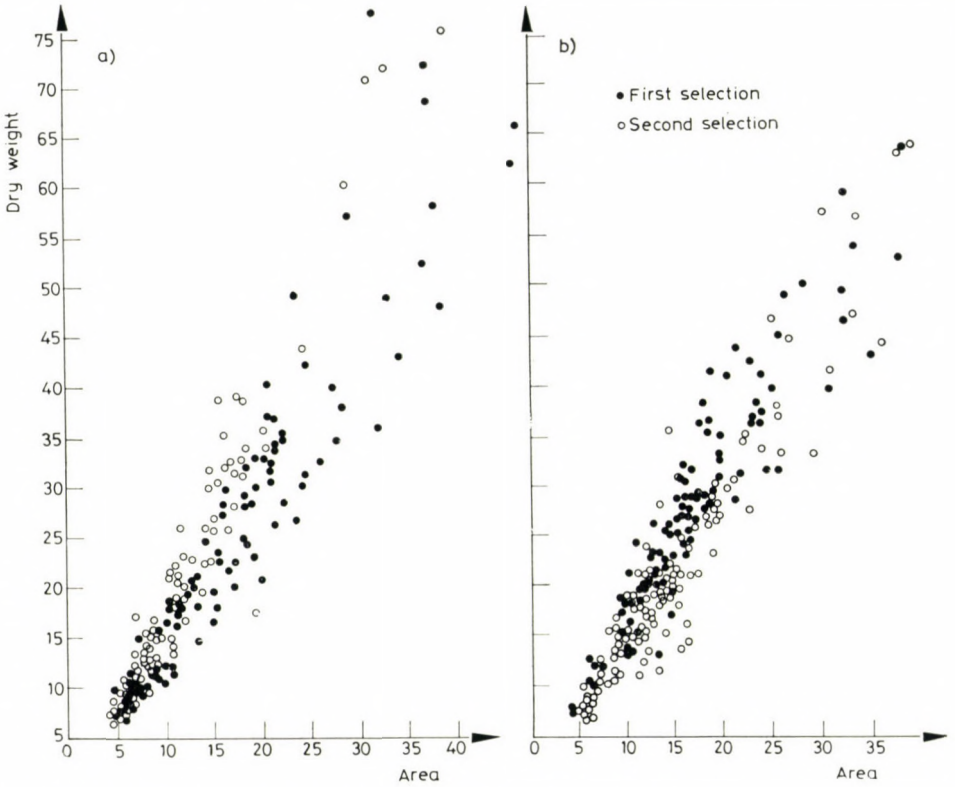

Fig. 2. The area and dry weight of *Anodonta* cerebral ganglia neurons in the active state for 30 min (a) and 5 h (b). Abscissa: cell area; ordinate: dry weight of the same cells in arbitrary units (explanation in the text)

(variation coefficient while measuring a single specimen) on our object equalled ±8%. Thus, the isolated cell groups differed with high reliability. The same conclusion was made after checking the means of cell selection: two selections of neurons from different sections of a ganglion gave similar distributions (Fig. 2).

The results of histogram analysis shown in Fig. 1 are presented in Table 1 and interpreted in the following:

(1) The weight of neurons depends neither on that of the mollusc nor of the ganglion.

(2) The ratio between light and heavy neurons in the cerebral ganglion varies in molluscs with the duration of activity. The number of light cells decreases, that of moderate and heavy cells increases correspondingly during the first 2—3-h activity. By 5 h the distribution of cells by weight tended to the initial one observed 15—30 min after the valves had opened. These changes seem not to be concerned with individual variability of ganglia of different mollusc specimens: the ganglia of 3-h activity of two molluscs showed similar cell distribution (Table 1).

Table 1

Comparison of neurons of the cerebral ganglion of Anodonta cygnea L. by the weight of perikaryon

Duration of active state	Light neurons		Moderate neurons		Heavy neurons	
	number	per cent	number	per cent	number	per cent
15 min	104	89	11	9	2	2
30 min	82	81	15	15	4	4
30 min (second selection)	82	77	22	20	3	3
2 h	75	73	23	22	5	5
3 h	71	65	27	25	11	10
3 h (second aminal)	65	62	25	24	15	14
5 h	91	84	13	12	4	4
Duration of the rest state						
15 min	70	65	28	26	9	9
30 min	68	73	18	19	7	8

(3) A great number of heavy neurons is characteristic of the two studied periods of the inactive state. As for the ratio between cells of different weight, the cerebral ganglia 15 and 30 min after the closure of valves did not differ from those having been continuously active for 2—3 h. Consequently, if the cell ratio depends on the state of animals, it changes not only during active, but also during the rest period.

(4) Neuron dimensions in general are in conformity with their weight; the heavier the cell, the larger it is. But cell dimensions may change unproportionally to the change of the cell weight. Hence the ganglia of 30-min and 5-h activity did not practically differ as for the ratio of the cell weights, though the cell dimensions were smaller in the second case (Fig. 2).

Serotonin content in the cerebral ganglia (Table 2) is different during activity and rest, but it varies independently of the changes in the cell weight. Both a low (2-h activity) and high (rest of 15 min) levels of serotonin correspond to the same weight distribution, while similar serotonin concentrations (30-min and 3-h activity) were measured in ganglia with clearly different pericarya parameters.

Table 2

Serotonin content (5HT μg/g wet weight) in the cerebral ganglia of Anodonta at different duration of activity and rest (average values of three measurements (±S.E.M.)

	15 min	30 min	2 h	3 h	5 h
Active state	46.7±15.8	41.7±15.88	36.1±7.57	43.4±6.69	47.7±4.53
Rest state	51.5±16.8	50.1±9.66	35.6±4.51	43.9±12.23	52.7±20.41

As possible cause of the changes in the dry weight of *Anodonta* neurons serotonin accumulation in the ganglion, suggested earlier by SALÁNKI and HIRIPI [4], may be ruled out. Variability of the dry weight of neurons in different ganglia seems to be also little connected with protein transport from perikaryons into cell axons. The shift of the optical path difference in the neuropile, proportional to protein concentration in axon, did not differ at 30-min, 2-h, 3-h and 5-h activity of the experimental animals.

Our observations enable a suggestion concerning the periodical changes of the intensity of protein synthesis in neurons of the cerebral ganglion. At the constant velocity of protein transport into the axon, changes in the intensity of the synthesis might govern, the variations in the dry weight of perikaryons. Since, the nature of such changes is unclear yet, the hypothesis should be checked by isotope analysis.

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ROSETTE FORMATION OF CONCANAVALIN A--TREATED ERYTHROCYTES AROUND POLYMORPHONUCLEAR LEUCOCYTES AND LYMPHOCYTES

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Abstract

Concanavalin A (Con A) induces rosette formation of erythrocytes around polymorphonuclear leucocytes and lymphocytes in cell suspensions of autologous human blood cells. The effect which is most characteristic in a concentration between 25 and 50 μg/ml is due to Con A bound on the erythrocyte membrane. A similar effect, although less pronounced, was observed with phytohaemagglutinin at concentrations of 10 and 25 μg/ml. The treated erythrocytes showed a higher affinity to polymorphonuclears when compared with lymphocytes. At the contact area, the membrane of the erythrocyte became highly folded while its free surface was smooth and spherical. The effect of the local concentration and immobilization of the lectin on the erythrocyte membrane and the similarity of the contact pattern to that of erythrophagocytosis are discussed.

Introduction

In the last few years interest has been centred on the action of concanavalin A (Con A) on the plasma membrane of various cell types. At neutral pH, Con A is a tetramer with four subunits each of which exhibits one sugar-binding site [5] by which it is specifically bound to α-D-glycosyl residues [6, 7]. After having bound the Con A, the sugar-containing molecules of the plasma membrane move in lateral direction to form cap-like aggregates on one pole of lymphocytes and polymorphonuclear leucocytes [2, 11]. The binding of Con A on the plasma membrane can initiate important biological events, viz. blastic transformation and mitotic division of lymphocytes [1, 4, 8, 13, 17, 21 *inter alia*]. Con A is able to agglutinate certain transformed and malignant cells while it is without appreciable effect on the parent cell lines [9, 10, 19].

Without protease treatment, human erythrocytes are loosely agglutinated even at high Con A concentrations. We observed, however, that Con A-treated erythrocytes are avidly bound to autologous leucocytes and form characteristic rosettes around them. This phenomenon is accompanied by strong deformation of the erythrocytes participating in the rosettes.

Material and method

Blood was taken from healthy human individuals with citrate or heparin in quantities ranging from 20 to 150 ml.

Separation of erythrocytes and leucocytes

The blood was layered on top of a 3% gelatin solution dissolved in Parker's medium in a glass cylinder at 37 °C. The cylinder was tilted to 45, 60 and 90 degrees for 6, 8 and 10 min, respectively. The upper part of the solution containing mainly leucocytes was diluted with Parker's medium and centrifuged (1200 rev/min, 20 min). After two washes in Parker's medium the cell suspension was laid on the top of a Ficoll solution consisting of 1.91 g Ficoll (Sigma), 4 ml 75% Uromiro (Bracco, Milano) and 26.08 ml Parker's medium and centrifuged at 1300 rev/min for 10 min. The cell suspension above the sedimented erythrocytes was washed two times with Parker's medium. The last resuspension was made to reach a cell concentration of 4×10^7 per ml. Erythrocytes were generally present in the same number as leucocytes. The cell suspension was kept until mixed with erythrocytes at 37 °C in an atmosphere containing 5% CO_2 (usually one hour).

For a better preservation of the cells for electron microscopy, the isolation procedure was shortened in several cases by omitting the sedimentation in gelatin. In such cases the erythrocyte: leucocyte ratio was generally 2 : 1.

The erythrocyte suspension was gained from the same blood sample (autologous) by taking out samples from the sedimented erythrocyte mass and washing it 5 times with PBS or Parker's medium (pH 7.2, 2000 rev/min, 5 min). After the last centrifugation the erythrocytes were resuspended in PBS to reach an erythrocyte concentration of 5×10^8 per ml.

Treatment of erythrocytes and leucocytes with Con A

In the initial part of this study we wanted to clarify which of the two cell types must be exposed to Con A to obtain characteristic rosette formation. For this purpose half quantity of the erythrocyte or leucocyte suspension was treated with 100 μg/ml Con A (purified with Sephadex chromatography, frozen dried, Pharmacia, Uppsala) at 24 °C for 20 min and washed two times in Parker's medium. The other half of the cell suspensions was left intact and the four cell suspensions were paired with one another in the possible four combinations. After 20 min at 24 °C, samples were taken for light microscopy while the remaining part of the mixtures was fixed and processed for electron microscopy.

The optimal lectin concentration

The erythrocyte suspension was distributed in centrifuge tubes in quantities of 1ml/tube, centrifuged, and the cells were resuspended with 1.8 ml of Con A or phytohaemagglutinin P (PHA, Difco) solution. The tubes were left to stand at 24 °C for 20 min. The concentrations used for the lectins were: 1, 2, 5, 7, 10, 25, 50 and 100 μg/ml. The cells were washed two times with PBS or Parker's medium and then centrifuged.

The erythrocytes previously exposed to the lectin were resuspended with 0.75 ml of the leucocyte suspension in each tube and were left at 24 °C for 30 min meanwhile the tubes were gently shaken at intervals. The cell mixtures were fixed with large quantities of the glutaraldehyde fixative; little samples were taken out for light microscopic examination while the remaining part was further processed for electron microscopy.

Con A-treated and glutaraldehyde-fixed erythrocytes

In one case the effect of glutaraldehyde fixation was observed on the rosette formation of Con A-treated erythrocytes. The erythrocyte suspension exposed to 50 μg/ml Con A was fixed with 1% glutaraldehyde in Millonig phosphate buffer at 24 °C for 15 min, washed two times with PBS and Parker's medium and subsequently mixed with the living leucocytes.

Light and electron microscopy

One drop of the cell suspensions, either fixed or unfixed, was examined between slide and cover glass without staining. Other part of the light-microscopic material were semithin sections stained with toluidine blue and Azure II. All light micrographs were taken with an Opton (Zeiss, Oberkochen) 40 × planapochromate immersion objective.

For electron microscopy, the cell suspensions were fixed with 8 ml of 1.5% glutaraldehyde in Millonig phosphate buffer at 24 °C for 2 h, washed with 0.15 M cacodylate buffer and postfixed with 1% osmium tetroxide in the same buffer at 4 °C. In several cases 1% tannic acid was added to the glutaraldehyde fixative to increase the contrast of the proteinous structures. The osmium-fixed and buffer-washed cells were mixed with 2.5% agar-agar or 10% gelatin to form blocks which were subsequently dehydrated and embedded in araldite (Durcupan ACM, Fluka). Thin sections made with a Reichert OMU—2 ultramicrotome were stained with a 2% solution of uranyl acetate in water and lead citrate and examined in a JEM 6C or JEM 100B electron microscope.

Results

Mixtures of erythrocytes and leucocytes treated with Con A

When mixtures of erythrocytes and leucocytes were treated with Con A concentrations of 50 or 100 μg/ml, an intense aggregation of erythrocytes around leucocytes was observed. The erythrocytes surrounded the leucocytes in form of rosettes (Figs 2, 3) or formed occasionally large cellular aggregates with the leucocytes. Among the rosettes and aggregates, the erythrocytes were generally free and nearly normal in shape and only a mild tendency for aggregation between the erythrocytes was observed.

To determine which of the cell types had to be treated with Con A to form rosettes, we combined cell suspensions exposed or not exposed to Con A (Table 1). Rosette formation was present only if erythrocytes were pretreated with Con A. There was no rosette formation when only the leucocytes were in contact with the lectin.

Table 1

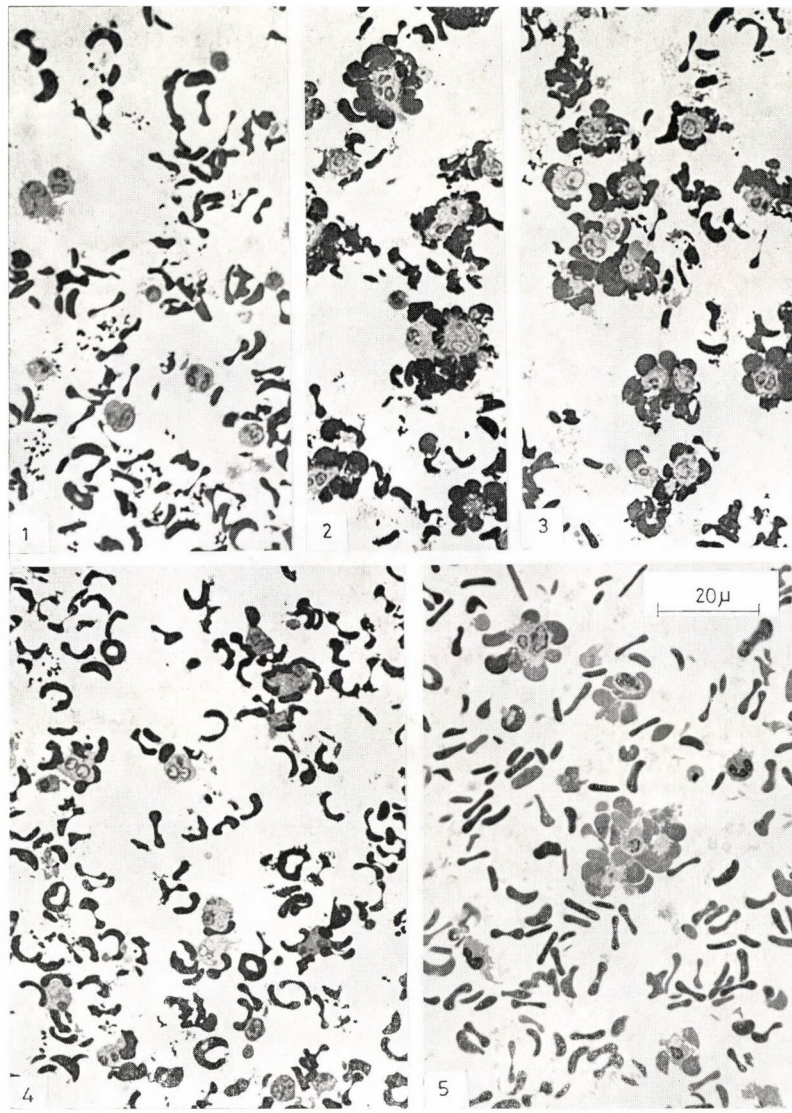
Effect of Con A treatment of separated erythrocytes and leucocytes on rosette formation

	Con A-treated leucocytes	Untreated leucocytes
Con A-treated erythrocytes	+	+
Untreated erythrocytes	—	—

Isolated suspensions of erythrocytes and leucocytes were exposed to 50 μg/ml Con A for 30 min at 24°C and recombined with each other or with untreated cell suspensions.
+ and — indicate presence or absence of rosettes.

The optimal lectin concentration

Among the Con A concentrations studied, the most intense rosette formation occurred at a concentration between 25 and 50 μg/ml. Rosettes were seen in lower numbers at 7 and 10 μg/ml and were practically absent at 1, 2 and 5 μg/ml. At higher concentrations, the rosette formation was similar to that seen at lower concentrations except that it was associated with a more pronounced aggregation tendency of the erythrocytes.



Figs 1—5. Light micrographs from semithin sections; × 840

Fig. 1. Untreated erythrocytes and leucocytes. Cells are free and no rosette formation can be observed

Fig. 2. Erythrocytes exposed to 50 μg/ml Con A. Characteristic rosettes are formed around polymorphonuclear leucocytes

Fig. 3. Erythrocytes exposed to 50 μg/ml Con A. On this part of the specimen erythrocyte rosettes were mainly formed around lymphocytes

Fig. 4. Erythrocytes treated with 50 μg/ml Con A and mildly fixed with glutaraldehyde. Erythrocytes participating in the rosettes retain their form to some extent. Rosettes are less numerous.

Fig. 5. Erythrocytes exposed to 25 μg/ml PHA. Rosettes are similar to those of Con A-treated cells but occur less frequently

With the PHA-treated erythrocytes the optimal concentrations proved to be 10 and 25 μg/ml (Fig. 5). At higher concentrations (50 and 100 μg/ml) the erythrocytes were strongly agglutinated and many of them haemolyzed.

Frequency of rosette formation

In semithin sections of one of the 50 μg/ml Con A treated material we found that from 1 000 leucocytes 90% of the polymorphonuclear leucocytes and 40% of the lymphocytes were associated with treated erythrocytes. The largest rosettes and the most intensive contact was observed in the case of the polymorphonuclear leucocytes.

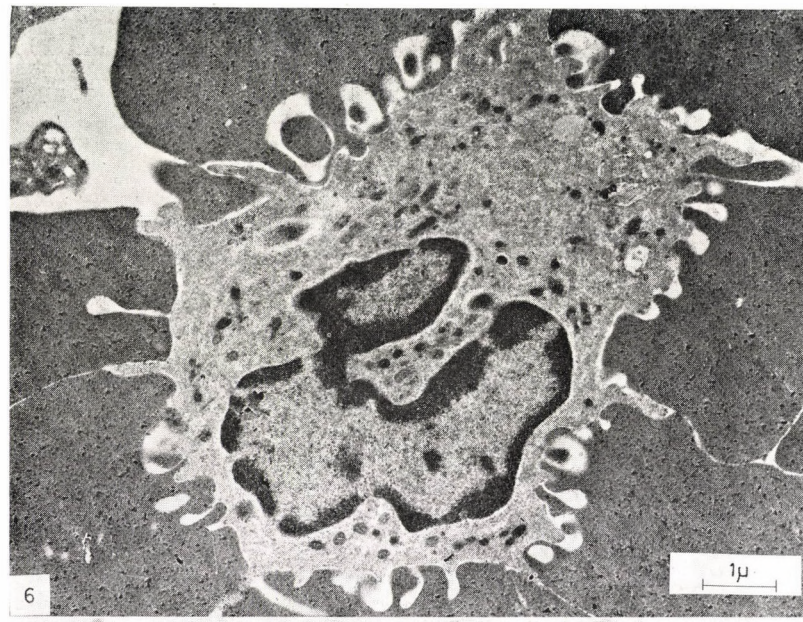
Electron microscopy

Rosettes formed by Con A-treated erythrocytes around leucocytes showed interesting ultrastructural characteristics. The free surface of the red cell participating in the rosette bulged out spherically (Figs 2, 3) while its surface contacting the leucocyte became highly folded (Figs 6, 7). The latter surface generally behaved in two ways. If the membrane of the leucocyte remained relatively smooth, the folded erythrocyte surface gave the impression as if the red cell would stand on the leucocyte by "feet" (Figs 6, 7) similarly to those of the podocytes in the renal glomeruli. In other instances the leucocyte membrane was also folded so that the processes of the contacting cells were interdigitated (Fig. 8). The avidity of the erythrocytes to leucocytes was so high that many erythrocytes not having enough space due to numerous other erythrocytes taking part in the rosette formation reached their contact with the leucocyte by an elongated process.

The ultrastructural pattern was essentially similar when not only the erythrocytes but also the leucocytes had been treated with Con A, with the exception that the leucocyte surface was more folded and they were frequently associated with one another by complicated cell processes.

Erythrocytes previously treated with 50 μg/ml Con A and shortly fixed in glutaraldehyde were also able to form rosettes. However, the number of such cells participating in the rosette formation and the frequency of the rosettes were lower (Fig. 4). The fixative-induced rigidity of the erythrocyte did not allow folding of the membrane at the contact surfaces; the large contact area was reached in such cases by the leucocytes sending long processes between the red cells.

To increase the contrast of protein structures, we examined the contact between the membranes of the erythrocyte and the leucocyte at higher resolution on specimens fixed with glutaraldehyde and tannic acid (Figs 8, 9). In the closest contacts there was an intercellular space of about 10 —20 nm which



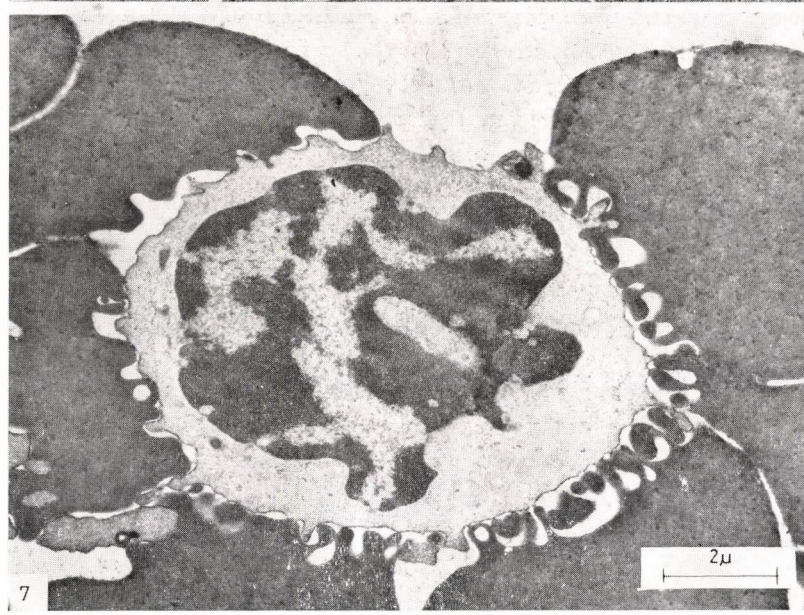
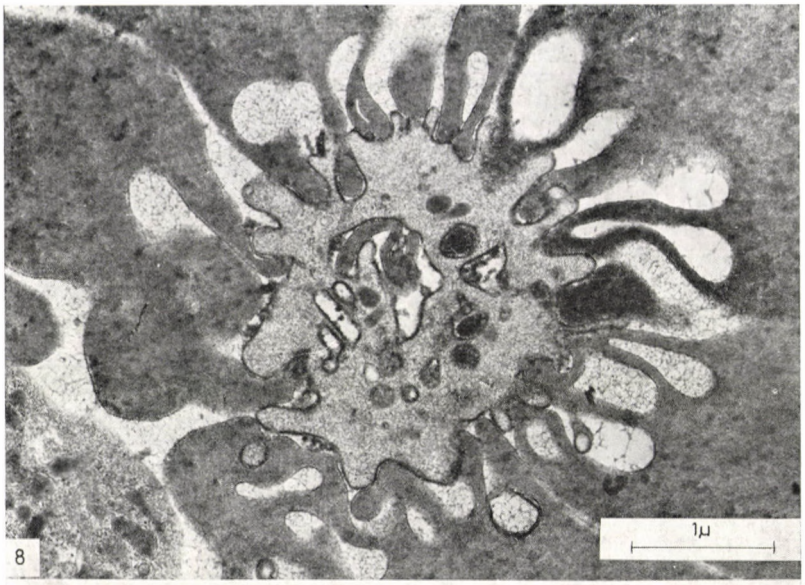


Fig. 6. Ultrastructural details of a rosette around a polymorphonuclear leucocyte. The membrane of the erythrocytes at the contact surface is highly folded in contrast to the smooth membrane at other areas of the erythrocyte surface. Some of the leucocyte processes interdigitate with those of the erythrocytes. Erythrocyte suspension previously exposed to 50 μg/ml Con A; × 12 000

Fig. 7. A lymphocyte enclosed by the ring of rosette-forming erythrocytes. Note the highly folded appearance of the erythrocyte membranes contacting the lymphocyte. Erythrocytes exposed to 50 μg/ml Con A; × 20 000



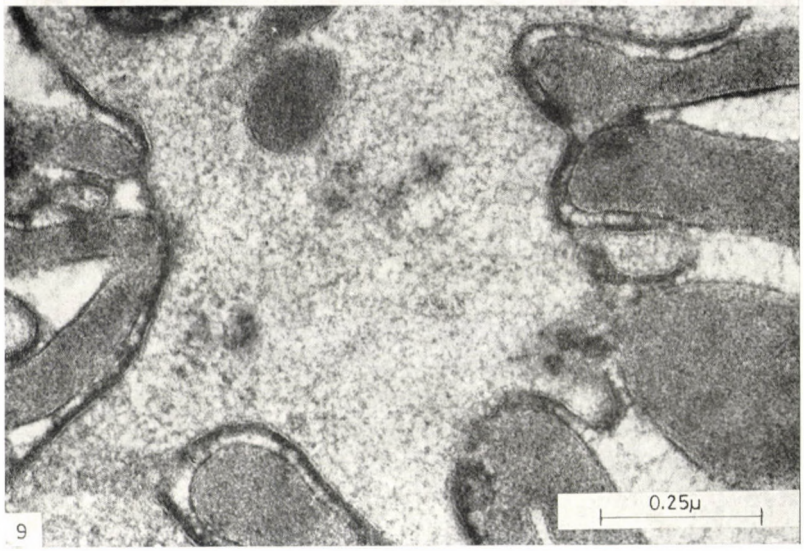


Fig. 8. Centre of a rosette showing the detail of a polymorphonuclear leucocyte. The erythrocyte surface is deeply folded and interdigitates with the leucocyte processes. Erythrocytes exposed to 50 μg/ml Con A, Specimen fixed with a mixture of glutaraldehyde and tannic acid, × 24 000

Fig. 9. Centre of a rosette at high magnification to illustrate membrane details. The erythrocyte membrane is separated from the leucocyte membrane by a 20 nm gap which is partly filled with a dense substance or traversed by dense bridge-like structures. The dense substance in the gap may partly represent the external coats of both cell membranes; × 115 000

was partly or entirely filled by a dense material (Fig. 9). It should be mentioned, however, that a dense coat, which was somewhat thicker on the leucocyte, was observed on the control cells as well.

The frequency of rosette formation with erythrocytes treated with 10 and 25 μg/ml PHA was lower when compared with that of the Con A-erythrocytes, and fewer red cells were participating in the rosettes. The ultrastructural characteristics of the rosettes were however essentially similar.

Discussion

As was seen from the results, the essential criterion of the rosette formation was the binding of the lectin to the erythrocyte membrane. The latter is well known to contain large amounts of glycoproteins, mainly sialoglycoprotein or glycophorin, consisting of about 60% sugar moiety in its molecule [3, 14, 18, 22, see 20 for references]. The lectins, known to specifically bind to certain sugar molecules, have ample opportunity to be bound to the erythrocyte membrane and to reach a considerable surface concentration. Since Con A exhibits four sugar binding sites at neutral pH [5], it may be suitable for cross-binding of the two membranes in the rosettes.

The question why the Con A-modified erythrocyte membrane prefers the leucocyte membrane for binding is unclear. An essential factor in this respect may be the peculiar molecular pattern of the two cell membrane surfaces (density of the ligands, their actual dynamic state, conformational changes of either the ligands or the lectins, etc. [12]). It is worth mentioning that the lectin immobilized and concentrated on a glass surface is very effective in lymphocyte transformation [1, 8].

The erythrocyte surface contacting the leucocyte in the rosette undergoes considerable changes: the erythrocyte membrane is usually deeply infolded into the erythrocyte body. Such folding of the erythrocyte membrane was never observed either spontaneously on the lectin-treated erythrocyte, or on the free surface of the erythrocytes in the rosette or on erythrocytes mildly agglutinated at large Con A concentrations. We think therefore that a specific contact between the lectin-treated erythrocyte and the leucocyte membrane is essential. The alteration of the erythrocyte shape seems to be important since it affects the fairly rigid erythrocyte which changes its form only due to drastic effects (osmotic stress, lysolicithin, etc.). Such multiple deep infoldings at the contact surfaces may indicate local collapses of the forces maintaining the normal membrane structure of the erythrocyte.

Con A-treated erythrocytes are bound to leucocytes with different affinity: rosette formation is the most intense around polymorphonuclear leucocytes. However, even with these leucocytes a certain percentage is completely devoid of rosette-forming erythrocytes. Since erythrocytes were available in sufficient quantity for rosette formation, it is unlikely that the lack of rosette was due to lack of erythrocytes. Differences in the surface properties

of the leucocytes may better explain this interesting finding. Similar differences in the Con A binding of lymphocytes have been reported [2].

The topographical relation of the red cells to the leucocyte has similar features to that of the erythrocyte-macrophage contact in erythrophagocytosis [15, 16]. The adsorption of the erythrocyte to the macrophage surface and the irregular folding of its membrane at the contact area are similar in both instances. It would be worth while studying whether the binding of the Con A-treated erythrocyte to the leucocyte is an analogous process to the adsorption phase of endocytosis and wheth er such erythrocytes can be phagocytosed by the cell.

Acknowledgements

The valuable help of Mrs. K. Ónodi (National Institute of Haematology and Blood Transfusion, Budapest) in separating the blood cells is gratefully acknowledged. Thanks are due to Pharmacia, Uppsala for the generous gift of concanavalin A.

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LOW-DENSITY LIPOPROTEIN COMPLEXES WITH HISTONES

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(Received 1976—03—15)

Abstract

Low-density lipoproteins (LDL) form soluble and insoluble complexes with the total histone and its fractions. The bulk of these complexes is formed by the arginine-rich histone fraction. LDL are bound with histones by means of ion binding.

Introduction

Low-density lipoproteins (LDL) [10, 14] as well as histones [17, 18] show a remarkable ability to form complexes with other chemical compounds. Formation of such complexes is of essential biological importance.

It is believed that histone-DNA complexes play an important role in the process of genetic control [1, 6]. Complexes of basic proteins [7, 19] and LDL [11] with fibrin and some other proteins are characterized by increased resistance against proteolysis. Complex formation with basic protein of heparin neutralizes the latter's anticoagulation properties [8].

Formation of LDL and basic protein complexes with different macromolecules plays an important role in the pathomechanism of some diseases [20, 21].

Demonstration of increased content of LDL [9, 15] and arginine-rich proteins [13] in arterial vessels changed by atherosclerosis suggests the possibility of an interaction between these components and has stimulated the author to examine the problem *in vitro*.

Material and method

LDL was obtained from calf thymus according to the method of BURSTEIN and co-workers [2]. Total histone, lysine-rich histone (F1), slightly lysine-rich histone (F2b) and arginine-rich histone (F3) had been produced by Polish Chemical Factory, Gliwice, Poland. Universal buffer of Britton and Robinson 0.2 mol/l, pH 2—10 was used throughout.

One ml of total histone (1 mg/ml) or one fraction of histone of the same concentration was added to 1 ml LDL to show formation of lipoprotein complexes with basic proteins. The pH of the medium was 7.5. After 60-min incubation at +20 °C, the mixture was centri-

fuged at 15 000 g for 10 min. The sediment consisting of insoluble LDL-histone complexes was washed with buffer of pH 7.5 and suspended in 0.5 ml of the buffer. In the suspension cholesterol [3] and arginine [4] were determined.

1 ml LDL of different concentrations (1, 4, 8, 12, 16 and 20 mg/ml) was added to 1 ml histone of constant concentration (4 mg/ml) or 1 ml total histone of different concentrations (1, 4, 8, 12, 16 and 20 mg/ml) was added to 1 ml LDL of constant concentration (20 mg/ml) to examine the influence of the concentration of the two proteins on the formation LDL-histone complexes. The same procedure was applied as above.

The influence of pH on the formation of insoluble LDL-histone complexes was examined for the pH range of 2—10 at one-unit pH intervals. The LDL concentration was 15 mg/ml and the histone concentration was 4 mg/ml.

One ml buffer of pH 7.5 or NaCl or urea solution of different concentrations (1, 2, 4, and 8 mol/l) was added to the obtained insoluble complexes formed from 0.5 ml of LDL and 0.5 ml of histone to establish the type of chemical binding between LDL and histone.

The presence of LDL in insoluble complexes with histone was examined by the method of double immunodiffusion on Ouchterlony plates using a monovalent antiserum (Behring, West Germany). The complexes were dissolved in 1 mol/l NaCl. The protein was stained with amido black 10B and the lipids with oil red 0.

Formation of insoluble LDL-histone complexes was examined electrophoretically. The total histone mixture with LDL and appropriate control systems were prepared in the buffer of pH 8 in the way described above. Thirty μl of supernatant of LDL-histone mixture were placed on Whatman filter paper No 3. The electrophoresis was run in veronal-medinal buffer of pH 8.6 and ion strength 0.06 for 14 h. The protein was stained with bromophenol blue [5], arginine on the basis of Sakaguchi's reaction [4] and LDL with Sudan black [16].

Results

It was shown that LDL form insoluble complexes with the total histone and its fractions and the arginine-rich fraction formed the greatest amount of the complexes. This was followed in amount by the slightly lysine-rich fraction and, at last, by the lysine-rich fraction (Fig. 1).

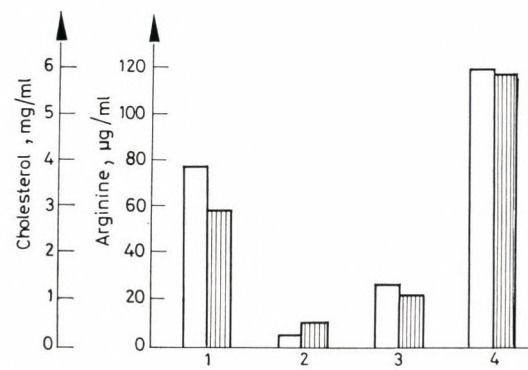

Fig. 1. Formation of insoluble LDL-complexes with total histone and its fractions measured by the amount of arginine and cholesterol. 1 = total histone, 2 = lysine-rich fraction (F1). 3 = slightly lysine-rich fraction (F2b), 4 = arginine-rich fraction (F3). □ arginine, ▥ cholesterol

The amount of insoluble LDL-total histone complexes depends on the concentration of the two proteins (Fig. 2). As Fig. 2 shows the amount of insoluble complexes of the two proteins grows with the increase of LDL con-

centration when the histone concentration is constant, and its maximum is reached at the final LDL concentration of 6 mg/ml. When the LDL concentration is constant, the greatest amount of insoluble complexes is formed at the histone concentration of 4 mg/ml. Further increase in the concentration of

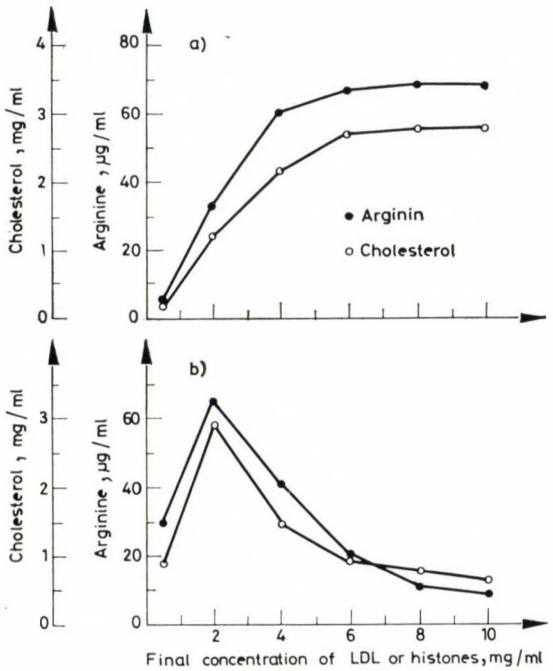

Fig. 2. Formation of insoluble LDL-total histone complexes. (a) = Constant histone concentration (4 mg/ml) and varying concentration of LDL. (b) = constant LDL concentration (20 mg/ml) and varying concentration of histone

this protein results in a decrease of the formation of insoluble complexes (Fig. 2B).

The amount of insoluble LDL-total histone complexes also depends on pH. At pH below 5 the complexes are not formed at all. Their amount grows rapidly with growing pH, reaching the maximum in the pH ranges between 8 and 10 (Fig. 3).

Precipitating lines on Ouchterlony plates have proved the presence of LDL in insoluble complexes (Fig. 4).

Fig. 5 shows that insoluble LDL-histone complexes easily dissociate in the presence of NaCl, but are highly resistant to the action of urea. Nearly complete dissociation of the complexes needs 0.5 mol/l NaCl (final concentration) whereas the same concentration of urea reduces the complex content only by 30%. On the other hand, complete dissociation requires 4 mol/l urea.

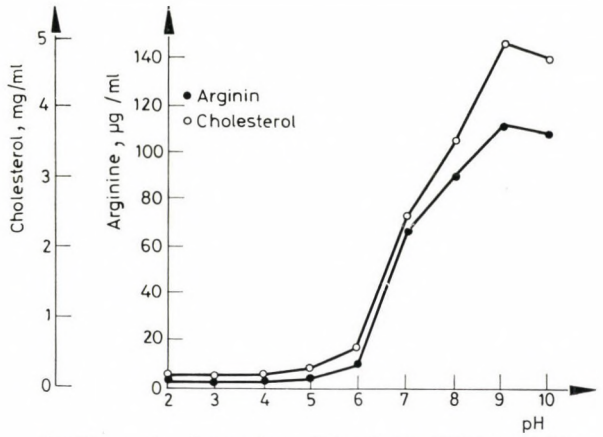

Fig. 3. Influence of pH on the formation of insoluble LDL-total histone complexes

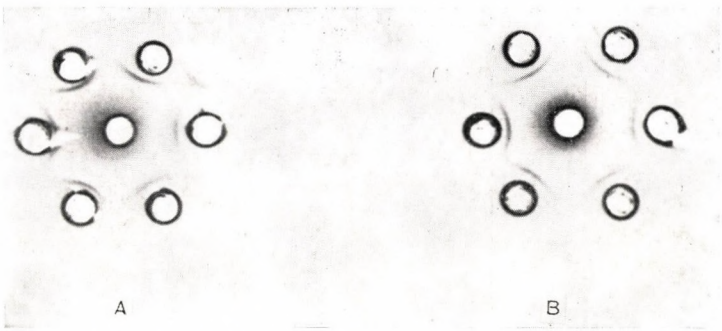

Fig. 4. Immunodiffusion of LDL-total histone complexes. A = Staining for protein; B = staining for lipids

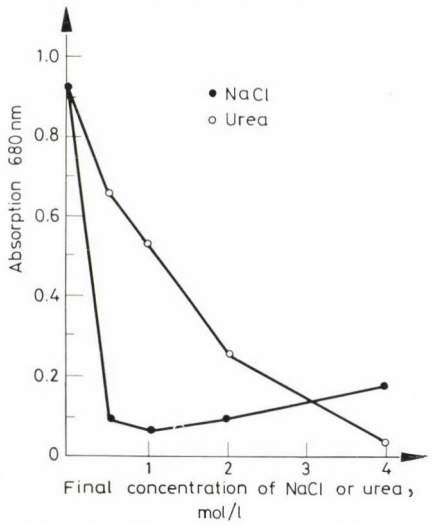

Fig. 5. Dissociation of insoluble LDL-total histone complexes by different concentrations of NaCl and urea

Electrophoretic examinations have shown that LDL and histone also form soluble complexes (Fig. 6). LDL produce one electrophoretic stripe of anode motility which stains with bromophenol blue, Sakaguchi's reagent and Sudan black. The total histone produces two stripes of cathode motility which stains with bromophenol blue and Sakaguchi's reagent. The electrophoreto-

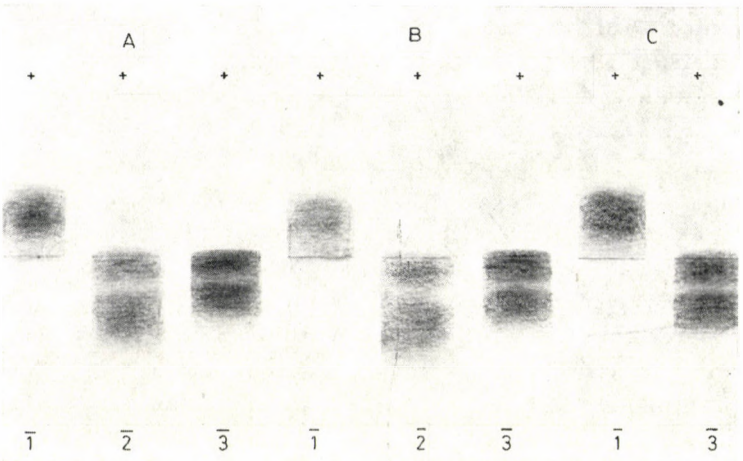

Fig. 6. Electrophoretogram of LDL (1), total histone (2) and the mixture of LDL with total histone (3). A = staining for protein; B = staining for arginine; C = staining for lipids

gram of LDL–histone mixtures is different from the above ones. Namely, one protein stripe is found at the starting line and an other is of cathode motility and both stripes stain with bromophenol blue, Sakaguchi's reagent and Sudan black, thus showing that they are LDL-histone complexes. But there is no stripe of anode motility responding to free LDL.

Discussion

It seems that the amount of LDL-histone complexes depends on the kind and number of basic amino acids included in the molecule. Since LDL form more complexes with arginine-rich histone than with lysine-rich histone it may be concluded that the presence of arginine residue in the molecule specifically promotes the interaction with LDL.

Yet it cannot be excluded that the amount of complexes also depends on the molecule conformation. Owing to the appropriate steric conformation, the basic amino acids may be found on the molecule surface, thus being accessible for an interaction with LDL. But other conformations may close these residues inside the molecule, thus making the residues inaccessible.

The different localizations inside the molecule may be explained by the fact that slightly lysine-rich histone forms more complexes than lysine-rich histone.

Since the amount of insoluble LDL-histone complexes depends on the ratio of concentrations, it may be concluded that LDL bind histone in different molecular proportions and their solubility depends on the ratio of histone molecules to LDL molecules.

Prevalence of the histone molecules over the LDL molecules makes the complexes soluble and this may explain the small amount of insoluble complexes in systems including larger amounts of histones (Fig. 2b).

Formation of such soluble complexes was shown electrophoretically.

The marked pH dependence of the formation of insoluble LDL-histone complexes, determining the electrical charge of molecules and thus their interaction, show ionic-bond character of LDL-histone complexes.

Phosphoric acid residues included in LDL are carriers of negative charges, whereas in histones the carriers are dissociated residues of basic amino acids. The suggestion that the formation of LDL-histone complexes is determined by ion binding has been confirmed by the experiment with NaCl solution which, being able to disrupt ionic bonds dissociates the complexes in concentrations as low as 0.5 mol/l. Contrary to NaCl, urea solutions, which disrupt hydrogen binding, evoke decomposition of LDL-histone complexes only in very high concentrations. Probably, the decomposition in this case is due to the effect of urea on the LDL molecule. It seems that LDL-histone complexes may serve as a good model of examining the intermolecular action. Since the preliminary investigations show the presence of such complexes in the walls of arterial vessels changed by atherosclerosis [12], their formation may play some role in pathomechanism of these changes.

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AXONAL LABYRINTHS IN THE RAT SPINAL CORD: A CONSEQUENCE OF DEGENERATIVE ATROPHY

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Abstract

Transection, crush or local colchicine treatment of a peripheral nerve induces degenerative atrophy of central terminals of primary sensory neurons in the Rolando substance of the rat spinal cord. In addition to osmiophilic alterations that occur in the course of degenerative processes in general, degenerative atrophy is characterized by the appearance of spectacular labyrinthine formations. Electron-microscopic analysis reveals that these consist of flattened axonal profiles. Axonal labyrinths are interpreted as signs of futile regenerative efforts of axon terminals undergoing degenerative atrophy. Labyrinths disappear from the Rolando substance several months after peripheral nerve injury, when degenerative atrophy of the central terminal is replaced by regenerative proliferation.

Introduction

Degenerative atrophy, a peculiar trophical feature of primary sensory neurons, has recently been discovered in this laboratory [1]. Accordingly, injury of a peripheral nerve that causes Wallerian degeneration of the distal stump and chromatolysis of the parent nerve cells, induces degenerative alterations also in the central terminals. The same effect was observed after local treatment of peripheral nerves with metaphase-arrest drugs, like colchicine or vinblastine [2]. In order to distinguish this phenomenon, obviously necessitating an amendment of the neurone doctrine, from the „regular" Wallerian degeneration, the term "degenerative atrophy" has been coined to this process. One of the consequences of degenerative atrophy is the rapid disappearance of fluoride-resistant acid-phosphatase activity from nociceptive central terminals of the ipsilateral Rolando substance. At the electronmicroscopic level, degenerative atrophy is characterized by accumulation of cytolysomes, whorl-like bodies and similar osmiophilic debris in impaired axon terminals. In addition, conspicuous, large labyrinthine structures were observed in the Rolando substance, similar in many respects to some of the axonal alterations described in normal material by SOTELO and PALAY [7] and interpreted as concentric laminar membranes of endoplasmic cisterns. The objective of this study was to elucidate the fine structure and the way of formation of these labyrinths, and thus to distinguish them from variations observed under normal conditions.

Material and method

Investigations were performed on 12 albino rats of both sexes, 150—200 g body weight. In 5 animals, the left sciatic nerve was transected; in 3 animals, the left sciatic was crushed with watchmaker's tweezers; in 2 animals, a 4 × 1 × 1 mm Gelaspon fibrin sponge, soaked in 0.05% colchicine (dissolved in saline) was applied around the left sciatic for 30 min; and in 2 animals, a similar cuff, but soaked in 0.1% vinblastine (G. Richter, Budapest; dissolved in saline) was applied for 30 min. After 4—42 days survival, the rats were subjected to perfusion with Karnovsky's aldehyde fixative through the aorta. The lumbosacral spinal cord was dissected, post-fixed in the same fixative for 12 hours at 4 °C and small tissue blocks from the left and the right Rolando substance (substantia gelatinosa) were excised. These samples were post-fixed in Millonig's osmic acid solution (1%), dehydrated in graded alcohols, processed through propylene oxide and embedded in Durcupan (Fluka). Thin sections (silver interference colour) were obtained on a Reichert Ultrotome, using glass knives. Sections were stained with lead citrate and uranyl acetate. Tesla 513 B and JEM 100 B electron microscopes were used.

Results

In addition to disappearance of acid phosphatase activity from the ipsilateral Rolando substance, ultrastructural signs of disorganization were identical in all three groups, i.e. after transection, crush or local colchicine (or vinblastine) treatment of the sciatic nerve. Accumulation of whorl-like bodies, cytolysomes and osmiophilic particles can be seen in every specimen as described earlier [1, 2, 6]. Lysis of synaptic vesicles (Fig. 1) results in a homogeneous ground substance within the terminal axoplasm. From the 10th postoperative day on, there begins formation of axonal labyrinths.

In order to gain more insight into the mechanism of axonal labyrinth formation, we collected and analysed a number of electron micrographs. Apparently, the onset and speed of labyrinth formation show considerable individual variations, therefore, it is impossible to establish decisive, distinctive states for each consecutive postoperative day; in fact, more or less complicated formations were seen in every section. Since it is impossible to follow the fate of the very same labyrinth, "it is very difficult to reconstruct a dynamic process from the static pictures provided by the elctron microscope" [7]. There remains a leading sign if we suppose that more complex formations are derived from less complex ones, viz., the degree of intricacy.

Taking into account all the above restrictions, it appears that labyrinth formation begins with a sheet-like apposition of flattened axonal profiles — usually around the perikaryal surface membranes of substantia gelatinosa cells (Fig. 2). In order to facilitate analysis of these structures, we painted identical profiles with different colours in Fig. 11, which is a multicoloured line diagram corresponding to Figs 2–7. Such pictures are especially useful in analysing complex formations.

The next step is characterized by bending of the axonal sheets. Due to the longitudinal growth of the axons, this is bound to result in interdigitations (Fig. 3). In many cases, it is hard to say whether all the profiles participating in the formation of such bent systems are actually axons. Serial sections,

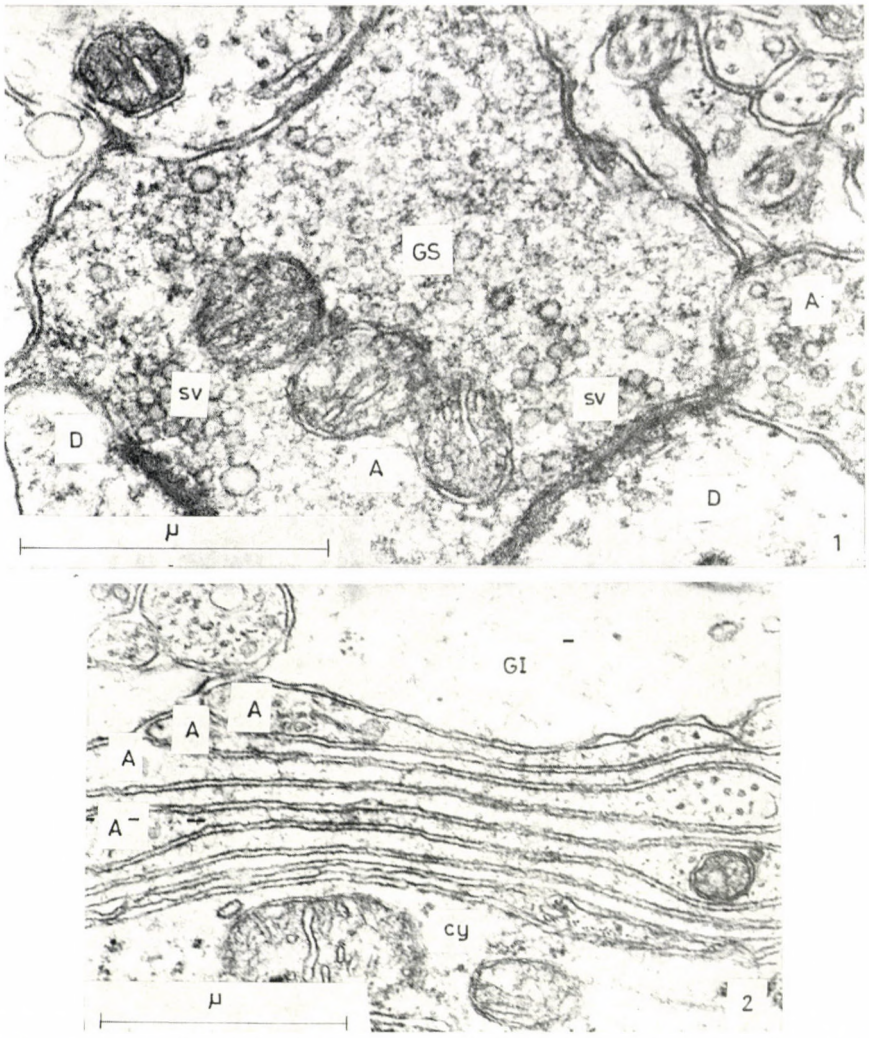


Fig. 1. Lysis of synaptic vesicles in an axon terminal (A) undergoing degenerative atrophy. Note persistence of synaptic vesicles in active presynaptic areas (sv) and the homogeneous, slightly electron-opaque axoplasmic ground substance (GS) resulting from destruction of synaptic vesicles in the central regions of the axon terminal. Synaptic vesicles in the adjacent axon terminal (A'), which is not affected by the process of degenerative atrophy, are entirely normal. D = dendrites. Rolando substance, segment L_5, 9 days after local vinblastine treatment of the ipsilateral sciatic nerve

Fig. 2. Earliest stage of labyrinth formation: sheet-like apposition of flattened axonal profiles. Surrounded by glial cell process (Gl), flattened axonal profiles (A) snuggle up to the cytoplasm of a substantia gelatinosa cell (cy). Rolando substance of the rat (L_6) 12 days after transection of the ipsilateral sciatic nerve

however, prove unequivocally that microstructural characteristics of axons are present in all of them. For instance, axons No. 1 and 2 contain large dense-core vesicles in Fig. 3, whereas in Fig. 4, a serial section of this same area about 1 μm apart, large dense-core vesicles are present in axons No. 3 and 4.

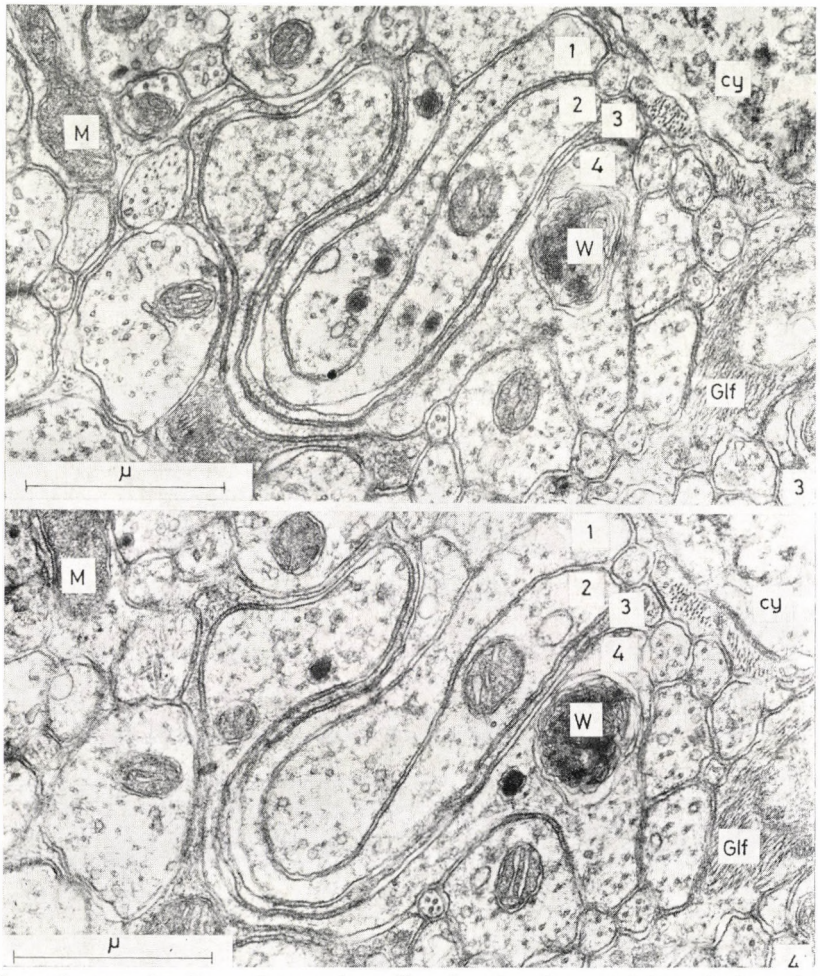


Fig. 3. Second stage: bending of axonal profiles. In the close vicinity of the substantia gelatinosa cell (cy), four axons, numbered 1, 2, 3, 4, can be seen. Dense-core vesicles are present in axons No. 1 and 2; in axon 4, a whorl-like body (W) is apparent. Note glial filaments (Glf) in the glial profile. M = mitochondrion

Fig. 4. Serial section to Fig. 3, about 1 μm apart. Numbering and lettering as above. Note the presence of dense-core vesicles in axons No. 3 and 4. Fifteen days after vinblastine treatment

The intricacy of this relatively simple axonal labyrinth is apparent in the multicoloured picture Fig. 11(b).

From this stage on, the electron-microscopic appearance of the axonal labyrinth depends mainly on the plane of section. Since the labyrinths are not spherosymmetrical bodies, there should be distinguished at least two major planes. One of them, represented by Figs 3 and 4, can conveniently be called "transversal" or meridional cross-section, since the sheets themselves can be envisaged as hemispherical surfaces. In more advanced forms, such sections (Figs 5 and 6) reveal that, in essence, axonal sheets surround an osmiophilic

"core" (C). Symmetricity of this arrangement is evident from the multi-coloured diagram 11(c), drawn to scale from Fig 6.

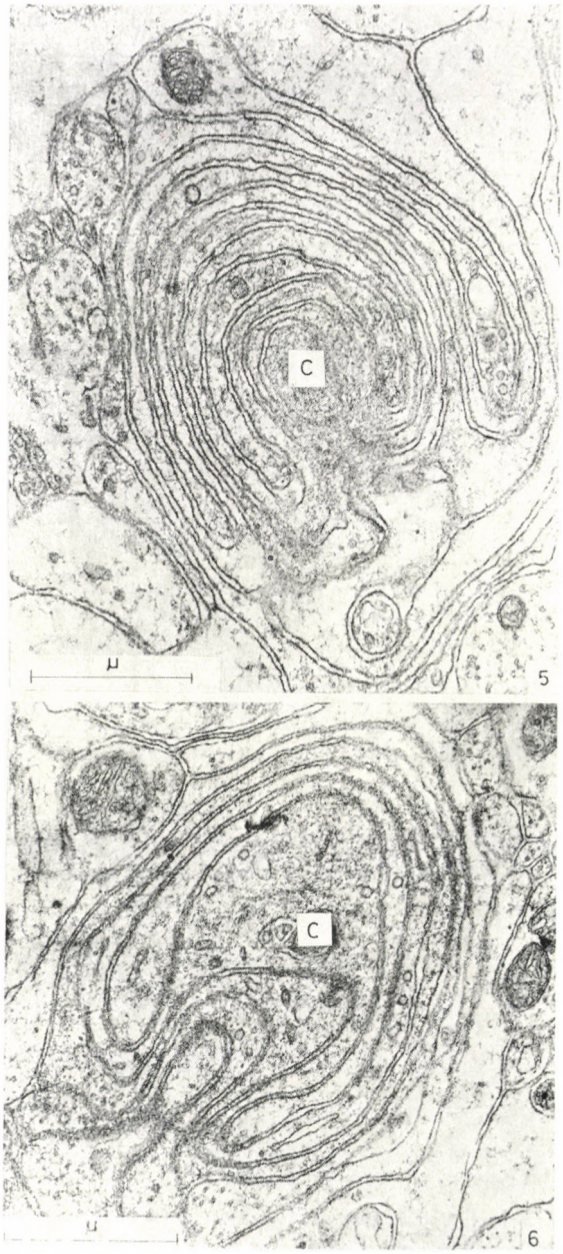


Fig. 5. Advanced stage of polyaxonal bending: labyrinth formation. Meridional (transversal) section. C = central osmiophilic body, probably primarily responsible for the alterations. 42nd postoperative day

Fig. 6. The same as in Fig. 5. Note the osmiophilic body (C) in the centre of the whole complex· Synaptic vesicles are present in all axonal profiles

On the other hand, if such hemispherical surface planes are sectioned *parallel to the equator* of the imaginary sphere, the patterns obtained reveal more or less concentric, though often highly deformed, rings of axonal sheets (Figs 7 and 8). In effect, the concentricity is in most cases only apparent; for

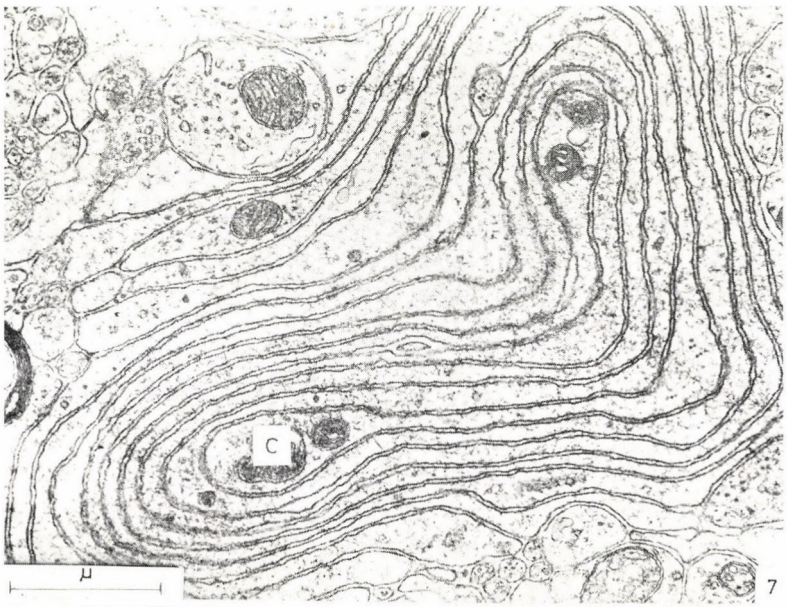
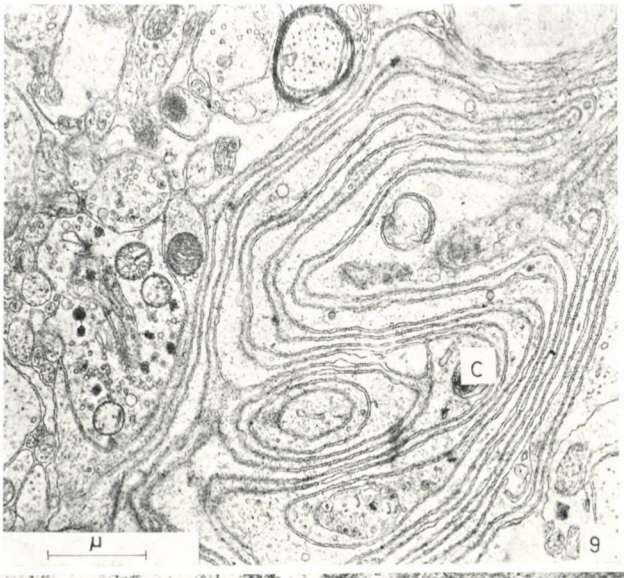


Fig. 7. Section of axonal labyrinth in the equatorial plane. In this plane, the individual axons taking part in labyrinth formation outline concentric (or in this case: L-shaped) rings. C = central osmiophilic body; 42nd postoperative day

Fig. 8. The same as in Fig. 7. Note ovoid rings of concentric axonal layers (A), separated from each other only by thin sheets of extracellular space (E). The central osmiophilic body (C) is apparent

instance, the multicoloured pattern corresponding to Fig. 7 (Fig. 11(d)) reveals that the black profile takes two consecutive turns while forming the labyrinth; the same is true for the red and the green axonal sheets. In these samples, too, a central core (C) can be seen.



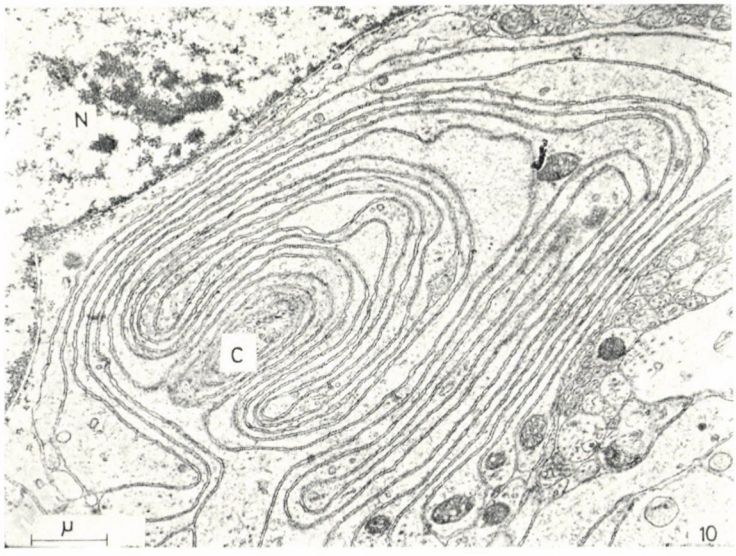


Fig. 9. Advanced stage of labyrinth formation; oblique section. Within the apparently normal neuropil, the labyrinth occupies a considerable area. C = central osmiophilic body; 42nd postoperative day

Fig. 10. Nucleus-sized axonal labyrinth. Note the central osmiophilic body (C) and the nucleus of the adjacent substantia gelatinosa cell (N); 42nd postoperative day

The final stage of labyrinth formation (Figs 9 and 10) is characterized by the utmost intricacy of the axonal sheets. In oblique sections (Fig. 9) hemispherical symmetry and concentricity are intermingled, whereas in sections cut "transversely", (in the meridional plane) the symmetry of the

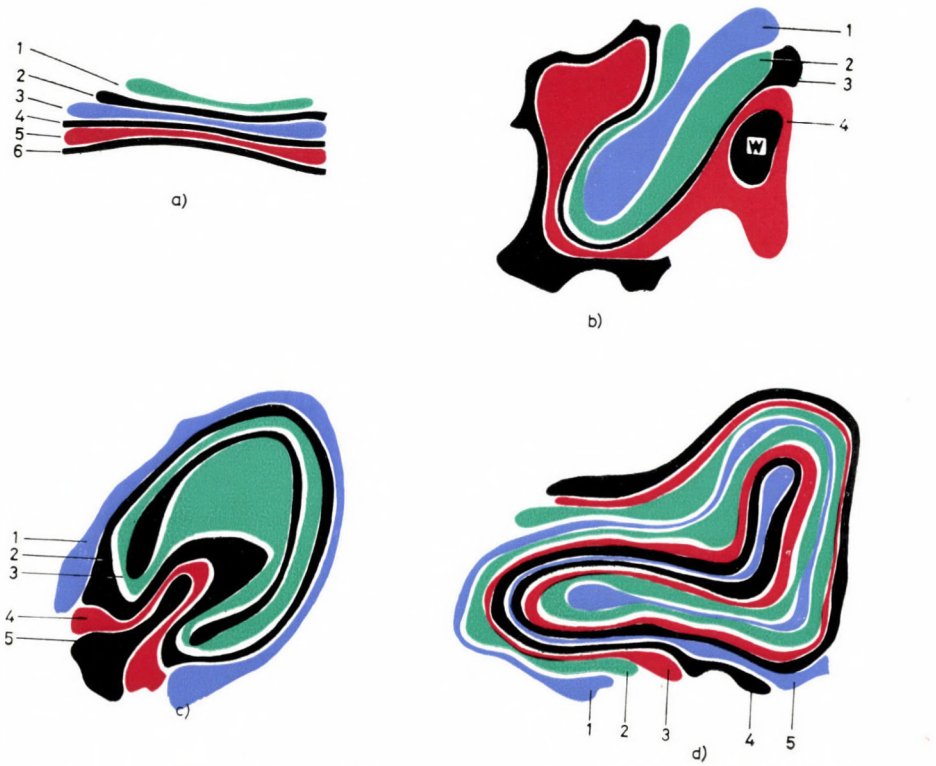


Fig. 11. Multicoloured line diagrams of axonal labyrinths. (a) = identical with Fig. 2; (b) = identical with Fig. 3; (c) = identical with Fig. 6; (d) = identical with Fig. 7. Numbering of axons in (b) is the same as in Fig. 3

pattern, though distorted by unequal growth of the individual axonal elements, is still apparent (Fig. 10). Also characteristic of these labyrinths is their location in the close vicinity of substantia gelatinosa cells.

Discussion

According to the ultrastructural analysis, labyrinths characterizing the Rolando substance in more advanced stages of degenerative atrophy consist mainly, if not entirely, of flattened axonal profiles. Due to the destructive effect of vesiculolysis, synaptic vesicles can only be identified exceptionally; dense-core vesicles, however, withstand the lytic process, thus axonal profiles can be identified. The process of labyrinth formation begins with a stratified

apposition of flattened axonal profiles, usually in the very neighbourhood of substantia gelatinosa cells. By means of lateral expansion and subsequent bending of these axonal sheets, brought about by a continuous growth of the axoplasm, more and more intricate forms are produced. Finally, these become complicated to such an extent that graphical reconstruction of the individual layers needs a very careful analysis.

In an extensive study, SOTELO and PALAY [7] called attention to the ultrastructural alterations in the lateral vestibular nucleus of normal rats. Some of these formations are extremely similar to the axonal labyrinths observed in the course of degenerative atrophy. It could be argued that, in fact, the patterns observed by SOTELO and PALAY represent signs of a "physiological degeneration." Many of the ultrastructural peculiarities depicted by SOTELO and PALAY are undoubtedly identical with those observed in the course of a Wallerian degeneration. However, in spite of the similarity between the labyrinths reported in this study and the Figs 4 and 5 in SOTELO and PALAY's paper, it seems impossible for us to accept the interpretation given by these authors. A careful analysis of the electron micrograms and, especially, inspection of the multicoloured line diagrams proves that these labyrinths are not, as supposed by SOTELO and PALAY, whorls of endoplasmic cisternal membranes, rather they consist of individual, flattened axonal profiles, with a possible participation of dendritic and glial elements.

Axonal labyrinths, similar to those observed in these studies can often be found close to the transected stump of peripheral nerves; such patterns are believed to represent futile regenerative efforts of the transected axons. It is reasonable to assume a similar genesis of labyrinth formation in the Rolando substance. In fact, axonal labyrinths in the Rolando substance persist for several months after transection of the respective spinal nerve; they disappear, however, in the course of regenerative proliferation shortly after functional regeneration of the peripheral nerve has been completed [1].

GRANT [3, 5] and, more recently, WESTRUM and co-workers [8] have described degenerative debris in central terminals of the vestibulocochlear and trigeminal nerves, after transection of the respective peripheral truncs. On the basis of light-microscopic studies, performed by means of the Fink-Heimer impregnation technique, these authors assume that transection of the peripheral process of a bipolar or pseudounipolar cell induces "transganglionic degeneration" of the central axonal process, proximal to the ganglion. We have pointed out in a recent paper [1] that the term "transganglionic degeneration" is slightly misleading since in such cases, no Wallerian degeneration occurs in the central axon; and, though the signs of degenerative atrophy are similar, the speed and the outcome of this process considerably differs from a straightforward Wallerian degeneration. On the other hand, it can be assumed that the frequent occurrence of axonal labyrinths in degenerative atrophy

might be at least partly responsible for the positive Fink-Heimer degeneration patterns obtained by GRANT and by WESTRUM and co-workers if we suppose that axonal labyrinths exert a high degree of argyrophilia.

As regards the functional value of axonal labyrinths, no definite conclusion can be drawn, since appropriate electrophysiological studies have not been performed as yet. Indirect evidence suggests that axonal labyrinths are functionally inert.

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SOME HISTOCHEMICAL REACTIONS ON THE NEUROSECRETORY CELLS OF RIVULOGAMMARUS SYRIACUS CHEVREUX (CRUSTACEA, AMPHIPODA)

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Abstract

The histochemistry of neurosecretory material in neurosecretory cells of *Rivulogammarus syriacus* has been investigated. Histochemically, these cells contain different neurosecretory substances. The material in A and C cells consists of mucopolysaccharides and lipids, that in B, B' and D cells there are a protein containing cystine S—S group, mucopolysaccharides and lipids, and that in E cell contains a weak protein with cystine S—S group, a substance showing β metachromasia and lipids. The lipids are found in all the cells.

Introduction

The anatomy and cytology of the neurosecretory system of *Rivulogammarus syriacus* has been described [4]. By the use of the paraldehyde-fuchsin (PF) technique and chromealum haematoxylin phloxine (CHP) technique, the neurosecretory cells found in the cephalic region of *Rivulogammarus* may be classified into six types (A, B, B', C, D and E) according to their staining reactions.

Few systematic histochemical studies have been carried out on neurosecretion in Crustacea. The majority of the histochemical studies have been supplementary to morphological studies on neurosecretion. Most of them have been made on decapods. GABE [13, 14] observed the presence of PAS-positive material in the organs of Hastrom and sinus glands of Oniscoid isopods and in the organs of Bellonci of the isopod, *Sphaeroma serratum*. MIYAWAKI [24, 25] reported the presence of PAS-positive material "probably neurosecretory" in the neurosecretory cells of the cephalic and thoracic ganglia of the crab, *Telmessus cheiragonus*, and later he [27, 28] reported the presence of cytoplasmic globules in the nerve cells of crabs, rich in RNA, cerebrosides and phospholipids, which were presumably sites of neurosecretory material synthesis. MIYAWAKI [26] found PAS-positive neurosecretory cells of the cerebral ganglion of the isopod, *Idotea japonica*. Five types of proteinaceous neurosecretory materials in the sinus gland of *Carcinus maenas* have been described [35].

Few observations on the histochemistry of the neurosecretory system in the entomostracan groups have been published so far [5, 22, 23].

Recently, studies on the histochemistry of the neurosecretory material have progressed, with the use of more or less rigorous histochemical techniques. The information obtained by these methods shows some divergence. Probably this depends on the techniques, or is related to differences in the physiological state of the animals used, or may express genuine differences in the chemical composition of the neurosecretory material in different species of Crustacea.

The present investigation is concerned with the nature of the neurosecretory material in the cephalic nervous system of *Rivulogammarus* as revealed by the different histochemical tests.

Material and method

Mature specimens of *Rivulogammarus syriacus* of both sexes were used. The average size of the female specimens was 6.5 mm, ranging from 5.0 mm to 9.0 mm and that of the male was 7.0 mm, ranging from 5.0 mm to 11.0 mm. Whole specimens were fixed in aqueous Bouin, Helly and Carnoy fixatives. The anterior region was decapitated and after dehydration in alcohol and clearing in xylene, was embedded in paraffin, serial horizontal sections of 8 μm thickness were obtained. All histochemical tests were performed according to PEARSE [31].

The histochemical tests used include:

(1) the performic acid-alcian blue (PAAB) technique for disulphide groups [1];
(2) the permanganate-oxidation-Alcian Blue (POAB) technique [3];
(3) the periodic acid Schiff (PAS) technique [21] for 1.2 glycol groups and a variety of other materials. For this reaction, two series of serial sections were treated, one in each of the following ways:
   a — the standard method for PAS reactive groups;
   b — diastase digestion (2 h at 26 °C) followed by the standard method for the detection of glycogen.
(4) toluidine blue (TB) technique [20];
(5) Sudan Black B (SBB) technique [31];
(6) the alloxan-Schiff technique for protein-bound amino groups [38].

Analysis of the various results was subjective; the responses were scored on a four-point scale ranging from (−) to very strongly positive (+++).

Results

The responses of the various neurosecretory cells of *Rivulogammarus syriacus* to the various histochemical tests, CHP and PF techniques are summarized in Table 1.

In detail, the responses are as follows:

PAAB technique and POAB. The results of both of these tests for disulphide groups are basically similar. A weak background response is noted in the cytoplasm of most neurosecretory cells. The B, B' and D cells are strongly positive and the PAAB-positive material is demonstrated in the vacuoles of the cells. It indicates the presence of disulphide bonds in the secretions of B,

Table 1

Histochemical reactions of different neurosecretory cells of Rivulogammarus syriacus

Test	Group demonstrated	Neurosecretory cells					
		A	B	B'	C	D	E
PAAB and permanganate oxidation Alcian Blue	S—S groups	—	+++	+++	—	+++	+
PAS	Carbohydrate	+++	+++	+++	+++	+++	—
PAS (diastase)	Clycogen	—	—	—	—	—	—
Toluidine blue	Metachromasia	+	+	+	++	++	+++
Sudan Black B	Lipid	++	++	++	++	++	+++
Alloxan-Schiff blue	Protein bound NH2 groups	+	+	+	+	+	+

+++ = strongly positive, ++ = positive; + = weakly positive; — = negative

B' and D cells (Figs 1 and 2). The secretions of E cells are weakly positive, while the secretions of A and C cells are completely negative (Fig. 3).

PAS technique. All the neurosecretory cells except E cells are either positive or strongly positive. The PAS-positive material is demonstrated in the

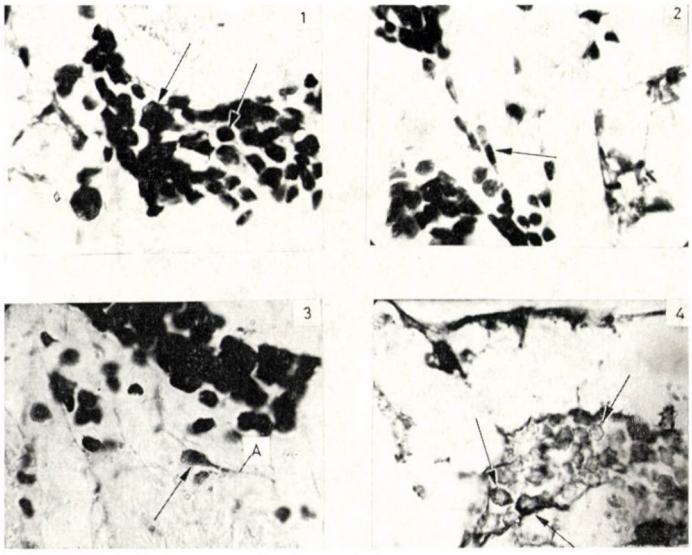

Fig. 1. Horizontal section through the brain of *Rivulogammarus syriacus.* Bouin, AB. Note the presence of strongly positive alcian blue material in different neurosecretory cells (arrows). Magnification for all figures is × 1250

Fig. 2. Horizontal section through the brain of *Rivulogammarus syriacus.* Bouin, AB. Note the presence of positive alcian blue material in D cell (arrow)

Fig. 3. Horizontal section through the brain of *Rivulogammarus syriacus.* Bouin, SBB. Note C cell (arrow); A = axon; NSM = Neurosecretory material

Fig. 4. Horizontal section through the brain of *Rivulogammarus syriacus.* Helly, PAS. Note the presence of strongly positive material in A and E cells (arrows) and the absence of PAS material in other E cell (arrow)

cytoplasmic region of the cells (Figs 4 and 5) or just outside the Nissl zone even after digestion with saliva (diastase digestion) for 30 min at 37°C. This result indicates, therefore, that the material is not glycogen, but composed of neutral mucopolysaccharides, mucoproteins or glycolipids.

TB technique. When the Helly-fixed sections are stained with alcoholic TB, a dark blue staining occurs in the cytoplasm of all the neurosecretory cells. The C cells are strongly positive while E cells show two kinds of reactions: one group of less active cells with blue background response in cytoplasm and the other group of cells with violet background response (Fig. 8). The violet granular mass indicates β metachromasia.

SBB technique. All types of neurosecretory cells show some degree of response to this test. Small granules scattering in the cytoplasm are stained with SBB (Figs 6 and 7). The vacuoles do not show sudanophilic granules, indicating that there is no appreciable amount of lipids in the vacuoles.

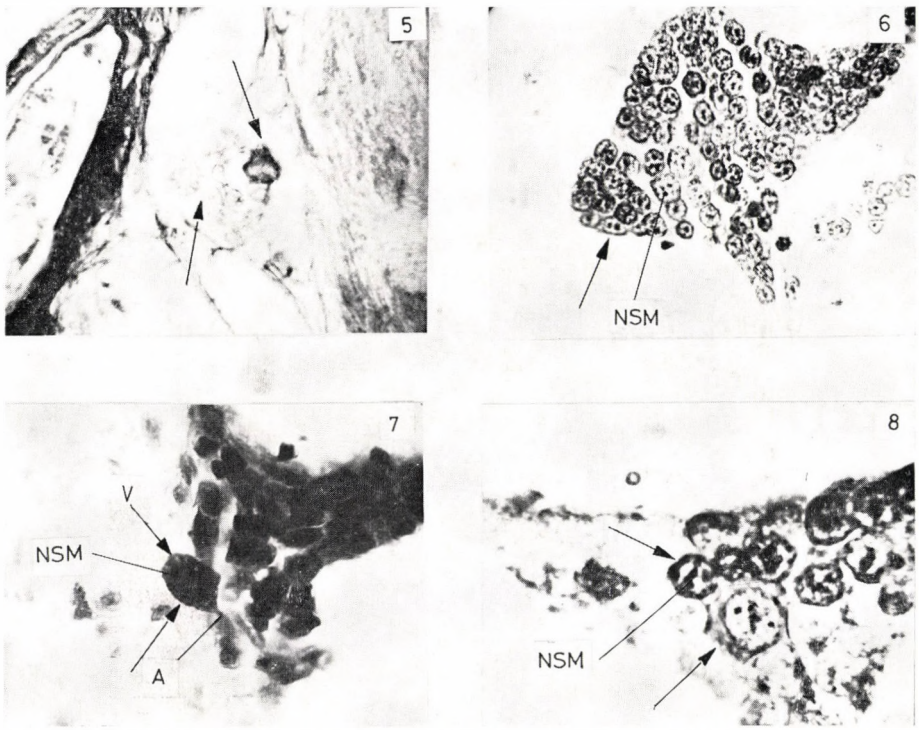

Fig. 5. Horizontal section through the brain of *Rivulogammarus syriacus.* Helly, PAS. Note the presence of strongly PAS-positive material in B cell (arrow) and the absence of PAS material in E cell (arrow)

Fig. 6. Horizontal section through the brain of *Rivulogammarus syriacus.* Helly, SBB. Note the presence of strongly SBB-positive material in E cell (arrow)

Fig. 7. Horizontal section through the brain of *Rivulogammarus syriacus.* Bouin, SBB. Note A cell (arrow); A = Axon; NSM = Neurosecretory material; V = Vacuole

Fig. 8. Horizontal section through the brain of *Rivulogammarus syriacus.* Helly, TB. Note the presence of strongly TB-positive material in E cell (arrow) and positive material in B cell (arrow); NSM = Neurosecretory material

Alloxan-Schiff technique. All types of neurosecretory cells show mild response to this test indicating the presence of protein.

Discussion

The results of the histochemical tests indicate that the neurosecretory cells contain histochemically different substances. The PAAB reaction for the demonstration of protein bound S–S- and SH-groups, is positive in the B, B^- and D cells. The PAS reaction is not influenced by saliva treatment, indicating that the substance contains no glycogen. It seems to be composed of neutral mucopolysaccharides, mucoprotein or glycolipids. With the TB reaction all cells are positive, C and E cells even strongly positive. All the cells show SBB positive reaction.

In arthropods, several workers have reported the presence of neurosecretory material rich in disulphide groups; in the crab, *Carcinus maenas*, disulphide groups in axon endings in the sinus glands have been reported [35]. Cystine has been found [30] in the chromactivating substances extracted from the thoracic ganglion of the crab, *Eriocheir japonicus*, by using electrophoresis and paper chromatography. It was suggested that these substances are polypeptides with S–S bonds. A similar result has been reported in connection with cystine-rich neurosecretory materials of *Chirocephalus diaphanus* [22].

The presence of neurosecretory material rich in disulphide groups has been reported in insects [3, 8, 9, 18, 32, 36, 37]. As to other invertebrate groups, the presence of neurosecretory materials rich in cystine has been reported in leeches [19] and in basommatophoran molluscs [6]. The disulphide-rich neurosecretory material of *Rivulogammarus syriacus* is also the PF-positive material.

The presence of a PAS-positive component in the neurosecretory material of crustaceans has been reported [5, 13, 26—28, 35]; in insects [3, 17, 32]; in polychaetes [2, 10, 11, 15]. In *Rivulogammarus syriacus* it appears that the neurosecretory material contains a PAS-positive fraction and it seems that the production of the neurosecretory material in the neurosecretory cells is associated with the glycolipids.

There is [5, 35] little or no staining of the neurosecretory material in their species of crustaceans with SBB, however, the neurosecretory material in a number of species of crustaceans were reactive to Sudan Black and Luxol fast blue [22, 23]. The lipid contents in the neurosecretory material in insects have been reported [7, 12, 16, 17, 29, 33, 34].

As a conclusion, it may be established that the neurosecretory cells of *Rivulogammarus syriacus* contain histochemically different substances. The material of A and C cells is mucopolysaccharide and lipid. The neurosecretory material of B, B' and D cells is a protein containing cystine SS bonds. It also

contains mucopolysaccharides and lipids. The material of E cells contains a weak protein cystine SS group, β metachromasia and lipids.

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PANCREATIC AND INTESTINAL AMYLASE LEVEL IN FRUGIVOROUS, CARNIVOROUS AND OMNIVOROUS BIRDS

SHORT COMMUNICATION

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Pancreatic amylase has been demonstrated in *Passer domesticus* by LANGENDORFF [6], in domestic fowl and pigeon by FARNER [3] and HEWITT and SCHELKOPF [5]. FARNER [3] and HEWITT and SCHELKOPF [5] demonstrated the presence of intestinal amylase in ducks. Influence of NaCl and pH on biliary amylase in some vertebrates was examined by BHATTACHARYA and GHOSE [2]. Although the pancreas and the intestine are the main sites of amylase secretion, very limited information is available on pancreatic and intestinal enzymes in birds in relation to their feeding habits. The present studies were undertaken to elucidate this relationship.

Psittacula krameri, SCOPOLI (Fam: Psittaculidae) a frugivore, *Lanius schach* LINNAEUS (Fam: Lanidae) a carnivore, and *Acridotheres tristis* LINNAEUS (Fam: Sturnidae) an omnivore species [1] were selected for this investigation. The birds were kept in the laboratory on their normal diet. Six specimens of each species were used each time for enzyme estimations. The pancreas and the intestine were removed immediately after dissection and washed thoroughly with double-glass-distilled water and dried with Whatman No. 1 filter paper. The tissue was homogenized in 5.0 ml of glass-distilled ice-cold water and the homogenate was centrifuged for 15 min at 3 000 rev/min. The supernatant was collected in a test tube and kept at 0 °C. The estimation of α-amylase was done by the method of SMITH and ROE [8]; 1.2% solution of soluble starch (Merck's, Germany) was used as substrate in 0.2 mol/l barbitone buffer. The optimum pH was determined in the same buffer, in a pH range from 6.4 to 8.8. The extinction was recorded in a Bausch and Lomb spectronic-20 at 620 nm. One unit of the enzyme was defined as "the amount of enzyme that under the conditions of this procedure, with 60 mg of starch present, will hydrolyze 10 mg of starch in 30 minutes". The protein in the tissue extracts was estimated by the method of LOWRY and co-workers [7], using bovine serum albumin (Sigma, U.S.A.) as standard. The specific activity was expressed in terms of units/mg protein.

Fig. 1 represents the pH activity curve for amylase. The optimum pH for starch breakdown by pancreatic amylase was 7.6, 7.2 and 7.6 in *P. krameri*, *L. schach* and *A. tristis*, respectively.

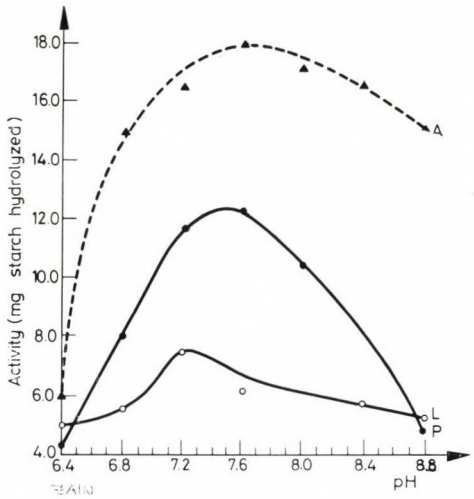

Fig. 1. pH optimum curve for amylase P. krameri (P); L. *schach* (L) and A. *tristis* (A)

Table 1

Amylase activity in pancreatic and intestinal tissue extract of birds

Species of birds	Pancreatic amylase		Intestinal amylase	
	activity/ml tissue extract (amylase units)	specific activity (amylase units/mg protein)	activity/ml tissue extract (amylase units)	specific activity (amylase units/mg protein)
P. krameri	1232	350	14.67	4.58
L. schach	11.25	2.7	0.0576	0.01
A. tristis	5.45	284	3.207	1.41

Table 1 shows the activity of pancreatic and intestinal amylase and their specific activity. Both activities were very high in *P. krameri*, the least in *L. schach* and moderate in *A. tristis*. The optimum pH for the activity of intestinal amylase was at 7.2, in all the three species.

The amylase activity in the intestine of all the three species was very low, suggesting that the pancreas is the main site for amylase secretion. The exceptionally low amylase activity in the intestinal extract from *L. schach* indicates that a true intestinal amylase in this case may be virtually absent and the starch digestion in this species may be the function of the pancreatic amylase alone. In *P. krameri* and *A. tristis* on the other hand, observable

specific activities in the intestinal extracts may be taken as conclusive evidence for the presence of amylase of intestinal origin. However, the results obtained with pancreas tissue extract show much higher amylase levels and suggest that the pancreatic amylase plays the main role in the digestion of starch in the granivore birds, too. These observations are similar to those of FARNER [3] and HEWITT and SCHELKOPF [5] in domestic fowl. These authors stated that an intestinal amylase, if actually exists, has a little role in the digestion of starch. The high pancreatic amylase activity in case of *P. krameri* and *A. tristis* observed during the present study agrees with the feeding habits of these birds very well.

The specific activity values are less conclusive since the enzyme preparation used by us was not pure. Still, the remarkably high specific activity values in case of pancreatic extracts from *P. krameri* and *A. tristis*, which are granivore birds, suggest either a highly active nature of pancreatic amylase, or relatively higher proportions of the enzyme in the pancreatic secretion of these birds.

Further studies with pure enzyme preparations are in progress to explain these observations in greater detail.

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OESTRADIOL UPTAKE BY INTACT AND SYMPATHECTOMIZED GENITAL TRACT OF THE FEMALE RABBIT*

SHORT COMMUNICATION

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Bilateral electrolytic lesions in the anterior hypothalamus of the rabbit induced enlargement of the endometrial glands to an extent similar to that seen in human glandular cystic endometrial hyperplasia [4]. The presence of cystically dilated endometrial glands in the uterine horns, as well as histological signs of hypersecretory activity in the abdominal part of the oviducts indicated an increased oestrogen action on the female genital tract in the anterior hypothalamus-lesioned rabbits. However, the histological signs of the oestrogen-induced, increased hypersecretory activity were completely absent in the sympathectomized oviduct of the anterior hypothalamus-lesioned rabbits. Also, the oestrogen-induced uterine hypertrophy appeared only to a minor degree on the denervated side compared to the uterine horn having normal sympathetic innervation. It seemed very likely that the tissues of the female genital tract without sympathetic nerve supply were less sensitive to hormonal actions than the tissues with normal innervation [4]. Similar observations have been reported by ÁRVAY and NAGY [1]. These experimental findings raised the question whether catecholamine input from sympathetic nerve endings was necessary for the maintenance of normal synthesis of oestrogen-binding proteins in the tissues of the female genital tract. If so, the reduction of norepinephrine content and the probable consecutive decrease in oestrogen binding sites after sympathectomy could account for the reduced oestrogen-sensitivity in the denervated female genital tract of the anterior hypothalamus-lesioned rabbits. This assumption seemed to be supported by CARDINALI and co-workers [2], who showed that oestradiol was avidly taken up *in vivo* and *in vitro* by the innervated pineal gland, while pineal denervation by superior cervical ganglionectomy caused pineal oestradiol uptake to decrease significantly.

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We investigated the possibility mentioned above by measuring the oestradiol uptake by denervated and non-denervated uterine horns of rabbits.

On six female rabbits of 2.5 kg body weight, lumbosacral sympathectomy was performed by bilateral removal of the sympathetic chain from L_3 to the S_2 levels and by the removal of the inferior mesenteric ganglion, together with the hypogastric nerves. In addition, by transecting the vagina below the uterus any adrenergic nerves ascending in, or along, the vaginal muscle coat from the vaginal adrenergic ganglia [5] were severed. Five intact rabbits of the same body weight as the sympathectomized ones served as controls. Experimental and control animals were spayed 14 days after the operation of the experimental animals, and received 1 μg/kg body weight oestradiol propionate (Akrofollin, Chinoin, Budapest) daily for the consecutive five days. Experimental and control rabbits were killed by a blow on the neck nineteen days after sympathectomy. Three pieces of tissue were excised from the middle part of each uterine horn and divided into two parts, one for measuring oestradiol uptake, the other for determining norepinephrine content.

For measuring oestradiol uptake, pieces of uterine tissues were collected in ice-cold Krebs-Ringer solution and labelled *in vitro* by incubating at 37 °C for 1 h in 4 ml Krebs-Ringer saline II (3) containing 5.9 nmol/l (2—4—6—7—^{3}H)-oestradiol (The Radiochemical Centre, Amersham, England), spec. act.: 85 Ci/mmol/l. The tissues were washed three times with 4 ml cold, unlabelled Krebs-Ringer saline. Each wash was for 3 min. Then, the tissues were homogenized in 5 ml 0.01 mol/l-tris, 1 mmol/l EDTA, 1.5 mmol/l mercapto-ethanol, pH 7.4, with the aid of an ULTRA-TURAX homogenizer and centrifuged at 1 000 *g* for 15 min. The 1 000 *g* pellet will be called the nuclear fraction pellet although it was an impure fraction. Specifically bound ^{3}H-oestradiol was extracted from the nuclear pellet with 0.3 mol/l KCl for 15 min. Specifically bound ^{3}H-oestradiol was separeted from non-specific material in the cytoplasmic fraction by dextran-coated charcoal suspension. Tritium was measured in a Tritium Scintillation Counter (Beckman L. S.—100) with 2 ml dioxan and 8 ml scintillation fluid containing 4 g of PPO and 0.04 g dimethyl-POPOP in 1 000 ml toluene.

To determine norepinephrine content, pieces of uterine tissues were homogenized in 2.5 ml ice-cold acidified butanol. The homogenizer tubes were washed out with the same quantity of the acidified butanol. The homogenates were centrifuged at 2 000 *g* for 15 min, and the supernatants were added to the mixtures of 0.2 ml 0.01 N-HCl and 5 ml heptane. After shaking for 20 sec, the mixture was centrifuged at 2 000 *g* for 10 min. The upper phase was aspirated away, and 0.2 ml of the water phase was transferred to test tubes and completed to 0.5 ml with 0.5 mol/l phosphate buffer, pH 6.5. Then, the following reagents were added in order: (1) 0.05 ml of 0.9 mol/l EDTA, pH 6.5; (2) 0.05 ml of 0.1 N-iodine; in 3 min, (3) 0.1 ml of fresh alkaline sulphite solution.

After standing for 2 min at room temperature, each tube received 0.1 ml of 6 N-acetic acid, and was kept in water bath at 100°C for 2 min. Then, the tubes were quickly cooled, and received 0.5 ml of distilled water. The fluorescence was measured by a Locarte MK 4 spectrophotofluorimeter.

Table 1

Norepinephrine content (μg/g wet tissue) of, and uptake of ^{3}H-oestradiol in vitro (expressed as d. p. m/mg wet tissue) by, intact and sympathectomized rabbit uterus

	No. of rabbits	Norepinephrine[a]	(2 – 4 – 6 – 7 – ^{3}H)-oestradiol[a]	
			specifically bound by *cytoplasmic* binding sites[b]	specifically bound by *nuclear* binding sites[c]
Intact uterine horns	5	0.74±0.05	133±15	540± 6
		0.86±0.08	109± 8	460±19
		0.44±0.01	108±12	390± 6
		0.67±0.03	87± 6	530± 4
		0.53±0.04	96± 7	510± 5
		mean: 0.65±0.075[d]	mean: 107± 7.75[e]	mean: 486±27.68[e]
Sympathecto-mized uterine horns	6	0.32±0.02	175±12	507±13
		0.44±0.05	201±14	423± 7
		0.37±0.03	124±18	493± 5
		0.54±0.02	113±13	507± 8
		0.42±0.04	111± 7	585± 6
		0.63±0.07	116± 7	565±19
		mean: 0.45±0.047[d]	mean: 140±15.65[e]	mean: 513±23.42[e]

[a]Results expressed as mean ± S.E.M. of 6 tissue pieces taken from the same uterus.

[b]Separated by dextran-coated charcoal suspension from 1000 *g* supernatant.

[c]0.3 M-KCl extract of 1000 *g* nuclear fraction pellet.

[d]$P < 0.05$

[e]N.S.

The Student's *t* test was used for statistical analysis.

As shown in Table 1, the norepinephrine content of the sympathectomized uterine horns was significantly ($P<0.05$) reduced compared to that of the intact uterine horns of the control rabbits. There was, however, no significant difference between the contents of ^{3}H-oestradiol bound specifically either by cytoplasmic or by nuclear binding sites of the intact and sympathectomized uterine horns. This finding does not support the hypothesis that the reduction of norepinephrine content subsequent to sympathectomy could induce a decrease in the oestrogen-binding sites of the uterus; consequently, a decrease in oestrogen-binding sites cannot account for the reduced oestrogen-sensitivity observed in the denervated female genital tract of anterior-hypothalamus-lesioned rabbits.

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HETEROGENEITY IN THE FIBRES OF THE BODY WALL MUSCLES OF THE EARTHWORM

SHORT COMMUNICATION

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The body wall in various species of earthworm has two components of somatic muscle layers, an outer thinner circular muscle layer, which is only 1/6th to 1/8th the thickness of the inner longitudinal muscle layer [1, 4, 5]. The ultrastructural features of the muscle fibres of these layers have been studied in great detail [5, 6, 7] and, based on the nature of the arrangement of myofilaments, these have been classified as obliquely striated fibres [11]. Physiological studies, though difficult to make, on a material like this, wherein the muscle sheets are exceedingly small and difficult to separate from each other, have also been attempted. The earliest work reported is that of BUDDINGTON [2]. This author recorded two types of contractions after stimulating body wall strips of earthworms, viz., a fast spike-like contraction and a subsequent, slower wave, both showing summation with repetitive stimulation. TASHIRO [9] and TASHIRO and YAMAMOTO [10] also tried to record the mechanical responses from the individual layers. They reported that in the longitudinal muscle layer, field stimulation elicited two distinct waves of tension development: phasic and tonic contraction. They further reported that in the circular muscle layer these two components were not distinguishable and they wondered whether this was due to the fact that the tension obtained from the circular strip resulted not only from the contraction of the circular muscle layer alone, but also partly from the longitudinal muscle layer. DREWS and PAX [3], on the other hand, after stimulating longitudinal and circular muscle layers with a prolonged repetitive stimulation, obtained a response which consisted of two distinct phases of tension development, an initial rapidly developing peak followed by a slow decline to about 1/2 peak tension, the latter forming the second phase. Although, only one morphological type of muscle fibre has been recognized in the body wall musculature by earlier workers [5, 6, 7] the physiological behaviour of the two muscle layers suggested the possibility of there being two separate zones or sublayers within each of the layers, the two zones being composed of cytologically, cytochemically and physiologically differing units. The present work was therefore undertaken to elucidate the validity of this assumption.

Fresh frozen cryostat sections passing through different regions of the body such as buccal cavity, pharynx, gizzard, stomach, intestine and the posterior segments of the earthworm *Megascolex* spp. were taken and treated histochemically for demonstrating succinic dehydrogenase [8].

On examining body wall muscles from the various regions of the body, it became obvious that both the circular and the longitudinal muscle layers were in fact composed of two distinct zones or sublayers. In each muscle layer, the peripheral zone stood out prominently because of a high deposition of for-

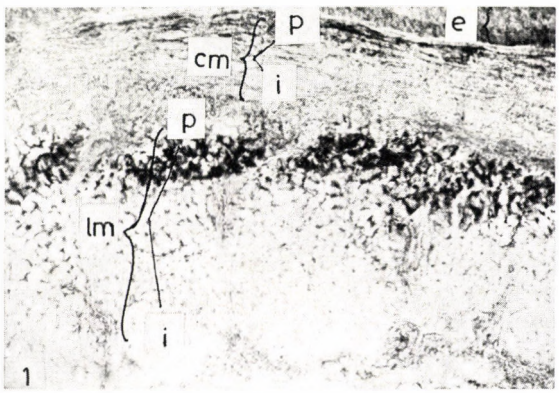

Fig. 1. Photomicrograph of T. S. of body wall of earthworm showing the localization of succinic dehydrogenase. The three layers of the body wall are outer single layered epidermis (e) the middle thinner circular muscle layer (c. m.) and the inner thicker longitudinal muscle layer (l. m.). The formazan deposition is more abundant in the outer or peripheral zone (p), and scanty in the inner zone (i) of each muscle layer. × 250

mazan granules in the component muscle fibres in contrast to the inner zone, which, due to the scanty deposition of formazan, appeared very pale (Fig. 1). This distinction was more striking in the inner layer than in the outer one.

The enzyme succinic dehydrogenase is a mitochondrial enzyme [8]. It is known from the studies of MILL and KNAPP [7] that the obliquely striated fibres of the earthworm have their mitochondria placed near the peripheral portion of the fibre, located in the sarcoplasm immediately below the sarcolemma. As regards the heavy deposition of formazan granules seen in fibres of the peripheral zone of each layer, it is not clear whether the deposition denotes the presence of exceedingly large mitochondria or a cluster of several smaller mitochondria. Whatever be the size of this organelle, the fact which emerges from this histochemical study is that superimposition of the phasic contraction on the tonic contractions maintained at a steady level by repetitive stimulation as recorded with the longitudinal muscle layer [9, 10] could be really due to the combined effect of the activities of two morphophysiologically dissimilar zones of this muscle layer. The fibres of the peripheral zone, which are abundantly supplied with mitochondria and which would therefore have a greater

oxidative capacity, should be metabolically and physiologically different from the fibres of the inner zone. This is supported by the work of TASHIRO [9] and TASHIRO and YAMAMOTO [10], who observed responses to varied set of conditions; there would occur enhancement of the phasic and abolition of the tonic contraction in sodium-free solution and disappearance of the tonic contraction at 30 °C but the phasic contraction remaining unaffected at this temperature. A further detailed electrophysiological study is, therefore, necessary to obtain dissociation of the two components of tension, seen due to superimposition of phasic and tonic contractions of the two sublayers. Here it may be added that, since the circular muscle layer shows a similar regionalization it should also yield a similar pattern when tested electrophysiologically.

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RECENSIONES

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 the next 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 three chapters 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 for (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 author 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)

Nitrogen fixation by free-living microorganisms
Ed. W. D. P. STEWART. *International Biological Programme 6.*
Cambridge University Press, London (1975), pp. 471, £22.

Symbiotic nitrogen fixation in plants E
Ed. P. S. NUTMAN. *International Biological Programme 7.*
Cambridge University Press, London (1976), pp. 584, £22.

The International Biological Programme as "The Biological Basis of Productivity and Human Welfare" was conducted between 1964—1974. Of its seven sections four were concerned with the study of biological productivity — among them — the nitrogen fixation. The volumes (6 and 7) include the material of the Nitrogen Fixation and the Biosphere International Synthesis Meeting of PP — N Section of IBP held in Edinburgh in 1973.

The 6th volume dealing with (1) nitrogen fixation by free-living bacteria, (2) nitrogen fixation by free-living blue-green algae, (3) the acetylene reduction technique, (4) the biochemistry of nitrogen fixation contains 29 lectures by 58 representatives of 17 countries of 5 continents.

As regards the 7th volume, there are interdisciplinary discussions of symbiotic nitrogen fixation which are divided into the following parts: (1) General aspects and taxonomy, (2) Quality of legume inoculations, (3) Field experiments of nitrogen fixation by nodulated legumes, (4) Legume nitrogen fixation and the environment, (5) Nitrogen fixing symbiosis in non-leguminous plants. 41 lectures are presented by 62 researchers from 22 countries of 5 continents.

Of course, the two volumes cannot contain all the results of the ten years' work of the national and international IBP, but it provides a good survey of the problems of both fields of nitrogen fixation and starting points for the future work. These achievements are important from all ecological and agronomic aspects of nitrogen fixation and for the process itself.

For this reason, the books are useful not only for biologists, microbiologists, geneticists, chemists, biochemists and agronomists but other specialists could find the wide-ranging fields of nitrogen fixation of interest, as well.

M. KECSKÉS (Budapest)

RUMSEY, F. and DUERR, W. A.: **Social sciences in forestry: a book of readings**
W. B. Saunders Co., Philadelphia—London—Toronto (1975), pp. 408

This book is edited for those interested in forestry, especially in forest economy and other social sciences applied and needed in forest management. It may serve good for teachers of forestry economics and management or even students and forest managers as well. The book might serve them in two ways; as a set of topics for graduate or senior discussion courses in forest economy, or as a supplementary literature source of forest economy and social sciences. The book consists of five parts. Each is comprised of articles by renown authors on social science applications to successive aspects or subdivisions of forestry. Within each of the parts the story proceeds from the more general subjects to the more specific. First emphasis is given to economic, mostly because this is the field with which the authors and the would-be readers are the most familiar. Before an article there is a short biography of author(s), an editor's summary and only for educational purposes a short list of questions to consider when reading. At the end of the articles detailed references help reader to find more information about the topic. Part I, on economics and other social sciences of forestry at large, is organized so as to bring out both the subject's historical development and its concern for a range of interdisciplinary combinations. The selected articles, among others, deal with cultural anthropology, social ecology, psychology, education, sociology, political and management sciences, etc. Part II comprises articles on the main classes of inputs into forestry. These involve studies on forest land-use, capital and investment, labour and productivity. Here is a strikingly informative study by R. Keninston, who reviews the literature and advocates more application of sociology and psychology in ownership studies. Part III concerns with forest resource management. It opens with three items on multiple-use and general land-management considerations. These are followed by more specific articles on forest recreation and one on upstream watershed management. The remaining items deal mostly with timber: goal setting, forest regulation, and alternative silvicultural practices. Part IV touches upon different aspects of wood-using industry: of lumber industry, of plywood and of pulp and paper. Part V concerns, in a sence, the market: quantities, prices, trade of primary timber, marketing and trends in the lumber market. The book then ends two articles on trade in logs and in lumber futures.

L. DALA (Budapest)

GUENTHER, W. B.: **Chemical equilibrium**
A practical introduction for the physical and life sciences
Plenum Press, New York—London (1975), pp. 246 + 2, figs 88, $ 23.40.

This textbook offers a mathematical treatment of aqueous chemical equilibria.

The book is divided into 12 chapters covering the monoprotic and polyprotic acid-base equilibria and titration curves, metal ion ligend systems, solubility equilibria and redox equilibria. A special emphasis is laid upon the clear formulation of the basic ideas which are further elucidated by a rich and adequately chosen collection of examples. These latter are compiled from different fields including geochemistry and biochemistry among others. The chief endeavour of the book is to deal with real systems and to avoid as possible the rather complicated ideas of the theory of ionic solutions of moderate ionic strength.

For this purpose the appendix provides a table for activity coefficients after KIELLAND and DAVIES, which are applicable up to 0.2 M.

A further outstanding property of the book is the abundant use of graphs, graphical solution methods, different kinds of plots including some new methods which contribute to the clarity of the volume. Gran plots are critically described firstly in the literature.

The aim to adhere closely to standard IUPAC symbolism is recognizable everywhere. The complexities are introduced gradually, the limitations of the calculational procedures are clearly set. In the appendix a limited number of computer programmes in FORTRAN can be found which are capable of calculating some 40 equilibrium systems. The chapter ends are provided by problems to solve.

Let us see several examples from the content: the calciumphosphate system; bone and mineral formation; the citrate system; some physiological pH effects; effects of pH on reaction rates; the Al^{3+}-F system; the Cu(II)-NH_3-EDTA system; amino acid complexes of Cu^{2+} and Co^{2+}; the sea water problem, possible equilibria in blood fluids; $CaCO_3$ solubility in open systems; ampholyte solubility; E-pH diagram for iron, etc.

The book is destined to assist research workers and students in solving pratical problems in chemistry, biochemistry, geochemistry and environmental interdisciplinary fields.

K. Raksányi (Budapest)

Biomembranes: Structure and Function
FEBS Proceedings of the Ninth Meeting, Budapest, 1974, Vol. 35. Ed. Gárdos, G., Szász, I. Academic Press, New York (1975), pp. 317, figs 155.

Contributions of the FEBS proceedings have been edited by two excellent workers of the subject. The contributions are discussed in 4 main part, *viz.* (1) Elements of the membrane and ultrastructure (5 lectures); (2) Mechanism of sugar and amino-acid transport (6 lectures); (3) Ion transport and related membrane phenomena (7 lectures); (4) Mitochondrion bioenergetics (3 lectures).

In all contributions excellent workers of the field report the newest results of physical and chemical structure research. Numerous interesting contributions deal with the actual problems of membrane research and present a cross-section of the work performed in this field. The molecular and dynamic mechanisms of membrane transport are discussed with special attention to its relation to energy, certain general and specific truths are revealed.

Editors express their hope that the volume will promote further work on membrane research. The contributions are accompanied by excellent illustrations and well-arranged tables. The exhibition of the volume is very good and is a credit to the Academic Press.

E. Báthori (Budapest)

Mechanism of action and regulation of enzymes.
FEBS Proceedings of the Ninth Meeting, Budapest 1974. Vol. 32. Ed. Keleti, T. Akadémiai Kiadó, Budapest (1975), pp. 265.

All the 18 contributions made on the mechanism of action and regulation of enzymes are presented. The subject is divided into three groups, viz. (1) Kinetics and structural basis of enzyme action and regulation (8 lectures), (2) Multienzyme systems and the interactions of enzymes (4 lectures), (3) In vivo regulation of enzyme systems (6 lectures).

In the introduction, the editor refers to the historical value of the volume. This means that the book presents the reader with a certain period of historical development of the subject. Results presented provide plentiful information and will serve as a source of information for both experienced and unexperienced research worker. In the last 20 years, enzymologic research has changed basically. Today the problem of regulation is timely: enzyme-kinetic methods refined in a complicated way, thermodynamic approach, physicochemical, chemical methods, sequence-analyses, etc. render the investigation in this field more and more practicable.

The presented contributions report the results achieved with the aid of the most modern methods. The illustrations are excellent, the tables well arranged. The contributions are completed with a detailed list of references.

The beautiful exhibition of the volume praises the work of the Akadémiai Kiadó.

L. Soltész (Budapest)

Proteins of the Contractile Systems
Proceedings of the Ninth FEBS Meeting. Vol. 31. Ed. Biró, E. N. A.
Akadémiai Kiadó, Budapest (1975), pp. 225.

The acquirement of scientific information has ever been an undulating proceeding. This has been so in research work on the contractile apparatus of the muscle. New ideas and new techniques have developed faster in one research field than in others but stimulated the goal and instrumentation of the others. The development of muscle biochemistry preceded the ultrastructural analysis of the contractile system, however, the advent of electron microscopy and X-ray analysis of the myofibrillar components brought on avalanche of new information about structures which had not been studied with biochemical methods. During the last years a great deal of new information accumulated on the chemical components and function of the newly-discovered myofibrillar structures. Thus, the Federation of European Biochemical Societies organized a symposium at the best moment to consolidate the relevant knowledge concerning the structure and function of contractile proteins. The papers presented at the symposium have been collected in a well-illustrated volume. All the contributors are famous scientists in muscle biochemistry. The book consists of three main chapters covering all the major and general aspects of the contractile system. The first chapter is related with the ATPase mechanisms and actinomyosin interactions, the second with the regulatory systems of the I filaments, and the third with enzymatic phosphorylation and assembly of contractile proteins. Most of the authors used red and white skeletal muscle, and/or heart muscle, and/or preparation but some dealt with synthetic filaments, contractile proteins of platelet, amoeba and slime mold. This volume deserves the interest of biophysicists, physiologists, morphologists and cell biologists as well.

Sz. Virágh (Budapest)

Charles Darwin's **Natural selection being the second part of his big species book written from 1856 to 1858.**
Ed. Stauffer, R. C., Cambridge Univ. Press, London (1975), pp. 692, £ 20.

The volume is an extremely valuable documentation, especially for those working in the field of Darwinism. This manuscript, published for the first time in this book, displays Darwin's profoundness, conscientiousness and strictly critical attitude. His later work was based on this. The first two introductive chapters deal with the general principles and aspects followed by Darwin at the time of writing the manuscript and followed by the publisher when editing it. The compilation of these two chapters is documentation-like, in most parts the original wording is presented. Here and in some later parts, the editor has performed some abridgements for the sake of better arrangement but, even in these cases, the original text is used. The middle part presents a sketch of the manuscript "Natural selection". This entirely represents Darwin's wording. The following 9 chapters reflect the main points of Darwin's life-work. This is reflected by the titles: (1) Possibilities of crossing in living beings. (2) Natural variation. (3) Struggle for life. (4) Natural selection. (5) Rules of variation. (6) The difficulties of the theory. (7) Hybridism. (8) Intellectual capacity and instincts of animals. (9) Areal distribution of living beings.

All chapters are concise and clear. The editor has very cleverly arranged into each chapter the circumstances in which it was written, the related correspondence, comments, attitude as regards criticism. Later completions are presented in an "Appendix", in accordance with the original manuscript.

The alphabetical list of literary references is very valuable, this is the credit of the editor. The reader is also guided by the exact references to the page numbers in the "Origin of species" and the "Natural selection". Reading is further promoted by a rich alphabetical subject index.

The appearance of this work is a great event.

B. Faludi (Budapest)

Crop genetic resources for today and tomorrow
IBP 2. Ed. Frankel, O. H., Hawkes, J. G.
Cambridge Univ. Press, London (1975), pp. 491, £13.

In a volume of the IBP programme issued in 1970, the same authors reported on exploration of crop-genetic resources, the methods of their preservation and their prolonged storage, further on they discussed the purposeful application of the collected samples. This

volume reports on more recent work performed and new problems arisen since in this field. Six subjects are discussed.

In the first part, dealing with the genetic variability of crop population, it is analyzed by population genetic methods how the genetic variability of the cultured sample is influenced by the culture system, by isolation, and by the natural and artificial selection and the optimal sample size for storage is discussed.

The second part reports the results of expeditions made for collecting the variable crops and their ancestors and discusses further tasks. The USA and the Soviet Union has endeavoured to collect the genetic resources of their own and foreign countries, other countries, for example, Chile, Bolivia and Peru, make efforts in collecting the genetic resources of one species (potato) for protecting them from extinction. In other countries of South-America cacao varieties have been collected.

The third part demonstrates how the previously and newly collected culture samples are utilized for producing species resistant to certain pathogenic agents. In the Soviet Union drought-resistant species, in the USA and in West Germany species of higher protein and oil content are of interest in this respect.

The fourth part deals with the problems of prolonged storage. Many newly-emerged problems (pollen-storage, storage of the tropical root-tuberose plants) are discussed. Communications on the possibilities of the application of tissue culture methods and related problems are the most interesting in this part.

The fifth part presents informations on the requirements in the documentation of samples.

The last part gives information on the several large centres of genetic resources (rice, maize). The reader can be acquainted in detail with FRANKEL's suggestions concerning a world-wide coordination aimed at solving these problems.

The volume will be of interest to researchers working in this field as well as those working in practice. These later are provided with information on the new genetic resources available.

A. BÁLINT (Gödöllő)

Small mammals: their productivity and population dynamics
International Biological Programme 5. Ed. GOLLEY, F. B., PETRUSIEWICZ, K., RYSZKOWSKI, L. Cambridge University Press, London (1975), £12.

At present the IBP is one of the international scientific collaborations, serving the interests of mankind. (Research scientists of Hungary take part with major share in this work.) In this long-running scientific programme the trade literary information has an extraordinary important role. The latest book of this series has been written by several well-known authors; it deals with the significant questions of ecology.

This volume, published in English, specifies small mammals (rodents and insectivores).

The first part deals with the estimation of population density, dispersity and age determination, analyses the oecological relations of energy-circulation of small mammals as well as the theory of biological production and the method of its calculation.

From a practical point of view, the most outstanding chapters are those analysing the production-biological function of small mammals in temperate-zone forests, fields and ploughed fields, and in arctic and tropical oecosystems.

The final part of the book draws the attention to the public health importance of small mammals and describes in detail the applied research methods concerning the rodent control in urbanized regions and ploughed fields and produces-tores.

The exact name list of small mammals as an appendix deserves special attention. The 80 pages of the exact reference list and subject list help the readers to get absorbed by this topic. We may recommend this book to every mammalologist and biologist interested in the population biology and demoecology, especially in countries in which little attention has been given to the research of the oecology and population-biological laws of small mammals.

GY. FÁBIÁN (Gödöllő)

SCHMIDT-NIELSEN, K.: **Animal physiology**
Adaptation and environment
Cambridge University Press, London (1975), pp. 699, £ 7.25

This volume represents a special approach to comparative animal physiology. While in textbooks of usual construction physiology is treated on the basis of different functions (e.g. digestion, respiration, etc.), in this work the subjects are arranged according to environmental factors (oxygen, food, etc.) which the animals must find or avoid, obtain or escape, to live. This kind of approach, which helps the reader to understand how living organisms function, must necessarily be selective. Therefore, some topics (e.g. physiology of reproduction) have been omitted or treated very briefly.

The book consists of five parts. In the first part functions connected with oxygen, viz. respiration in water and air, blood and circulation are analysed. The second part is devoted to food and energy. This part presents information on feeding, digestion, nutrition and energy metabolism. The following chapters (Part III) deal with the effects of temperature on living organisms and with their temperature regulation. Part IV includes chapters on water and osmotic regulation and excretion. The last part of the volume covers the physiology of movement, information and integration.

At the end of each of the parts, a selected list of references helps the reader in obtaining further information on the subject. The volume is well written and excellently illustrated. The author has compiled the most important new information and ideas in a complete book. The book may be recommended for students at universities and for all those wishing to have new insight into comparative physiology.

J. KOVÁCS (Budapest)

Control mechanisms in development. Activation, differentiation and modulation in biological systems.
Ed. MEINTZ, R. M., DAVIES, E. Plenum Press, New York—London (1975), pp. 226, $23.40.

The book is the 62nd volume of the series Advances in Experimental Medicine and Biology, and contains the papers presented in a symposium held at the University of Nebraska—Lincoln in October 14—16, 1974.

Most of the papers deal with protein synthesis as an expression of the genetic machinery. According to their specific subject matter within this topic, the papers are grouped into four main sections. The first section, Activation in Biological Systems, comprises four papers on the genetic regulation of protein synthesis in embryonic and hybridized cells. When RNA synthesis is suppressed, protein synthesis is not impaired in early developing embryos, suggesting the utilization of genetic information that has been transcribed during oogenesis (A. MARCUS et al.). From a series of experiments of periodic enzyme synthesis in yeast cultures conclusions are drawn about the chromosomal location of genes for rRNA, and about the regulation of rRNA synthesis (C. SAUNDERS et al.). In experiments with nuclear transplantations in enucleated *Xenopus* eggs the accumulation of proteins in nuclei has been shown to be a specific process (J. B. GURDON). An elegant technique is shown for somatic hybridization by protoplast fusion and transformation to study the exchange of genetic information in plant cells (O. L. GAMBORG).

Papers in the second section, Differentiation in Biological Systems, give account of experiments studying the effect of hormones on enzyme synthesis in germinating barley seeds (J. E. VARNER), and on ovalbumin synthesis in chicken oviducts (R. T. SCHIMKE et al.). The latter study involves highly sophisticated techniques, like specific immunoprecipitation of proteins, and DNA—RNA and DNA—DNA hybridization; and is concluded with showing the primary effect of oestrogen on ovalbumin synthesis is to regulate the content of ovalbumin mRNA. The third paper in this section discussed the internally coordinated, programmed expression of specific genes during differentiation using as a model the formation of proteinaceous egg-shell of insects (F. C. KAFATOS).

The third section deals with Modulation in Biological Systems at the level of the genetic and cellular machinery. Through the metabolism and function of the juvenile hormone in insects, the first paper investigates the question of how the animals develop metabolic defenses even towards their own hormones (Y. AKAMATSU et al.). The challenging problem of how the subcellular fate of proteins is determined is investigated in the next paper, which contains a wealth of data on the protein synthesizing apparatus in the rough endoplasmic reticulum (D. D. SABATINI et al.). A fascinating "fluid mosaic model" for the organization of lipids and proteins in membranes is shown to explain a hypothesis about the reversible

aggregation of intramembrane protein particles giving rise of differential surface properties (S. J. SINGER). The fourth paper discusses the survival value of the genetic programme from the point of view of its functional design at the molecular level (D. E. ATKINSON).

The last section is devoted to Philosophical Implications. It contains a paper which gives a brief review of the great results of molecular and cellular biology in relation to human genetics, and shows the prospects of this exceedingly powerful field of research (TH. T. PUCK).

The book is of great value for students of general biology, molecular and cellular biology, genetics and related fields.

The remarkable fast and good quality publication of the subject matter of a very interesting symposium speaks for the good work of the Plenum Press.

G. SZÉKELY (Debrecen)

SENGEL, PH.: **Morphogenesis of skin.**
Developmental and cell biology 3. Cambridge University Press, London (1975), pp. 277, £14.

The book is an account of our present knowledge of the embryonic and postnatal development of skin. It describes the main steps of the construction of the integument in aminotes (reptiles, birds and mammals including man). The tissues and cellular interactions that are required for the building of the skin and its appendages (hair, feathers, scales) are also discussed.

Skin is the largest organ of the body and as such, its ontogenesis is subjected to the rules controlling the development of all organs. Skin is viewed as an experimental system so that a general understanding of cellular and subcellular mechanisms involved in morphogenesis is arrived at. The morphogenetic principles governing the development of the skin are applicable to the organs and organisms generally by establishing relationship between processes that are involved in that development.

There are at least three reasons why the skin should serve as a model for organogenesis. First, the skin is composed of two distinct tissues of different developmental origin. These two tissues are spatially separated from each another unlike organs in which epithelial buds protrude into a mesenchymal stroma. This particular anatomical feature is, by itself, an almost schematic illustration of dermo-epidermal tissue interdependence. Second, throughout adult life, at least part of the skin is continuously renewing. In this sense, the epidermal cells keep their embryonic character indefinitely, making the skin the model of an ever-developing embryonic system. Third, skin is the only organ that is immediately visible to the external observer. Any disease or deviation from normality are immediately detectable from the outside. Thus is the reason why skin diseases are so manyfold.

The account is divided into three parts preceded by a general introduction into skin development. Cell proliferation, cell differentiation and morphogenesis are considered sequentially. Pigmentation and differentiation of cutaneous glands have been omitted. It is done in the interest of homogeneity and more detailed account of the experimental data now available on the mechanism of production of hard horny appendages. This shortcoming, however, gives the feeling of a truncated image of skin as a morphogenetic system.

The author of the book is professor of zoology at the University of Medicine and Natural Sciences of Grenoble, having studied skin development for more than twenty years. The outlines, guidelines and essential ideas expressed in this book are the result of the continuous experience of the author and his collaborators with the topic raised. The book will interest developmental biologists and dermatologists.

K. KIRÁLY (Budapest)

Immunological tolerance
British Medical Bulletin, Vol. 32 (2), May, 1976.

Like most issues of the British Medical Bulletin, this collection of 14 contributions essentially covers the current knowledge of its subject. The topics have been successfully selected, most of which include most recent data and speculations put forward by the contributors, among them many of the world's leading immunobiologists.

The first article, a basic one, is by L. BRENT, C. G. BROOKS, P. B. MEDAWAR and E. SIMPSON, "Transplantation tolerance". The authors start with a survey of old and new theories, establishing that "there has been a tendency in recent publications to link the

'central failure' interpretation to the hypothesis that tolerance is the outcome of an elimination of a clone of specifically immunocompetent cells. Perhaps the important distinction is between tolerance as essential non-reactivity and tolerance as the consequence of an active suppression of the immune response". The authors conclude that "any complete theory of the immunological response must account for both immunity and tolerance. Although no interpretation of tolerance as an active suppression of the immune response is fully satisfactory, the direction of modern thought is unmistakable: tolerance is an extreme or limiting form of some process of which the normal function is to moderate immune reactivity and it is under this general rubric that we may classify interpretations that appeal to anti-idiotype antibodies and suppressor T cells".

The second article, by R. Y. CALNE, "Mechanisms in the acceptance of organ grafts" deals with the role of the major histocompatibility complex (MHC) and the variation in the behaviour of different tissues transplanted in the same species. The studies the author reported here have focussed mainly on the differences between skin and kidney, kidney and liver graft survival, respectively, across the MHC and non-MHC transplanatation antigen barrier with the conclusion that "it would seem unlikely that immunological tolerance can be applied to immunologically mature animals."

In the third article, by J. R. BATCHELOR and K. I. WELSH, "Mechanisms of enhancement of kidney allograft survival. A form of operational tolerance", the authors propose, as an hypothesis, that a defective function of antigen-specific T_1 lymphocytes is responsible for the inductive stage of enhancement and a subsequent depletion of a T_1 subpopulation for the maintenance of the steady stage with the major role of an auto-anti-idiotype immunity as a regulatory function to limit both humoral antibody formation and cellular immunity.

The next article by R. W. BALDWIN and R. A. ROBINS, "Factors interfering with immunological rejection of tumours" presents data concerning humoral factors modifying cell-mediated immunity in the tumour-bearing host, including blocking reactions at the tumour cell surface as well as inhibition of lymphoid cell reactivity. In addition, important recent findings about the role of humoral factors *in vivo* in the modification of tumour immune-rejection responses are reviewed.

There are seven articles which share as focus the role of the T cell—B cell system in immunological tolerance, These are, "Self-tolerance and autoimmunity" by A. C. ALLISON and A. M. DENMAN; "Break-down of tolerance", by S. H. LEECH and N. A. MITCHISON; "Generation and selection of specific reacting cells by antigen", by J. B. HAY and B. MORRIS; "Mechanisms of B-cell tolerance" by G. G. B. KLAUS, J. G. HOWARD and M. FELDMAN; "Suppressor cells in humoral immunity and tolerance" by R. B. TAYLOR and A. BASTEN; "Suppressor T cells in cell-mediated immunity" by G. L. ASHERSON and M. ZEMBALA; and finally, "Control mechanisms in delayed-type hypersensitivity" by J. L. TURK, L. POLAK and D. PARKER.

The work of ALLISON and DENMAN on self-tolerance and autoimmunity is a basic one, like that of BRENT et al. The authors first outline the discovery, made on many experimental and clinical material, of autoimmune responses mediated by autoantibodies and directed against body tissues, resulting in more or less severe self-destructions. Then, they tell the story of their theory of the Selective Induction of Unresponsiveness in T lymphocytes for the explanation of autoimmunity in general. This theory is supported by a set of independent experimental and clinical observations which have also confirmed the authors' second "prediction that T-lymphocyte-mediated suppressor functions control B-lymphocyte responses" in a variety of systems, including the induction of autoimmunity.

LEECH and MITCHISON present new data regarding the replacement kinetics of T and B cells: "high-zone tolerance", when B cells have become reactive but T cells remain tolerant, and the "low-zone tolerance" with events happening in the opposite direction.

Fascinating bits of information reach the reader from further reports such as that polymeric "T-independent" antigens can block resynthesis of surface receptors by B cells, (KLAUS et al., pp. 141—146); that some forms of tolerance are due to physiological interactions of helper and suppressor T cells during humoral (TAYLOR and BASTEN, pp. 152—157); and, that B cells, besides T cells, can, in certain circumstances, have suppressor activity in the regulation of the immune response (ASHERSON and ZEMBALA, pp. 158—164.). Furthermore, data in the article of TURK et al. (pp. 165—170), clearly indicate that, in contact sensitivity, B cells inhibit the generation of reactive T cells. This work also deals, in detail, with the influence of cyclophosphamide on delayed hypersensitivity and tolerance.

"*Tolerance inducation as a model for cell differentiation*" is the title of an article written by D. W. DRESSER. The reader can find here a model to suggest an order of signals in which the switching on or off of a cell by antigen can be regarded as an example of cell differentiation.

Properties of suppressor antibodies: the target cell for suppression: the synthetic burst induced with antibody against immunoglobulin determinants are the subjects of the article entitled "Suppression of immunoglobulin formation with antibody against, immunoglobulin", by B. CINADER and S. DUBISKI. This is an excellent summary of what is known of the key-role of antibodies against the allotypes and idiotypes of immunoglobulins in the mechanisms of tolerance.

Finally, in the last article, "Erasion of the immune response by parasites", B. M. OGILVIE and R. J. M. EILSON, discuss situations in which the host's immune response seems to be defective and describe features of parasites that enable them to survive immunological rejection by the host.

The issue ends with "Reflections" three pages in length, by J. H. HUMPHREY whose name hall-marked the whole issue not only by this critical, but also by the two-page introduction.

All things considered, this volume contains much information about the complex problem in question.

I. TÖRŐ, jr. (Budapest)

Limnology of shallow waters
Symposia Biologica Hungarica, Vol. 15. Ed. SALÁNKI, J., PONYI, J. E.
Akadémiai Kiadó, Budapest (1975), pp. 304.

One of the major problems of many areas of our world is the production of appropriate quantity and quality of food. On the basis of international cooperation the International Biological Program (IBP) has made efforts to answer questions in the field of organic substance production, i.e., the basic procedures of photosynthesis, or the anthropocentric transformation of these in order to increase the required yields.

Hence, the amount of research work elaborated all over the world on ecosystems and production biology is enormous.

Frequently, we just do not consider the future consequences of human activities aiming at increasing crop yields. The increased urbanization, the population of earth, air and water, the problems of natural and artificial environment of man tend to become matter of life and death. The appalling changes of environment, often its destruction and the pollution of waters threaten in the first place, the organisms living in lakes and rivers. Shallow waters are particularly threatened. Therefore, the enormous significance of the Symposium on the limnology of shallow waters, organized on the side of the largest shallow lake in Europe: the Lake Balaton, by the Tihany Institute of Biology of the Hungarian Academy of Sciences. This Institute deserved this honourable task as since its foundation in the twenties about 500 papers have been published by the workers of the Department of Hydrobiology which have involved the living beings, problems of the ecology production and pollution of the Balaton.

Twenty-nine national and international contributions were presented at the Symposium. As a result of excellent organization a comprehensive picture was formed of the primary bacterial and secondary production of shallow waters and of the protection of the biosphera. Accordingly, this beautiful book is divided into 3 main chapters, viz. (1) Primary production (11 papers by 13 authors); (2) Bacterial production and decomposition (7 papers by 9 authors); (3) Secondary production (11 papers by 15 authors). Observations in Hungarian, European and American waters are discussed.

The papers are followed by a valuable list of references.

The exhibition of the volume is excellent, synchronous to the up-to-date information presented. Many very demonstrative illustrations, photos, graphs and tables are presented.

The volume is warmly recommended to research workers of production, biology, water protection and environmental biology.

T. HORTOBÁGYI (Gödöllő)

General ecology, biocenology, hydrobiology. Vol. 1
Ed. KUZNETSOVA, Z. I. G. K. HALL and Co., Boston (1975), pp. 109, $ 21 + 10%.

One of the central problems of research in modern hydrobiology is biological productivity of waters. Complex studies are being performed all over the world on this subject, involving waters and their environments. The aim of these investigations is to explore the procedures of organic substance production, and, in the future to supply mankind with

quantitatively and qualitatively satisfactory food. These research works are necessitated by the increase of the socio-circulation of water, causing an increasing pollution and eutrophization of water.

The investigation of ecological systems in water are elaborated at a very high standard in the Soviet Union. For workers on hydrobiology Soviet literature is indispensable, therefore the publications of Soviet contributions on problems related to ecology, biocenology and hydrobiology in English in the series of the ITOGI is welcome. This volume appeared in 1973 in the Soviet Union, its English translation was published in 1974. According to the plan of the publishers the further volumes should appear simultaneously in English in Boston, to the Russian original. These ITOGI volumes serve the survey of research effectuated all over the world.

The first volume of the series contains three studies, viz., 1. Yu. I. SOROKIN: The Primary Production of the Seas and Oceans, summarizes results of work performed between 1965 and 1972. Problems related to methods, ecology, photosynthesis, cenosis and effectivity in the various parts of seas and oceans are discussed. The 20-page study is very concise. The profoundness of this work is supported by 215 literary references.

The second literary review (pp. 24), also by SOROKIN, involves bacterial production in continental and marine basins. Bacterial production results significant organic substance. Three papers of J. OLÁH on this subject are referred to among the 308 references.

The third, 23-page, study, written by V. N. GREZE, analyses by modern mathematical methods the secondary production of sea invertebrates. The production of pelagic and benthos invertebrates are discussed among others.

The general estimation of the production of zooplantations and zoobenthos for some areas of the Ocean is also presented. 65 literary references are listed.

The preface refers to the fact that the volume has been prepared for both graduates and students. Their enterprise has been successful. This first volume is a great profit to hydrobiology.

We are looking forward to the next volume.

T. HORTOBÁGYI (Gödöllő)

The pineal gland
Ciba Foundation Symposium. Ed. WOLSTENHOLME, G. E. W., KNIGHT, J. Churchill—Livingstone, Edinburgh—London (1971), pp. 401, £4.25.

The subjects of the sixteen papers are the structure and function of the pineal organ, comparative neuroanatomical investigations, data concerning the synthesis of indolamines in the pineal, reports on physiological effects of pineal extracts and relations of this organ to the brain and endocrine glands.

The following topics are generally discussed: role of serotonin in the pineal, effects of melatonin on serotoninergic systems, behavioural effects of pineal principles, role of the pineal in cold adaptation, control of circadian rhythm, light input and pineal.

The symposium presents information about the pineal on a research period when the organ was considered first of all to be a "gland". Also the title of the book demonstrates this trend which has been slightly modified lately. It is well known that in lower vertebrates the pineal is not a gland; it contains photoreceptor cells and there is a direct nervous pathway between these receptors and the brain. Furthermore, new data about the fine structure of the pineal organ of higher vertebrates have underlined the receptor character of the pinealocytes. Recently typical photoreceptor membranes have been demonstrated in birds and also the mammalian pinealocyte might be regarded structurally as a derivative of the photoreceptor cells of lower vertebrates. Thus even the pineal organ of higher vertebrates does not exclusively seem to be a gland as Kappers claims at this symposium.

This book refers to further exciting problems the solution of which may lead to a better understanding of this interesting organ.

B. VÍGH (Budapest)

BECKER: **Antiviral drugs: Mode of action and chemotherapy of viral infections of man**
Monographs in Virology, Vol. 11. S. Karger, Basel (1976), pp. 130, sFr 75.

The treatment of virus infections has been attempted by searching for an anti-viral substances specifically inhibiting virus multiplication in the infected organism. This volume systematically reviews the natural and synthesized substances that have been studied in

the last 25 years. The molecular-biological condition in which the substance has an inhibiting effect or is able to intervene into the replication cycle of the different viruses is discussed. The modes of action of the various drugs are discussed in detail. A separate chapter is involved with the few compounds which are today assumed to be effective in human therapy Well-arranged summarizing tables and the demonstration of chemical structure of the compounds helps understanding. Chapter XI is worth special mentioning. This chapter discusses the possibilities of the prevention and chemotherapy of human virus infections. The author gives a detailed description of experience obtained with three drugs, viz., doxuridine, cytarabine and ara-A, which are in use at present. In Chapter XII the subject of the book is excellently summarized on 6 pages. Chapter XIII involves 500 recent literary references to which it is worth drawing attention.

I. Szakolczai (Budapest)

Roberts, L. W.: **Cytodifferentiation in plants: Xylogenesis as a model system**
Developmental and cell biology series
Cambridge Univ. Press, London (1976), pp. 160, £8.

This book is not more and not less, than an up-to-date review of the results or cyto-differentiation sequence (CDS) in the explant and plant tissue culture.

The role of phyto-hormones in the cytodifferentiation, more exactly, in the differentiation of xylem elements is given in chronological order. This is followed by a good summarization (after Torrey) of the stages of cytodifferentiation and the possible hormonal or chemical regulation of these stages.

Unfortunately, the basic concept and terminology of cell cycle, together with the interpretation and diagram of the successive periods of the cell cycle, are not given until the 4th chapter.

In accordance with its importance, the regulation of secondary xylogenesis is dealt with in a large chapter. The reader will be acquainted with ultrastructural studies on differentiating xylem elements and, also, with dilemmas arising from them. The book, especially this chapter contains numerous photomicrographs of differentiating tracheary elements; some of the pictures are excellent. The role in the cytodifferentiation of carbohydrate nutrition, some environmental factors and chemical inhibitors is discussed in a critical manner. The more than 600 references are very useful for research workers.

Júlia Szujkó-Lacza (Budapest)

Thomsa, E., Davey, M. R.: **From single cells to plant**
Wykeham Publications Ltd., London (1975), pp. 172, £ 2.50.

This small handbook makes the reader acquainted with the history of culturing plant tissue.

After an interesting and informative historical part, we find very useful and precisely-described tissue culture techniques for growing of plant tissue cultures from different points of view (physiological, biochemical and genetical).

The laboratories, instruments, methods of preparations and results, i.e. the tissue culture originated from different plant parts are discussed and well illustrated.

An "Appendix" contains the prescription for 7 different basal media for culturing of root, callus, cell suspension, anther and isolated leaf protoplasts.

Only the lack of references is uncommon in this book. This handbook will have wide appeal for researchers, teachers, and students in field of plant tissue culture.

Júlia Szujkó-Lacza (Budapest)

Cell Genetics in Higher Plants

Proceedings of a UNDP/UNESCO/ICRO Training Course, July 1976

Edited by D. Dudits, G. L. Farkas and P. Maliga

Cell Genetics is one of the most rapidly developing areas of plant biology. Its theoretical and practical importance is becoming increasingly evident to the molecular biologist as well as to the plant breeder. The first part of the present volume contains the material of a symposium, i.e. articles written by leading scientists in their fields. The second part contains the protocols of the practicals and demonstrations, i.e. describes in detail most of the methods currently used in plant cell genetics.

In English · 251 pages · 58 figures · 12 tables · 17×25 cm · Cloth
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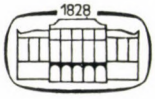

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