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REDIGIT

I. TÖRŐ

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AKADÉMIAI KIADÓ,
BUDAPEST 1967

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TOMUS XVIII

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HISTOCHEMICAL DEMONSTRATION OF BIOGENIC MONOAMINES IN THE CENTRAL NERVOUS SYSTEM OF THE LAMELLIBRANCH MOLLUSC ANODONTA CYGNEA L.

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(HEAD: JÁNOS SALÁNKI)

(Received January 6, 1966)

Synopsis

It was demonstrated by the fluorescence method developed by Falck that in the three ganglia of *Anodonta cygnea* L. like in *A. piscinalis* serotonin is present in the majority of nerve cells. Axons exhibiting fluorescence characteristic of serotonin were not found. After 20-hour pretreatment with iproniazide the golden yellow fluorescence characteristic of serotonin appears also in neuropile in scattered, granulated distribution, but it is localized to the cytoplasm of the glia cells. The intracellular localization of serotonin and its complete absence in the axons contradict the assumption that this agent may act as an interneuronal synaptic mediator. A minority of the nerve cells and the axons of the neuropile contain a primary catecholamine, most probably dopamine. Only partial depletion of catecholamine is effectuated even by large doses of metaraminol. In the course of the restitution process subsequent to reserpine-depletion catecholamine appears firstly in the nerve cells, in nearly every nerve cell, and only later in the axons. During this time the nerve cells do not contain serotonin. It is suggested that in Lamellibranchiates the interneuronal mediation is realized primarily by way of monoaminergic mechanisms in which most probably dopamine is predominant.

Introduction

As literary data have shown, both cholinergic and non-cholinergic mechanisms are involved in the transmission of the nervous impulse in molluscs [12, 13, 15, 21, 22]. Notwithstanding, several data support the assumption that in the central nervous system (CNS) of Lamellibranchiates non-cholinergic mediation is displayed predominantly, if not exclusively [7, 8, 19, 20, 23, 24, 26]. Consequently, the exact localization of biogenic amines in Lamellibranchiates deserves great interest.

Dahl and co-workers [7, 8] succeeded in demonstrating in the cerebral and visceral ganglia of *Anodonta piscinalis* serotonin (5 HT) and a catecholamine by Falck's fluorescent-histochemical method and a considerable amount of dopamine by spectrophotofluorimetry. They suggested on the basis of these findings, in agreement with Welsh and Moorhead [27], that besides catecholamines 5 HT may function as a transmitter. Examining the subcellular localization of 5 HT in the CNS of *Anodonta cygnea* and *Anodonta anatina* by differential centrifugation it was concluded that 5 HT is located intracellularly, in

the endoplasmic reticulum [25]. The fact that the soma of the nerve cell can be considered only as a postsynaptic structure seems to contradict the assumption that 5 HT may function as an interneuronal transmitter. Thus, it was thought reasonable to investigate also in *Anodonta cygnea* the location of the above biogenic monoamines to learn if there are in the ganglia axons containing 5 HT.

Another objective of these studies was to examine the effect produced by metaraminol, a specific noradrenaline-depleting agent, further to analyze the restitution process subsequent to reserpine administration. The restitution process was examined to clarify the first site of catecholamine production.

Material and method

In the experiments three pairs of ganglia: the cerebral, visceral and pedal ones of 15—20 cm specimens of *Anodonta cygnea* L. were used. The dissection of the ganglia took 1—2 minutes. They were frozen in isopentane, cooled by liquid air and subsequently freeze-dried in an apparatus type HVG 1 (Hochvakuum-Gefriertrocknungsanlage) with cooling by CO_2 snow at an end vacuum of 10^{-5} Torr. Freeze-drying of 6—9 ganglia lasted for 20—30 hours on every occasion.

The monoamines present in the freeze-dried material were converted into the respective fluorescent products with dry formaldehyde gas [10, 11]. The paraformaldehyde used in the formaldehyde treatment was stored at a relative humidity of 60 per cent [14] and the formaldehyde treatment lasted 1—3 hours at 80° C. The material was subsequently placed in paraffin for 10 minutes at 60° C. Serial sections of 6—8 μ were made, and spread on warm dry slides. Sections were examined uncovered and covered with paraffin oil or Entellan (Merck) + 10% xylol.

An adapted Zeiss Nfpk microscope served as fluorescence microscope. Light filters BG3, 4, and OG 1 were applied, "Zeiss Grosse Lumineszenzeinrichtung" served as light source. The colour photographs were made with Agfacolor photographic material and the figures presented here were under equal conditions as regards exposure time and development.

Autofluorescence examinations were carried out on (a) freshly-frozen sections of ganglia; (b) freeze-dried, paraffin-embedded sections not exposed to heat or formaldehyde treatment; (c) paraffin-embedded sections freeze-dried and heat-treated but not exposed to formaldehyde; (d) freeze-dried, heat- and formaldehyde-treated, deparaffinized sections reduced with $NaBH_4$, and exposed subsequently again to formaldehyde according to CORRODI and co-workers [4].

To demonstrate the location of fluorescent formations histologically the same sections covered with paraffin oil were deparaffinized, subsequently stained with haemalaun-eosin or paraldehyde-fuchsin and the respective areas were compared.

As monoaminooxidase (MAO) -inhibitor iproniazide was injected into the foot musculature, 100 or 400 mg/kg, in aqueous solution. Its effect was examined 20 hours after administration.

From among the specific noradrenaline-depleting agents metaraminol (aramine, alfa-methyl-beta-hydroxy-m-tyramine) was injected into the foot musculature, 1, 2 and 6 mg/kg body weight. Thereafter the animals were kept separately in vessels containing 3 litre water each, whilst the necessary oxygen supply was secured. Four, 24 and 44 hours later the ganglia were excised and the Falck-reaction was performed.

In order to deplete the monoamines, reserpine was administered, in the same way as metaraminol, 10 mg/kg body weight in the form of Rausedyl (0.25%) injection. According to MIROLLI and WELSH [16] the threshold dosis of reserpine for snails lies between 0.1—19.2 mg/kg. The 10 mg/kg dose used in these investigations was found effective. Falck reaction was performed 4, 24 and 60 hours after the injection of reserpine.

Results

The Falck-reaction gave yellow and green fluorescence in the sections of ganglia. The nerve cells exhibited an intensive yellow fluorescence (Fig. 1).

In UV-light this yellow fluorescence somewhat decreased in intensity and its yellow colour became darker.

Yellowish-fluorescing granules are observable sporadically also in the neuropile. This fluorescence is less intensive, and it is not changed by UV-light. It has proved to be identical with the fluorescent gliosomes of glia cells.

Green fluorescence is found in certain nerve cells. Such cells occur, though very seldom in all three ganglia. Occasionally a "green" process extends from the "green" cells (Fig. 2).

Green fluorescence is also exhibited by the whole mass of fibres in the neuropile (Fig. 3). In longitudinal sections axons are visible with fluorescing varix-like extensions. Moreover, parallel running axons are arranged in fascicles. The "green" processes penetrate also the cellular cortex.

No marked difference in intensity of fluorescence is demonstrable between sections uncovered and those covered either with paraffin oil or Entellan-xylol. Likewise, completely identical picture is obtained when the duration of formaldehyde treatment is changed within 1—3 hours.

Green fluorescence does not develop in ganglia freeze-dried but not exposed to heat and formaldehyde. The nerve cells and the glia cells of the neuropile display yellow fluorescence, which is, however, less intensive than that of ganglia treated with formaldehyde. It is darker yellow and its intensity does not change in UV light. Sections untreated with formaldehyde but exposed to heat do not differ in any respect from the former (Fig. 4). The autofluorescence is localized in granular structures and it is demonstrable also in freshly-frozen sections.

When sections are transfered into isopropanol (abs., 95, 90 and 80 per cent) subsequent to deparaffinization by xylol the formaldehyde-induced fluorescence does not extinguish. If, however, the sections, are kept for 5 minutes in 90 per cent isopropanol containing 0.1 per cent $NaBH_4$, the green fluorescence extinguishes completely and the intensity of the yellow one decreases practically to the level observed in ganglia not treated with formaldehyde. When formaldehyde treatment is repeated after reduction by $NaBH_4$, the original intensity of the specific green and yellow fluorescence is restored with unchanged localization.

Yellow fluorescence is in certain cells intensified by iproniazide. In the neuropile, granules are observable exhibiting yellow fluorescence, which in some places may be as intensive as the fluorescence of the cells. On the other hand, even serial sections failed to demonstrate axon-like yellow-fluorescent longitudinal, varix-like formations in the ganglia. The yellow fluorescent granules correspond to gliosomes (Fig. 5) also after iproniazide treatment.

Iproniazide treatment does not produce noticeable alteration in the intensity of green fluorescence, though in the cerebral ganglion a small increase in the amount of "green" cells was observable.

One or 2 mg/kg metaraminol does not affect the green fluorescence either in the cells or in the axons within 4 or 24 hours. Twenty-four and 44 hours after the administration of 6 mg/kg metaraminol the green fluorescence in the axons was found to be noticeably reduced but it did not disappear (Fig. 6). The animals remained capable of tonic contraction of the adductors.

Injection of 10 mg/kg reserpine into the foot muscles produces relaxation of the adductors. When the animal is killed, four hours later the adductors are not capable of tonic contraction. Reserpine produces the same effect even when applied on the surface of the ganglia [19]. The fluorescence in the ganglia was considerably altered by this agent. The yellow autofluorescence of cells persists so to say without any superposition of the specific yellow fluorescence. The intensity of green fluorescence in the "green" cells remains practically unchanged but it disappears almost completely from the neuropile of the cerebral ganglion (Fig. 7). Considerably less fluorescent spots are observable in the neuropile of the visceral and pedal ganglia, but the fluorescence of certain axon fascicles is even stronger than that of the neuropile in most of the normal animals (Fig. 8).

Twenty-four hours subsequent to the injection of reserpine the adductors of the animal are again capable of tonic contraction. In that state the three kinds of ganglia might be characterized by: complete absence of 5 HT-fluorescence, an autofluorescence paler than in the controls and restricted to a smaller portion of the cytoplasm. The axons do not exhibit green fluorescence. In the cerebral ganglion green fluorescence is displayed by the majority of nerve cells. Such cells are fewer in the visceral ganglion and are nearly absent in the pedal ganglion. The green fluorescence observed here is less intensive than in the "green" cells of normal ganglia.

This situation is modified to some extent by the 60th hour subsequent to the injection of reserpine, namely by this time autofluorescence has almost completely disappeared, whereas green fluorescence is exhibited by every nerve cell in the cerebral ganglion and by many in the visceral one. This fluorescence is very intensive (Fig. 9). In the neuropile of these two ganglia numerous "green" axons are present. In the pedal ganglion the number of "green" cells is still low and the fluorescence of the axons is pale.

Discussion

The fluorescence-histochemical method developed by FALCK [10] has been adapted with great success to vertebrates to examine the localization of biogenic amines [3, 6, 9]. The specificity of the reaction is undisputable when appropriate autofluorescence controls are used. The same method was used by DAHL and co-workers [7, 8] for the demonstration of monoamines in the nervous system of *Helix pomatia* and *Anodonta piscinalis*.

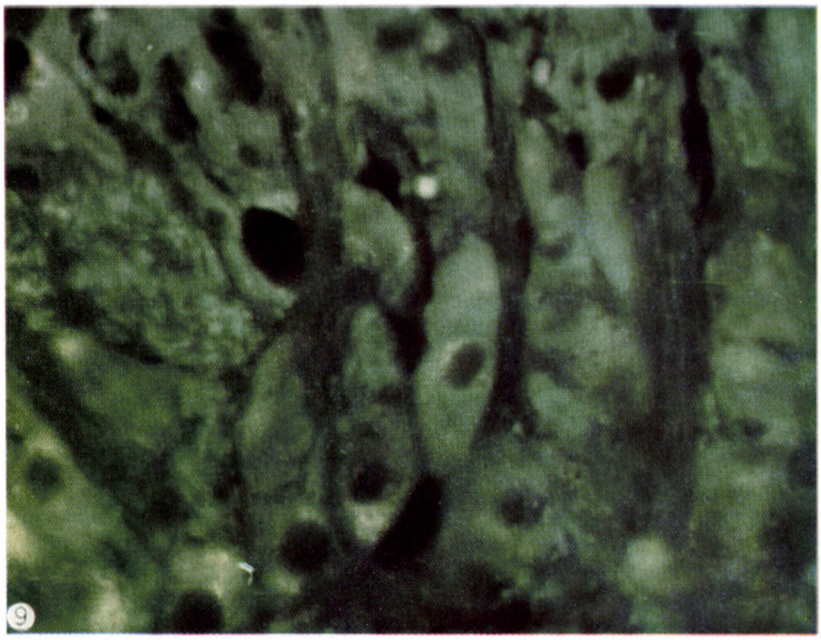

Fig. 9. Cerebral ganglion after 60 hours following the administration of 10 mg/kg reserpine. Two-hour formaldehyde treatment. Entellan + xylol covering, ×630

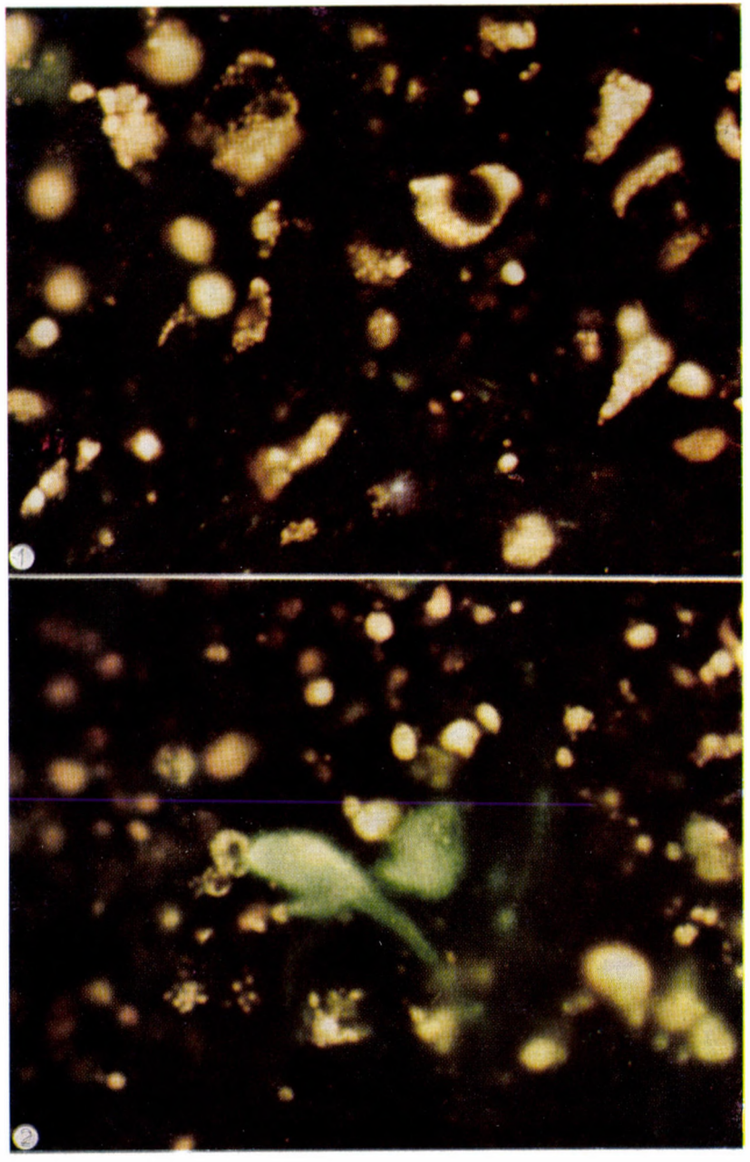

Fig. 1. Yellow-fluorescing nerve cells in the visceral ganglion. Two-hour formaldehyde treatment. Entellan + xylol covering × 630

Fig. 2. "Green" cells from the visceral ganglion. Two-hour formaldehyde treatment. Entellan + xylol covering, ×630

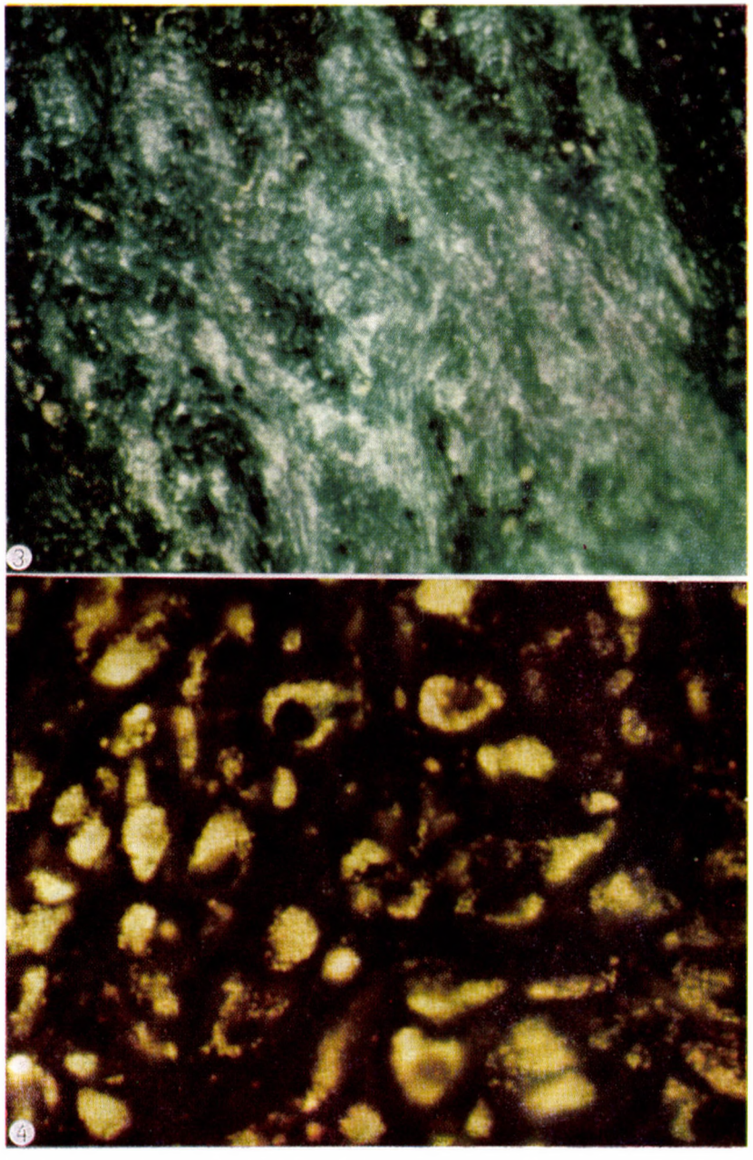

Fig. 3. A portion of the neuropile of a pedal ganglion. Two-hour formaldehyde treatment. Entellan + xylol covering, ×315

Fig. 4. The autofluorescence of the visceral ganglion, freeze-dried and heat-treated but not exposed to formaldehyde. Entellan + xylol covering, ×630

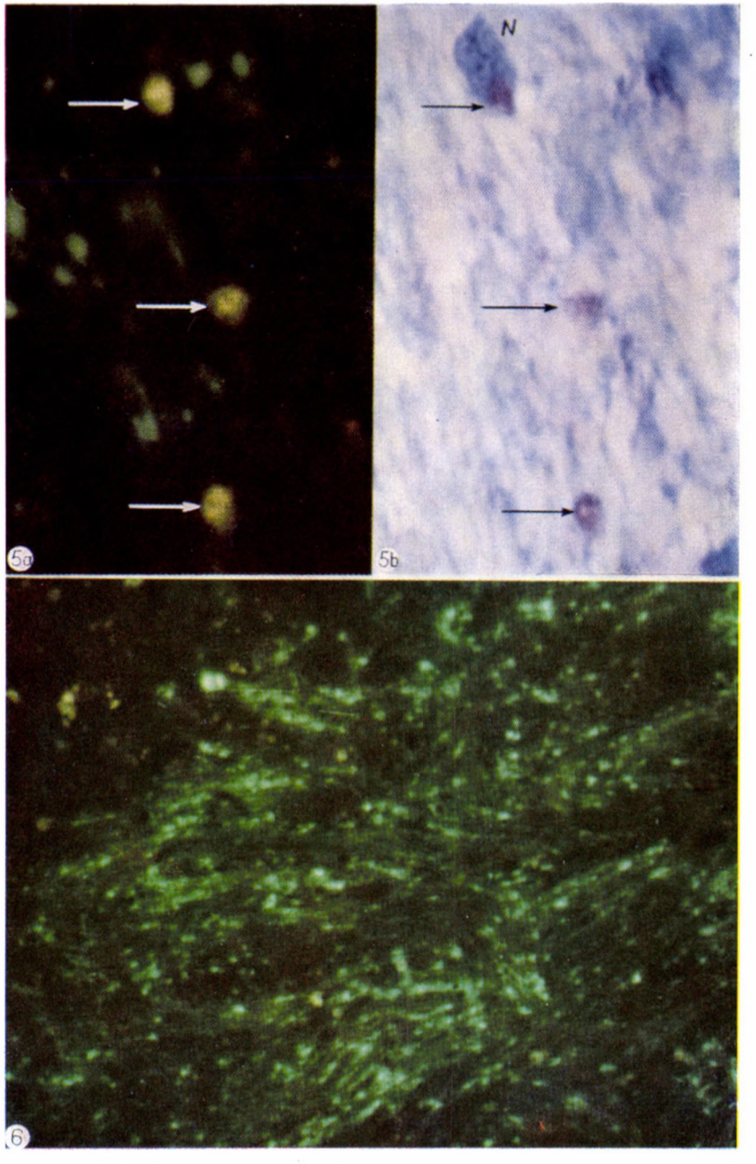

Fig. 5. The neuropile section of a cerebral ganglion 20 hrs subsequent to the administration of 100 mg/kg iproniazide.

a) Fluorescence-microscopic picture: "yellow" granules (arrows) among the "green" axons. Two-hour formaldehyde treatment, paraffin oil covering.

b) the same area stained with paraldehyde-fuchsin. The arrows are pointing to gliosomes identical with the "yellow" granules in localization in Fig. 5*a*. N = nucleus of glia cell ×1,260

Fig. 6. The neuropile-section of a cerebral ganglion 44 hrs after the addition of 6 mg/kg metaraminol. Two-hours formaldehyde treatment. Entellan + xylol covering, ×315

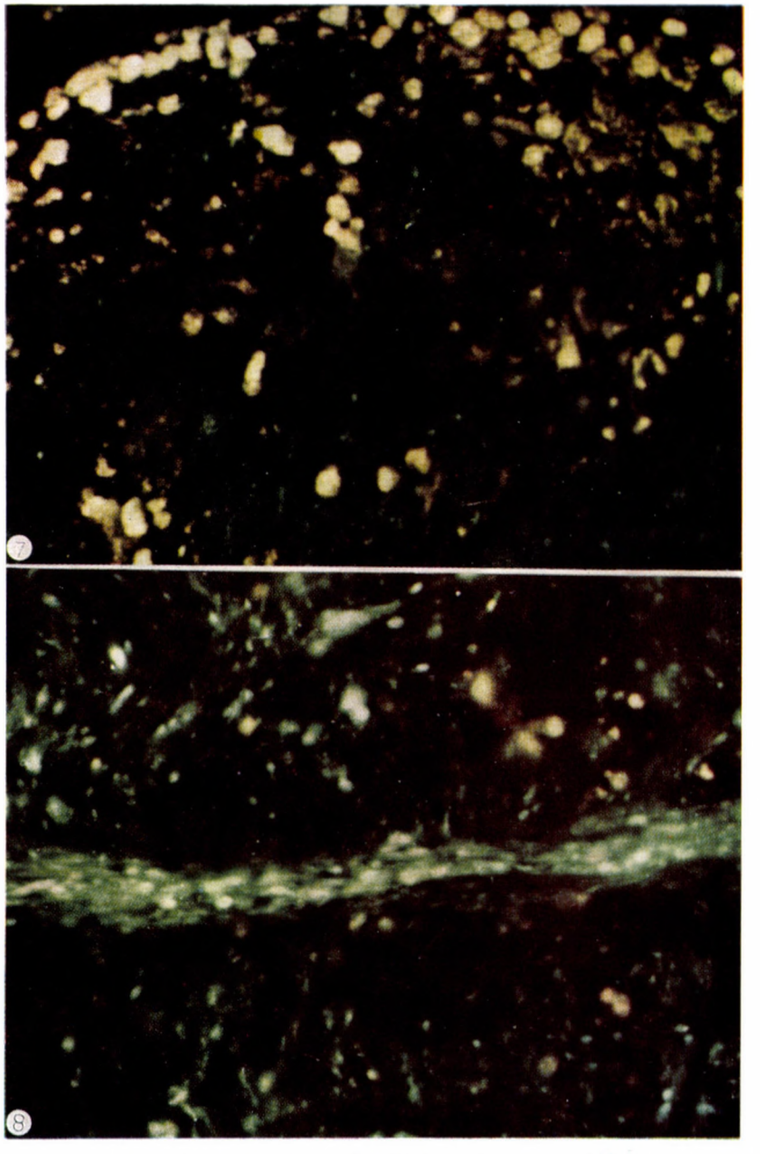

Fig. 7. The cerebral ganglion of an animal four hours after treatment with 10 mg/kg reserpine. Three-hour formaldehyde treatment. Entellan + xylol covering, ×315

Fig. 8. A portion from the visceral ganglion of the same animal, the cerebral ganglion of which is presented in Fig. 7. Three-hour formaldehyde treatment. Entellan + xylol covering, ×630

The majority of the nerve cells and glia cells in the three ganglia of *Anodonta cygnea* L. display an autofluorescence of granular localization which is demonstrable in sections either freshly frozen or freeze-dried and freeze-dried + heat-treated.

On the effect of formaldehyde the yellow fluorescence of the nerve cells becomes more intensive, but in UV-light it fades within several minutes to the level of autofluorescence. It is therefore assumed that the cells contain 5 HT or its precursor 5 HTP, from which, by means of formaldehyde treatment, an UV-labile beta-carboline is formed, which superposes its yellow fluorescence on the autofluorescence. This is verified also by the result of $NaBH_4$ reduction. These findings are in agreement in every respect with those obtained previously by the differential centrifugation method, namely 5 HT is localized on the endoplasmic reticulum [25].

DAHL and co-workers [7] considered the yellow-fluorescing granules in the neuropile observable especially after the addition of the MAO-inhibitor to be varicose dilatations of 5 HT-containing axons. The studies presented here do not substantiate this suggestion considering that these granules are identical in localization with gliosomes and they never occur in formations resembling longitudinal sections of axons. If these granules were the cross-sections of axons, their longitudinal picture would be by all means demonstrable in serial sections. The fluorescence of the formations increases after the addition of MAO-inhibitor, suggesting that also glia cells contain 5 HT. This is not surprising because this substance occurs also in other tissues of the Lamellibranchiates [1].

It seems, therefore, that 5 HT is not localized in the presynaptic structures of the ganglia of *Anodonta cygnea* and thus, its functioning as an interneuronal synaptic mediator is disputable. Due to its intracellular localization it is most probably involved in processes like the regulation of spontaneous activity.

There are reasons for believing that the green fluorescence observed in some cells, further in the processes constituting the neuropile is not autofluorescence. This is evidenced by control investigations. Green-fluorescing isoquinols are formed from catecholamines as a result of formaldehyde treatment [10]. In the present case the reaction product is insoluble in xylol and it develops even after a short, 1-hour-long formaldehyde treatment, suggesting a primer catecholamine [11]. Of these, first of all noradrenaline and dopamine may be taken into consideration. However, the efforts to differentiate these two substances histochemically have failed [5]. On the other hand, in the nervous system of molluscs dopamine and noradrenaline were demonstrable in large and very little quantities, respectively [21], further DAHL and co-workers [7, 8] established spectrofluorometrically the presence of dopamine also in the cerebral and visceral ganglia of *Anodonta piscinalis*. These observations suggest that the green-fluorescing reaction product yielded in the ganglia of *Anodonta cygnea* by formaldehyde treatment is a dopamine derivative.

Only partial depletion of the catecholamine was effectuated by metaraminol as shown by the residual green fluorescence even by 24 or 44 hours after the administration of 6 mg/kg body weight. Though there are no pharmacological data at disposal as regards the threshold dose of metaraminol, considering that a dose of 50 μg/kg is effective in vertebrates [17], 1—6 mg/kg may be considered to be over the threshold. Taking into account the noradrenaline-depleting effect [2] of alpha-methyl-m-tyrosine and its metabolites, the effect produced by metaraminol is also indicative of the presence of dopamine.

The present results concerning catecholamine are completely consistent with those obtained by DAHL and co-workers [7] on *A. piscinalis*.

On the basis of these data and those published by GERSCHENFELD [13] on the physiological effect of dopamine in snails, it is suggested that the bulk of the interneuronal transfer of nervous impulses is realized in Lamellibranchiates by means of monoaminergic mechanisms in which most probably dopamine is predominant.

According to some literary data, however, adrenaline and noradrenaline exist in the ganglia of the fresh-water mussel [18]. Attention must be called however to the fact that these data were obtained by methods unsuitable for differentiating dopamine from adrenaline and noradrenaline.

The processes of "green" cells also contain green-fluorescing material. Nevertheless, it is not to be questioned that the few green cells cannot be the source of the mass of the green-fluorescing fibres constituting the neuropile. It is questionable, however, if the cytoplasm of "yellow" cells are involved in the production of catecholamine or not. With regard to this, valuable data were furnished by the studies on the restitution process following depletion by reserpine. Depletion is nearly complete after 4 hours subsequent to the administration of reserpine with the exception of some fibre fascicles in the visceral and pedal ganglia. The pictures observed at the 24th and 60th hours after the injection of reserpine prove that after depletion catecholamine production begins in the nerve cells (in the majority of these). It appears only later in the axons of the neuropile. Thus it may be assumed that the "green" cells which are present also under normal conditions supplement the actually consumed catecholamines in the axons. This implies that the "yellow" cells are transformed periodically into "green" cells and conversely.

Parallel to the restitution process following reserpine effect the autofluorescence of the nerve cells is almost completely lost. The interpretation of this fact necessitates further investigation. Reference must be made, however, to AIELLO [1], who suggests that 5 HT metabolism in mussels is related to the formation of fluorescent compounds. It seems probable that the autofluorescent substance is a product of similar nature, which disappears from the nerve cells due to the reserpine-induced changes in 5 HT metabolism.

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HISTOCHEMISCHE UNTERSUCHUNGEN VON BIOGENEN MONOAMINEN IM ZENTRALNERVENSYSTEM DER *ANODONTA CYGNEA* L. (MOLLUSCA, LAMELLIBRANCHIATA)

Es wurde mit dem fluoreszenz-histochemischen Verfahren von FALCK bestätigt, daß die Mehrheit der Nervenzellen in allen Ganglien von *Anodonta cygnea* L., ebenso wie bei *Anodonta piscinalis*, Serotonin enthält. Es wurden keine Axone gefunden, welche eine für das Serotonin charakteristische Fluoreszenz gehabt hätten. Nach einer 20stündigen Vorbehandlung mit Iproniazid erscheinen auch in der Neuropyle zerstreute Granula mit einer für Serotonin charakteristischen goldgelben Fluoreszenz, diese sind aber im Zytoplasma der Gliazellen lokalisiert. Wegen der intrazellulären Lokalisation und des Fehlens in den Axonen kann das Serotonin als interneuronale synaptische Mediatorsubstanz kaum in Betracht kommen. Der kleinere Anteil von Nervenzellen und die Axone der Neuropyle enthalten ein primäres Catecholamin, welches wahrscheinlich Dopamin ist. Auch große Dosen von Metaraminol depletieren nur teilweise dieses Catecholamin. Während des Restitutionsprozesses nach der Depletion mit Reserpin erscheint dieses Catecholamin zuerst in den Nervenzellen, und zwar in fast allen Nervenzellen, und erst später in den Axonen. Um diese Zeit enthalten die Nervenzellen kein Serotonin. Auf Grund der erhaltenen Ergebnissen scheint es wahrscheinlich zu sein, daß die interneuronale Erregungsüberleitung in den Lamellibranchiaten hauptsächlich durch monoaminerge Mechanismen zustande kommt und Dopamin hier eine Hauptrolle spielt.

ГИСТОХИМИЧЕСКОЕ ИССЛЕДОВАНИЕ БИОГЕННЫХ МОНОАМИНОВ В ЦЕНТРАЛЬНОЙ НЕРВНОЙ СИСТЕМЕ БЕЗЗУБКИ *ANODONTA CYGNEA* L. (MOLLUSCA, LAMELLIBRANCHIATA)

При помощи флуоресцентного гистохимического метода Фалька было установлено, что во всех ганглиях *A. cygnea* также, как это описано в отношение *A. piscinalis* большинство нервных клеток содержит серотонин. Не наблюдалось аксонов, показающих флуоресцентности, характерную для серотонина. Через 20 часов после инъекции ипрониазида рассеянные золотистые зерна появляются и в нейропиле. Они обладают флуоресцентностью, характерной для серотонина, но локализуются в цитоплазме глиозных клеток. Локализация серотонина в телах нейронов и полное отсуствие его в аксонах указывает на то, что серотонин вряд ли может считаться межнейрональным синаптическим медиатором. Меньшая часть нервных клеток и аксоны нейропила содержат первичный катехоламин, вероятно допамин. Большие дозы метараминола лишь частично деплетируют этот катхеоламин. В ходе восстановления после деплетирования резерпином этот катехоламин сначала появляется в нервных клетках, именно почти во всех клетках, и только позже в аксонах. В этот период нервные клетки не содержат серотонина.

На основе полученных данных кажется вероятным, что межнейрональная медиация у пластинчатожаберных осуществляется в первую очередь по пути моноаминергических механизмов, в которых главную роль играет по-видимому допамин.

IMRE ZS.-NAGY: Tihany, Biológia, Hungary.

Acta Biol. Hung., **18** (1), 9—19 (1967)

STUDIES ON THE GOMORI-POSITIVE GLIAL CELLS

I. THE CHANGES IN THE PERIVENTRICULAR GOMORI-POSITIVE GLIAL CELLS IN RATS OF VARIOUS AGES

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Synopsis

Morphological conditions of the Gomori-positive glial cells arranged around the 3rd cerebral ventricle and aquaeductus Sylvii were examined in albino rats of various ages. Serial sections were made from the brain of newborn, suckling (about 3-week-old), adolescent (about 2-month-old), adult (about 5-month-old), and old (about 15-month-old) animals. The Gomori-positive glial cells cannot be demonstrated in the newborn: they appear for the first time in the suckling animal. The cells increase in the brain of adolescent animals. This increase in number keeps on as far as the old age. We observed the greatest number of such cells in old rats. The site of the appearance of the glial cells and their distribution around the ventricle is characteristic. The morphological appearance of the cells is different in young and old rats. On the basis of the present results a possible relationship between the Gomori-positive glial cells and a process of the nervous system changing parallel with the progress of age is discussed.

Introduction

Gomori-positive glial cells occur in the central nervous system (CNS) of both vertebrates and invertebrates of various degrees of evolution. The Gomori-positive substance is found in the cells in the form of granules and can be electively stained by the methods used for the demonstration of the neurosecretory material (aldehyde-fuchsin and chrome haematoxylin staining). In vertebrates Gomori-positive glial cells were first demonstrated by SMITH in 1951 [20], in various species by WISLOCKI and LEDUC [25, 26, 27], DIEPEN and co-workers [3], DIEPEN [4], FORD [5], NODA [13], LUNDBERG [12], LÖFGREN [11] and BARRY [2]. In connection with invertebrates the works of SCHARRER [18], PIPA [15, 16] NOVAK [14] and AROS [1] are known.

In the brain of vertebrates Gomori-positive glial cells are found first of all around the third ventricle (Fig. 1). They occur, however, also below the lateral ventricles, the aquaeductus Sylvii and the ependyma of the 4th ventricle as well as among the fibres of the nervus opticus, in the plexus chorioideus and in the meninges.

WISLOCKI and LEDUC on the basis of their morphological properties range the Gomori-positive cells in two groups: (1) cells found around the cerebral ventricles, particularly around the third ventricle and the aquaeductus Sylvii, below the ependyma epithelium, (2) macrophage-like cells arranged in the area

of the whole CNS and in the meninges, along the vessels. In our own examinations we called the first group periventricular, the second one perivasal glia.

Some authors regard the granules of glial cells as phagocytized neurosecretum [3, 4, 11, 12 13,], while others as a substance different from the neurosecretum [2, 7, 15, 21].

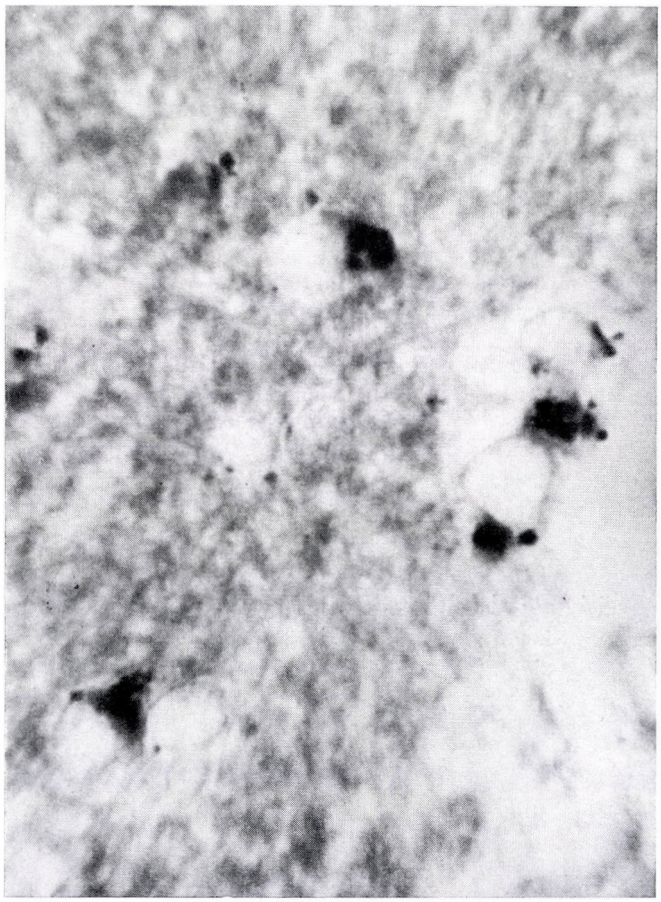

Fig. 1. Periventricular glial cells containing Gomori-positive granules. Aldehyde-fuchsin staining. Magnification ×240

In our earlier investigations [22, 23] in the study of the periventricular glia we demonstrated a relationship between the cells and the ependyma containing Gomori-positive granules. We demonstrated in anurae that the glial cells originate from the ependyma. With histochemical analysis we identified the Gomori-positive substance of the glia with that of ependyma and distinguished the neurosecretum from the Gomori-positive substance of the glial cells.

In the course of earlier observations we found that within a species the number of the periventricular glial cells shows individual variation, which seemed to be in relation first of all with the age of the animal.

In the present study we examined the age-dependence of the quantitative changes in the Gomori-positive glial cells of the periventricular type below the ependyma of the third cerebral ventricle.

Preliminary results have been published in a short abstract of a lecture [10].

Material and method

A total of 56 albino rats were involved in the study. The animals were ranged into 5 age groups:

(1) newborn	— 1-day-old	— 4— 6 g in weight
(2) suckling	— 21—28-day-old	— 35— 40 g in weight
(3) adolescent	— about 2-month-old	— 100—120 g in weight
(4) adult	— about 5-month-old	— 150—180 g in weight
(5) old	— about 15-month-old	— 350—500 g in weight

The age groups were established as recommended by HÁRSING [6] and KEMÉNY and BALÁZS [9].

The animals were killed by decapitation and the brains fixed in Helly or Bouin fixative. After paraffin embedding serial frontal sections of 5—7 micron thickness were prepared and stained with aldehyde-fuchsin and chrome haematoxylin-phloxin.

To establish quantitative data and to supplement the morphological examinations the glial cells were counted in four hypothalamic levels, viz. in [1] the chiasma opticum, [2] the sector of the tractus opticus up to the eminentia medialis, [3] the eminentia medialis and [4] the recessus mamillaris: in 9 cross-sections altogether. In each section the Gomori-positive glial cells surrounding the third ventricle within a distance equal diameter of the microscopic field, all around the ventricle were counted (magnification 15×40). The arithmetic mean of the nine values obtained was taken as glial cell count. Only the cells with visible nucleus were taken into consideration which, in a way characteristic of the periventricular glia, contained the Gomori-positive substance in a granular form.

Results

In brain sections obtained from *newborn* animals no Gomori-positive glial cells of the periventricular type characteristic of the adult animal were found around the third ventricle.

It should be noted that cells containing Gomori-positive substance but readily distinguishable from the periventricular glial cells can be found in newborn animals below the ependyma of the lateral ventricles as well as in the hypendymal substance of the subcommissural organ. At the latter site a flabelliform group of these cells surrounds the subcommissural organ; the spherical nucleus of the cells, containing small amount of chromatin, and lively-staining nucleolus. In their round cytoplasm shows a diffuse coloration and vacuoles. Such cells can be seen in serial sections also below the ependyma of the top of the aquaeductus Sylvii.

The Gomori-positive periventricular glial cells appear for the first time in the *suckling* animals, under the ependyma of the lower part of the third ventricle.

The cells are arranged in the substance of the eminentia medialis or laterally. In a low number they can be found also beside the lateral wall of the

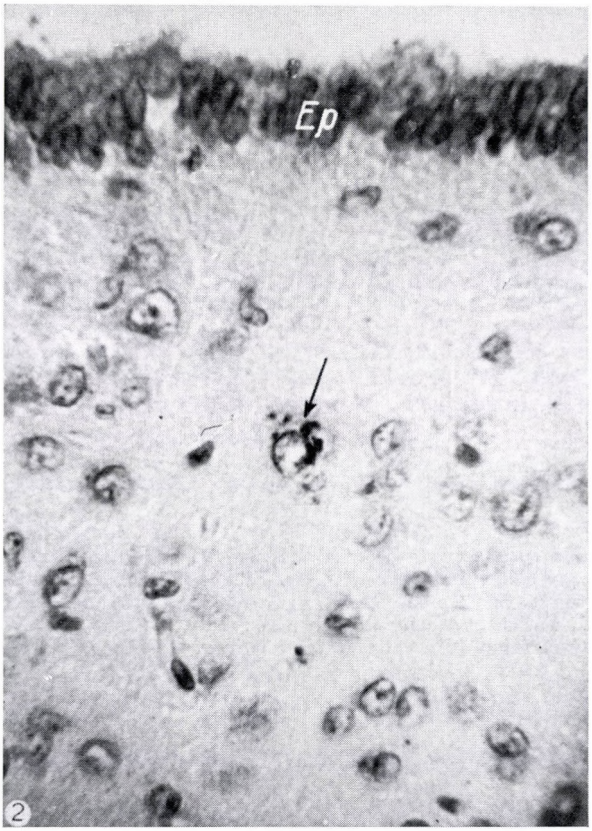

Fig. 2. Detail of the diencephalon from an adolescent animal; at the arrow a Gomori-positive glial cell. Chrome haematoxylin-phloxin staining. Magnification, ×1,200

ventricle but only at its lower portion. The nuclear structure of the cells is similar to the structure of the ependymal nuclei, the nucleus being oval and containing a medium amount of chromatin; several nucleoli are visible in it. In the narrow cytoplasm the Gomori-positive substance occurs in granular form, several granules being in each cell.

In the brain of the *adolescent* animals the Gomori-positive periventricular glial cells appear not only in the lowest part of the third ventricle but everywhere around the ventricle, below the ependyma (Fig. 2). The nuclei of the cells

show protrusions and retractions. The cytoplasm stains very faintly with the stains used; the cytoplasmic extensions are outlined by Gomori-positive granules smaller than 1 μ with sharp margin and characteristic shape. The Gomori-positive substance is frequently grouped on one side of the nucleus. The number of the cells increases in the anterior-posterior direction. Gomori-positive cells

Fig. 3. Characteristic glial cells arranged in the periventricular substance of old rat. Ep = ependyma. Chrome haematoxylin-phloxin staining. Magnification ×1,200

were found in each of the anatomical areas examined (area of chiasma opticum, retrochiasmatic area up to the eminentia medialis, eminentia medialis, recessus mamillaris). The number of the cells, however, is not equal in the various section levels. At the level of the chiasma opticum and of the tractus opticus only fewer cells (3 or 5 respectively, on the average) were found around the ventricle, then at the height of eminentia medialis and recessus mamillaris (17 or 33, on the average) in the posterior areas. In the two latter regions the arrangement of the cells is characteristic; they are evenly distributed around

the ventricle in the frontal plane intersecting the eminentia medialis but dorsally from the nucleus arcuatus they show characteristic accumulation. At the level of the recessus mamillaris accumulated Gomori-positive cells are found in the hypendyma around the subcommissural organ and the recessus.

The appearance of the periventricular glial cells shows rather considerable individual variation in this age group. In some animals very few glial cells

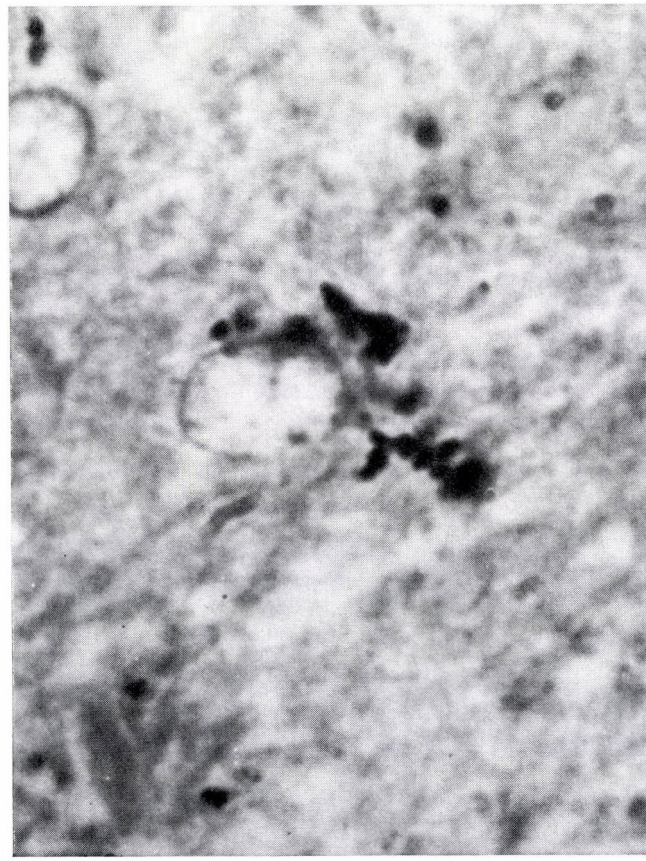

Fig. 4. Glial cell filled up with Gomori-positive granules, with branching appendage; old animal. Aldehyde-fuchsin staining. Magnification ×4,400

were found, often only in the area above the nucleus arcuatus and around the subcommissural organ while in others the number of the cells considerably exceeded the average.

In the *adult* animals the number of glial cells was higher all around the ventricle and the cells differ from those of the adolescent animals morphologically; they have more and larger extensions and their granulation is denser.

Gomori-positive glial cells occur in each of the areas in a higher number. (On the average 5 cells were counted at the chiasma opticum, 7 in the retrochiasmatic area, 23 at the eminentia medialis and 41 at the recessus mamillaris.)

In *old* rats the number of cells increases further (Fig. 3); at each section level more cells were counted than in corresponding area of the younger animals. (In the order of the regions examined the means are: 20, 31, 52, 97). The

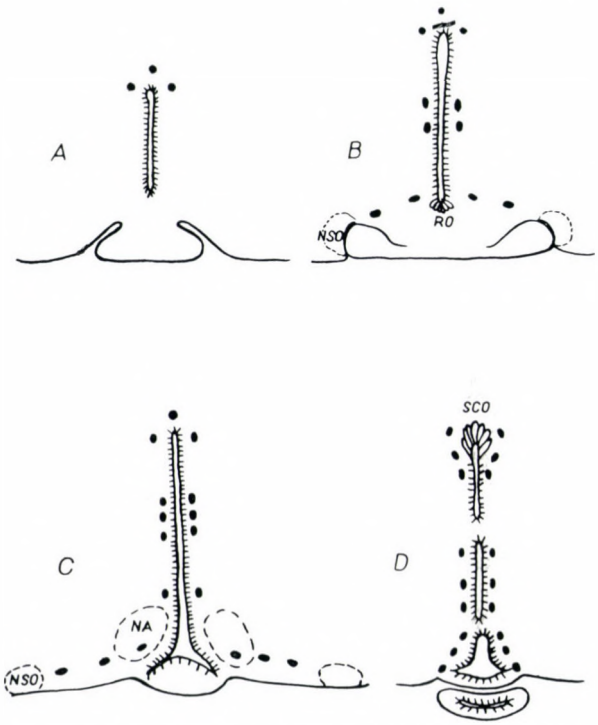

Fig. 5. Scheme of the accumulation areas of the Gomori-positive glial cells. Section levels: A = chiasma opticum, B = retrochiasmatic area, C = eminentia medialis, D = recessus mamillaris (NSO = nucleus supraopticus, RO = recessus organ, NA = nucleus arcuatus, SCO = subcommissural organ)

granulation of the cells is still denser and the number of extensions increases further (Fig. 4).

As to the distribution of the Gomori-positive glial cells around the ventricle it is generally characteristic, irrespective of age group, that they can be found everywhere along the third ventricle in cranio-caudal direction; in the anterior sections fewer than in the posterior ones. Under the ventricle ependyma at some levels — in all age groups alike — the glial cells are grouped in a characteristic way. At the chiasma opticum they surround the upper part of the ventricle in a fan-like arrangement. At the level of the tractus opticus they

are found above and at the midheight of the ventricle as well as around the ependyma organ, the so-called recessus organ. Some cells even occur farther away from the ventricle, toward the nucleus supraopticus. At the eminentia medialis, the cells above show fan-like arrangement while those at the midheight of the ventricle are arranged in a plane. A great number of these cells are also found at the lower third of the ventricle, among the cells of the nucleus arcuatus, extending toward the nucleus supraopticus and among the nerve fibre cross-sections of the eminentia medialis. At the recessus mamillaris the cells are evenly distributed; a remarkable accumulation can be found in the environment of the subcommissural organ and the habenula (Fig. 5).

Discussion

Concerning the nature of the Gomori-positive glial cells a number of divergent opinions exist. DIEPEN and co-workers [3], DIEPEN [4], NODA [13] and LÖFGREN [11] believe that the granules represent phagocytized neurosecretum. LUNDBERG [12] raises the idea that the phagocytized substance is decomposed and transformed by the glial cells. After a pretreatment with lipid solvent HILD [7] could demonstrate by staining procedures the glial granules but not the neurosecretum. In his opinion the Gomori-positive substance of the glial cells is not identical with the neurosecretum. BARRY [2] also points to the morphological difference between the substance of the secretory neurons and that of the glial cells and attributes to the glia an own secretory capacity. NOVAK [14], too, regards the Gomori-positive granules as gliosecretion. FORD and co-workers [5], concluding from the anatomical location of the Gomori-positive glial cells, reject the possibility of phagocytosis and prefer the theory of the active secretion by the glia. PIPA and co-workers [15, 16] found in insects histochemical difference between neuronal and glial granules. On the basis of electronmicroscopic examinations they believe that a group of Gomori-positive gliosomes might have derived from mitochondria. According to the electronmicroscopic examinations of RÖHLICH and co-workers [17] the glial granules are of a characteristic structure and may be compared to lysosome or lipofuscin granules.

In the course of our earlier investigations [22, 23, 24] we found a relationship between the Gomori-positive glial cells and the Gomori-positive granules in the ependyma cells, strictly speaking, the so-called ependymosecretory phenomena. Histochemically the substance of the granules was found to be glyco-mucoprotein [21].

In the present study we demonstrated the age-dependence of the number and appearance of the Gomori-positive glial cells. Such cells cannot be demonstrated at all in the hypendyma of the third cerebral ventricle in the new-

born animal. They appear for the first time in the suckling animal in the area of the eminentia medialis and in the lowest sector of the ventricle on both sides, further next to the basal surface of the brain in the immediate vicinity of the eminentia medialis in the direction of the nucleus supraopticus. In adult animals a considerable numerical increase of the cells can be observed. Obviously sexual maturity and accumulation of glial cells seem to be connected phenomena.

The number of the glial cells further increases in old animals. The Gomori-positive granulation of the cells also increases. In younger animals only slight accumulation of granules is found beside the nucleus but in adult animals the granules occupy more and more extensions until they fill up also the branching extensions of the glial cells of the old animal. All these observations support the view that the *glial cells may be involved in a process changing parallel with the progress of age*. The increased formation observed in old age fits well into the arborization trend manifest in the more developed stages of the CNS [8]. It is well-known that in the phylo- and ontogenetically later stages of the nervous system, beside the proliferation of cells, a more intensive formation of extensions and membranes is characteristic. This is one of the morphological substrates of the differentiation of the metabolism in the nervous system. It may be assumed that the morphological change, and the increase in number of these special glial cells are further manifestations of the above principle.

The characteristic periventricular, subependymal arrangement of the Gomori-positive glial cells is also remarkable. It is known from the works of SHIMIZU and co-workers [19] that the periventricular areas below the ependyma unlike other parts of the brain show a characteristic anaerobic metabolism. It is also well-known that the glial cells are the most important factors of the intermediary metabolism of the nervous tissue [8]. It is possible, therefore, that in this special metabolism the Gomori-positive glia is also involved.

Finally, it should be noted concerning the relationship between the Gomori-positive glia and neurosecretion or ependymosecretion that the Gomori-positive glial cells appear at first in the eminentia medialis, among the neurosecretory fibres. Subsequently, they can always be found in considerable number on this area and also toward the nucleus supraopticus. Their accumulation is also related to the ependymal organs, e.g. they accumulate under the subcommissural and in the subfornical organ.

All these data argue for the fact that a certain morphological relation exists between the systems referred to and the Gomori-positive glia, although a great number of glial cells can be also found in periventricular areas where neither neuro- nor ependymosecretory phenomena take place. The substance of the glial cells differs from the neurosecretum also histochemically [21] and it is different both from the neuro- and ependymosecretum in its ultrastructure [17]. Thus, the present examinations, which prove first of all the age-dependent

changes in the Gomori-positive glia, fail to decide whether the Gomori-positivity of the glial cells under study originates from phagocytized neuro- or ependymosecretum or these cells are independent "glio-secretory" cells. To elucidate this question, autoradiographic investigations are in progress.

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UNTERSUCHUNGEN ÜBER DIE GOMORI-POSITIVEN GLIAZELLEN

I. Veränderungen der periventrikulären Gomori-positiven Gliazellen in Ratten von verschiedenem Lebensalter

Die morphologischen Verhältnisse der Gomori-positiven Gliazellen, die sich um den III. Ventrikel und den Aquaeductus Sylvii herum lokalisieren, wurden in Ratten verschiedenen Lebensalters untersucht. Die Beobachtungen erfolgten an den Gehirnserienschnitten von Neugeborenen, Säuglingen (ungefähr 3 Wochen alt), Adolescenten (ungefähr 2 Monate alt), Erwachsenen (ungefähr 5 Monate alt) und an den von alten Tieren (ungefähr 15 Monate alt). Die Gomori-positiven Gliazellen sind in Neugeborenen nicht nachweisbar, sie erscheinen zuerst im Säugling. Die Gliazellen vermehren sich im Hypothalamus der Adolescenten. Diese quantitative Zunahme hält bis in das spätere Lebensalter an, so sind die meisten Gliazellen in alten Ratten sichtbar. Der Ort des Auftretens und die Verteilung der Gliazellen um den Ventrikel herum sind charakteristisch. Die morphologische Erscheinung der Zellen ist in der jungen und alten Ratte verschieden. Auf Grund vorliegender Ergebnisse wurde die Möglichkeit des Zusammenhanges der Zellen mit einem Prozeß der Veränderung des Nervensystems parallel zum zunehmenden Lebensalter behandelt.

ИССЛЕДОВАНИЕ ГОМОРИ-ПОЛОЖИТЕЛЬНЫХ ГЛИОЗНЫХ КЛЕТОК

I. Изменение количества перивентрикулярных глиозных клеток у крыс различного возраста

Изучались морфологические условия Гомори-положительных глиозных клеток, располагающихся около III желудочка головного мозга и Сильвиевого водопровода, у подопытных крыс-альбиносов различного возраста. Исследования проводились на серийных срезах, изготовленных из мозга новорожденных крыс, сосунков (около трехдельных животных), 2-месячных животных, взрослых крыс (около 5-месячных животных) и старых (около 15-месячных) животных. Гомори-положительные глиозные клетки впервые появились у сосунков. У новорожденных крыс их не удалось выявить. В мозге 2-х месячных крыс число Гомори-положительных клеток повышается. По мере старения это количественное повышение еще продолжается и, следовательно, больше всего глиозных клеток имеется у старых крыс. Характерны место появления глиозных клеток и их расположение около желудочка. Морфологические свойства отдельных клеток у молодых и старых крыс различны. На основании полученных данных выдвигается возможность существования связи между изменениями количества глиозных клеток и щиениемь нербной системы по мере старения.

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ON THE MECHANISM OF SEXUAL DIFFERENTIATION OF THE HYPOTHALAMUS

DECREASED HYPOTHALAMIC OESTROGEN SENSITIVITY IN ANDROGEN-STERILIZED FEMALE RATS

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Synopsis

Compensatory ovarian hypertrophy in hemispayed rats was significantly inhibited by oestradiol 0.1 μg/day administered for a period of one month. The inhibitory effect of the same dose of oestradiol was less marked and not significant in the hemispayed rats which had received a single injection of 1.0 mg testosterone one day after birth. The results suggest a desensitizing action of postnatal androgen on the sex-steroid-sensitive hypothalamic structures instrumental in the negative feedback action of sexual hormones.

Introduction

In a previous paper [7] it was reported that the secretion of gonadotrophic hormones (GTH) was more markedly inhibited by exogenous oestrogen in male rats castrated on the 2nd day of life than in those gonadectomized 60 days after birth. Based on these findings it was suggested that in the neonatal rat testicular androgen reduces the sex-steroid sensitivity of the hypothalamic structures necessary to the negative feedback process by which oestrogen inhibits GTH secretion. In rats castrated after puberty, where these structures are functionally inactive exogenous oestrogen did not inhibit the GTH output to the same degree as in animals castrated shortly after birth. In the latter animals the sex-steroid sensitivity of the hypothalamic structures in question remained intact, due to the absence of testicular androgen during the critical period of the first few days after birth.

The present experiment was designed to reveal whether a similar desensitizing effect of exogenous androgen exists in the female rat. The sex-steroid sensitivity of the hypothalamic structures was measured in the inhibition of the compensatory ovarian hypertrophy (COH) by oestrogen.

Material and method

The experiments were carried out in 88 female rats of our inbred strain kept under strictly uniform environmental conditions and on a standard diet. The animals were divided into two main groups.

(A)

Unilateral ovariectomy and oestrogen administration

The left ovaries of forty, 70-day-old rats were removed and then the animals were divided into four sub-groups. Ten hemispayed rats served as untreated controls, the remaining ones, in three sub-groups of 10 animals, received daily injections of oestradiol propionate (0.1, 2.0 and 10.0 μg, respectively, dissolved in 0.1 ml Oleum Helianthi) on the subsequent 30 days.

(B)

Pre-treatment with testosterone, unilateral ovariectomy and oestrogen administration

Forty-eight female rats were pre-treated with 1.0 mg testosterone phenylpropionate dissolved in 0.05 ml Oleum Helianthi, at the age of 2 days. Following the opening of the vaginal orifice vaginal smears were recorded daily. These revealed persistent cornification in all animals at least for a period of one month before the left ovaries have been removed at the age of 70 days. The animals were then distributed in four sub-groups. Twelve hemispayed rats remained without oestrogen treatment, thus served as control for the androgenized group. The remaining ones, in three sub-groups of 13, 10 and 13 animals, respectively, received the same treatment for the subsequent 30 days as the animals in the three oestrogen-treated sub-groups of Group A.

All the rats of both groups were killed on the 30th day of oestrogen administration, i.e. at 100 days of age. The pituitaries, thyroids, adrenals and the right ovaries were carefully freed from the surrounding connective tissues and fat, weighed by a torsion-wire balance and, similarly to the previously removed left ovaries, fixed in a 10% formalin solution. Serial frozen sections of both ovaries were stained with haematoxylin-Scharlach-R.

Results

The degree of the COH following treatment with different doses of oestradiol is diagrammatically shown in Fig. 1. Unilateral ovariectomy without administration of oestrogen resulted in a COH of considerable degree, whereas administration of a daily oestrogen dose of 2.0 or 10.0 μg abolished this effect completely in both groups. Treatment with daily 0.1 μg oestradiol gave, however, dichotomic results in the two groups. This dose of oestrogen inhibited significantly the COH in group A (ovarian weight: 51.2 mg versus 61.7 mg; $p < 0.02$) while in the androgenized animals (group B) resulted only in a slight, non-significant inhibition (ovarian weight: 30.3 mg versus 32.7 mg; $p < 0.20$).

The histological picture of the ovaries (Fig. 1) in the androgenized group was influenced neither by the unilateral ovariectomy nor by the oestrogen treatment. The ovaries in this group (B) contained only growing follicles and interstitial tissue but no corpora lutea.

In the weights of the other endocrine organs except the hypophysis there was no difference between the two groups following the above treatment. Table 1 shows that postnatal androgen administration markedly reduced the pituitary hypertrophy evoked by administration of higher doses of oestradiol (2.0 or 10.0 μg).

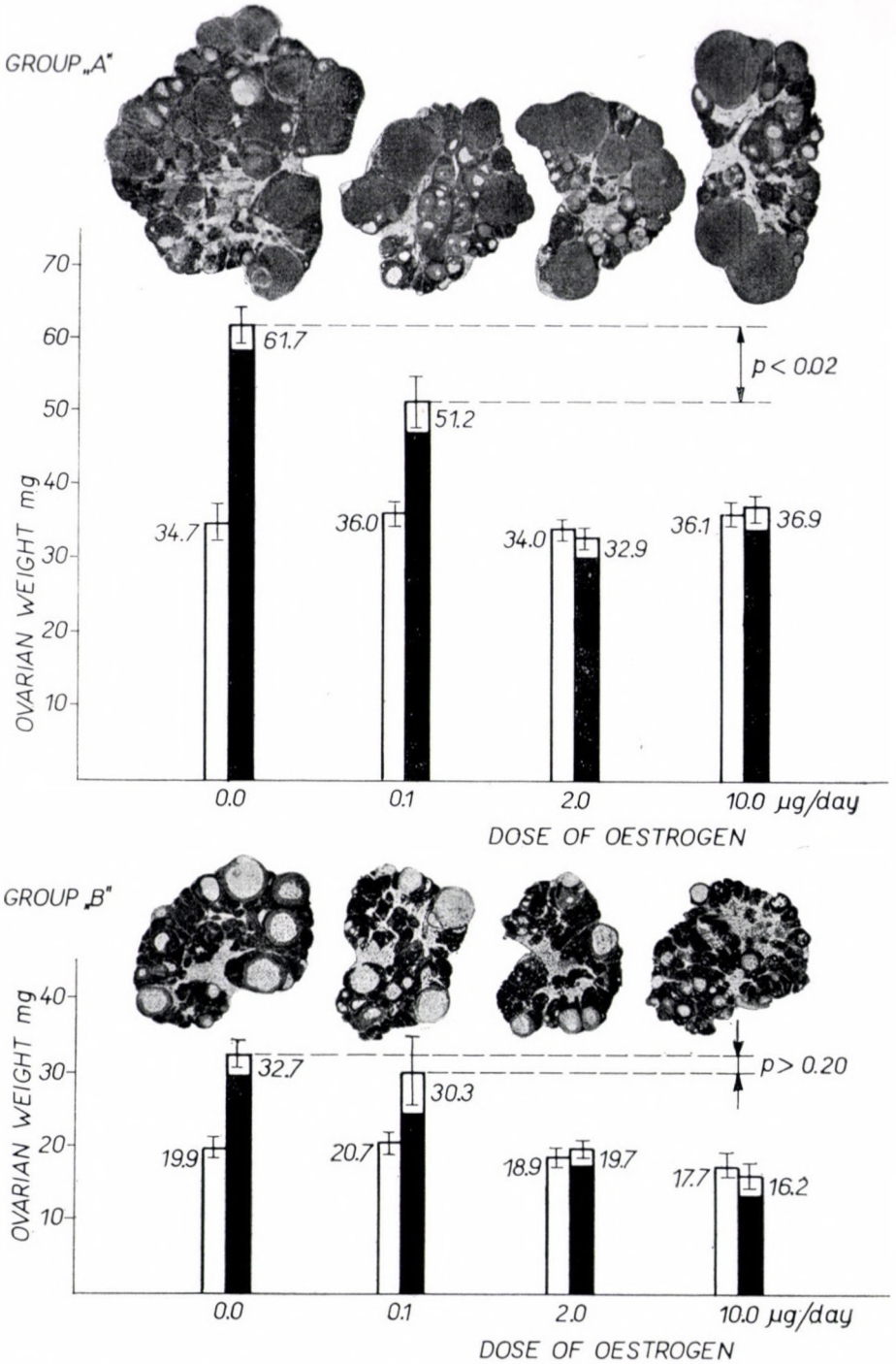

Fig. 1. Diagrammatic representation of compensatory ovarian hypertrophy and its inhibition by exogenous oestrogen in adult hemispayed rats. Group (A) without, group (B) with, pre-treatment with testosterone. Mean weights of the removed ovaries are represented by white, those of the remaining ones by black, columns. The first double columns represent the data of rats without oestrogen administration, the following ones indicate the data of rats treated with 0.1, 2.0 and 10.0 µg/day oestrogen, respectively. Average weights in mg and representative ovarian pictures are given above the columns. Note the absence of corpora lutea in ovaries of rats pre-treated with testosterone

Table 1

Mean body and pituitary weights of hemispayed and hemispayed + oestrogen-treated rats with (group B) and without (group A) pre-treatment with a single dose of 1.0 mg testosterone on the 2nd day after birth

	Sub-group	No. of rats	Treatment (μg oestrogen per day)	Body weight in g at killing	Pituitary weights in mg	
Group A (without androgen)	1	10	0.0	184 $\pm$ 5.1	14.4 $\pm$ 0.8	
	2	10	0.1	179 $\pm$ 3.8	14.1 $\pm$ 0.9	$p < 0.01$
	3	10	2.0	180 $\pm$ 3.3	19.1 $\pm$ 1.1	
	4	10	10.0	189 $\pm$ 4.2	37.4 $\pm$ 2.9	$p < 0.01$
Group B (with androgen)	1	12	0.0	185 $\pm$ 4.0	15.0 $\pm$ 0.2	
	2	13	0.1	182 $\pm$ 3.4	15.4 $\pm$ 0.9	$p < 0.05$
	3	10	2.0	196 $\pm$ 3.6	17.8 $\pm$ 1.2	
	4	13	10.0	174 $\pm$ 3.9	20.3 $\pm$ 2.4	$p < 0.05$

Discussion

In the androgen-sterilized persistent oestrous rats GORSKI and BARRACLOUGH [6], SWANSON and VAN DER WERFF TEN BOSCH [9] as well as SHAPIRO [8] failed to observe any change in the degree of COH following unilateral ovariectomy. These authors concluded that it is not necessary to postulate any imbalance in FSH secretion to explain the phenomenon of persistent oestrus, at least in the androgen-sterilized animals. In our opinion, however, from experiments comparing the degree of the mere COH of rats with and without postnatal androgen-administration, no conclusion can be drawn in the respect whether or not the negative oestrogen-FSH feedback was disturbed by this treatment. In this regard only a comparison between the degree of the *oestrogen-inhibiton* of the COH in androgenized and non-androgenized rats might furnish some evidence. Hence, in the present experiment the results of the subgroups with daily 0.1 μg oestrogen treatment appear to support the assumption that inactivity, or at least inadequate function, of hypothalamic structures instrumental in the negative feedback action of oestrogens [1, 4, 5] might also play a role in the disturbance of the GTH secretion following postnatal androgen treatment.

Various explanations for this inactivity or inadequate function might be advanced, the most simple being the assumption of a reduced sex-steroid sensitivity of the nerve cells which contribute to the preoptic and anterior hypothalamic mechanisms controlling LH and FSH release [2, 3].

Continuous administration of non-physiological amounts of oestrogen (more than 1.0 μg per day) inhibits GTH secretion by direct action on the ade-

nohypophysis, and this direct inhibiting action cannot be eliminated by destruction of the sex-steroid-sensitive, hypothalamic structures [2]. This is reflected also in this experiment by the fact that larger daily oestrogen doses (2.0 or 10.0 μg) inhibited FSH secretion to the same degree, as judged by the COH, both in rats with or without postnatal androgen treatment. On the other hand, the degree of pituitary hypertrophy following this oestradiol treatment (2.0 or 10.0 μg per day) was markedly different in the two groups. Being the enlargement of the hypophysis the result of a direct oestrogen-action on the pituitary tissue, one has to assume that the hypophysial tissue reactivity to oestrogen might have also been changed by the postnatal androgen treatment.

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DIE GESCHLECHTLICHE DIFFERENTIATION DES HYPOTHALAMUS; HERABGESETZTE HYPOTHALAMISCHE OESTROGENSENSITIVITÄT IN ANDROGEN-STERILISIERTEN RATTEN

Die kompensatorische Ovariumhypertrophie einseitig kastrierter Ratten wurde durch eine einen Monat lang dauernde Behandlung mit 0,1 μg Oestradiol pro Tag signifikanterweise verhindert. Der Hemmungseffekt derselben Oestradiolmenge war geringer und nicht signifikant in einseitig kastrierten Ratten, die am zweiten Lebenstage 1,0 mg Testosteron erhalten haben. Die Ergebnisse sprechen dafür, daß die gleich nach der Geburt verabreichten Androgene die sexualsteroidempfindliche, hypothalamische Nervenstrukturen, durch welche der negative Rückkoppelungseffekt der Sexualhormone auf die Gonadotrophinsekretion stattfindet, desensibilisieren.

О МЕХАНИЗМЕ ПОЛОВОЙ ДИФФЕРЕНЦИАЦИИ ГИПОТАЛАМУСА; УМЕНЬШЕНИЕ ЧУВСТВИТЕЛЬНОСТИ ГИПОТАЛАМУСА В ОТНОШЕНИЕ ЭСТРОГЕНА У КРЫС САМОК, СТЕРИЛИЗОВАННЫХ АНДРОГЕНОМ

Дача суточной дозы в 0,1 μг эстрадиола в течение месяца в значительной мере тормозила развитие компенсационной гипертрофии яичника, наступающей после удаления яичника одной стороны. Тормозящее действие одинаковой дозы эстрадиола было менее выраженным и не достоверным у крыс, лишенных яичника одной стороны, которым в день после рождения вводили разовой дозой 1,0 мг тестостерона. Результаты указывают на то, что андроген в случае введения немедленно после рождения оказывает десенсибилизирующее действие на чувствительные в отношении половых стероидов нервные элементы гипоталамуса, при посредстве которых осуществляется отрицательное действие обратной сигнализации (feedback) половых гормонов.

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ON THE MECHANISM OF SEXUAL DIFFERENTIATION OF THE HYPOTHALAMUS

FACTORS INFLUENCING THE "CRITICAL PERIOD" OF THE RAT

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Synopsis

0.5 mg testosterone propionate (TP) injected directly into the body of foetuses caused both external masculinization and anovulatory ovaries. — 5—16 mg TP given to pregnant rats in small daily doses during the last 3—7 days of pregnancy induced persistent vaginal cornification and anovulatory polyfollicular ovaries in the offsprings. These findings show that androgen-sterilization can be induced by the administration of TP to rat foetuses or to pregnant mothers. Ovarian hormones injected into newborn males during the critical period of the first 10 days of life prevented the masculinization of the hypothalamo-pituitary axis occurring normally in the male rat in this period of life.

Introduction

From YAZAKI's [21, 22] experimental results it appears that the hypothalamus has originally the ability both in male and female rats to maintain a cyclic secretion of gonadotrophic hormones (GTH). It is only on the first few postnatal days that, under the influence of either testicular or exogenous androgens, the hypothalamus of the rat develops into male direction losing the ability to maintain the cyclic release of GTH [3, 10, 11, 21, 22]. In the male, this is adequate for normal testicular function, but if it occurs in the female, then anovulatory state associated with persistent vaginal cornification and sterility is the result.

Sex-steroid-sensitive preoptic [1, 2, 15, 16] and anterior hypothalamic [4, 5, 6, 7] structures seem to be indispensable in the feedback mechanisms contributing to the maintenance of cyclic release of GTH. Inactivity or at least reduced functional capacity of these neural structures was the result of the early androgen action both in male [13] and female [14] rats. It was close at hand to assume, therefore, that the loss of hypothalamic feedback mechanisms may account for the establishment of the masculine, i.e. the non-cyclic pattern of gonadotrophin secretion in rats.

Various explanations for this inactivity could be advanced, the most simple, however, being the assumption of a reduction of sex steroid-sensitivity of the nerve cells contributing to the hypothalamic feedback mechanisms. This sensitivity either could not entirely be established or it was lost in the presence of androgen during the critical period of development.

It is becoming clear that a delineation of the critical period is marred by several variables as it was pointed out by SWANSON and VAN DER WERFF TEN BOSCH [18]. They showed that the full "early-androgen" syndrome may not exist at the time of puberty but may develop after an initial period of normal female ovarian activity [17, 18]. Secondly, the relation between the time of androgen administration and the effect on the brain (-pituitary-ovary axis) is greatly influenced by variations in the dose of androgen administered [18].

In a similar way of thinking we wondered whether (1) the "early-androgen" syndrome could be produced by administration of testosterone into the body of the female foetuses or to the mother and, (2) whether the "imprint" of the male pattern on the hypothalamus by early postnatal testicular androgen action could be prevented by administration of ovarian hormones to newborn male rats during the critical period of the first 10 days of life.

Material and method

Albino rats were used from our inbred strain derived from Wistar stock and kept under strictly uniform environmental conditions and on a standard diet.

Pre-natal days were calculated from the morning on which sperm were first found in the vaginal smear. This day was designated as the first day of pregnancy.

Doses of testosterone propionate (TP; Androfort, Kőbányai Gyógyszerárugyár), oestradiol propionate (Akrofollin, Chinoin) and progesterone (Glanducorpin, Kőbányai Gyógyszerárugyár) to be administered were dissolved in 0.1 ml oil.

Intrauterine injections were given subcutaneously, directly into the body of the foetuses through small incisions made on the uterine wall over each foetus under ether anaesthesia. The site of injection was sealed with collodium and the incisions were thread with silk.

Removal of the testes from ten-day-old rats was carried out through bilateral abdominal incisions under ether anaesthesia. The ovaries and vaginae to be grafted into the dorsal neck-muscles of the castrated rats were taken from 19—22 days old females.

The animals were killed by bleeding under ether anaesthesia. The reproductive organs and the ovarian and vaginal grafts were removed, weighed fresh and fixed in 10% formalin. Serial frozen sections of the *in situ* and grafted ovaries were stained with haematoxylin-Scharlach R. Paraffin sections of the vaginal grafts were stained with haematoxylin and eosin.

Results

Experiment 1. Testosterone propionate (TP) injection into foetuses

Nineteen females were anaesthetized on the 19th day of pregnancy and the uterus was exposed. All foetuses were injected with 0.5 mg TP. Thirteen out of the nineteen aborted shortly after the operation. The remaining six pregnants spontaneously delivered altogether fifteen females which were brought up by the mothers.

All these female offsprings had abnormal masculinized external genitalia, from which vaginal smears could not be taken. They were killed at the age of 100 days. The ovaries contained no corpora lutea but growing and cystically dilated follicles and interstitial tissue (Fig. 1).

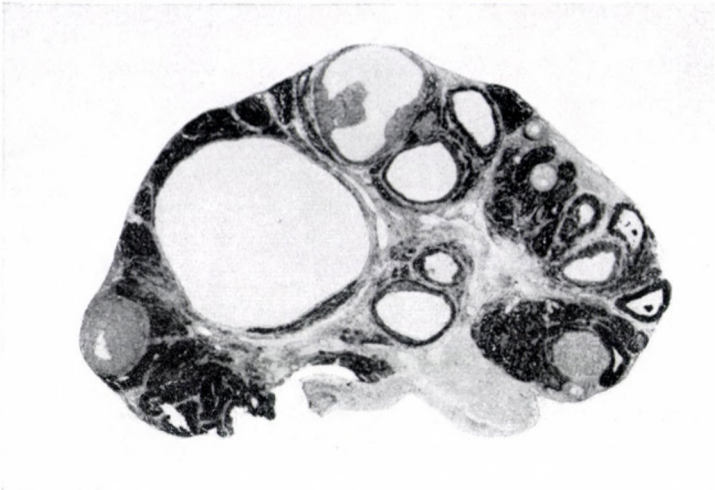

Fig. 1. Ovary of a 100 days old rat injected with a single dose of 0.5 mg testosterone propionate two days before birth. Polyfollicular ovary without corpus luteum. — Frozen section stained with haematoxylin-Scharlach R.

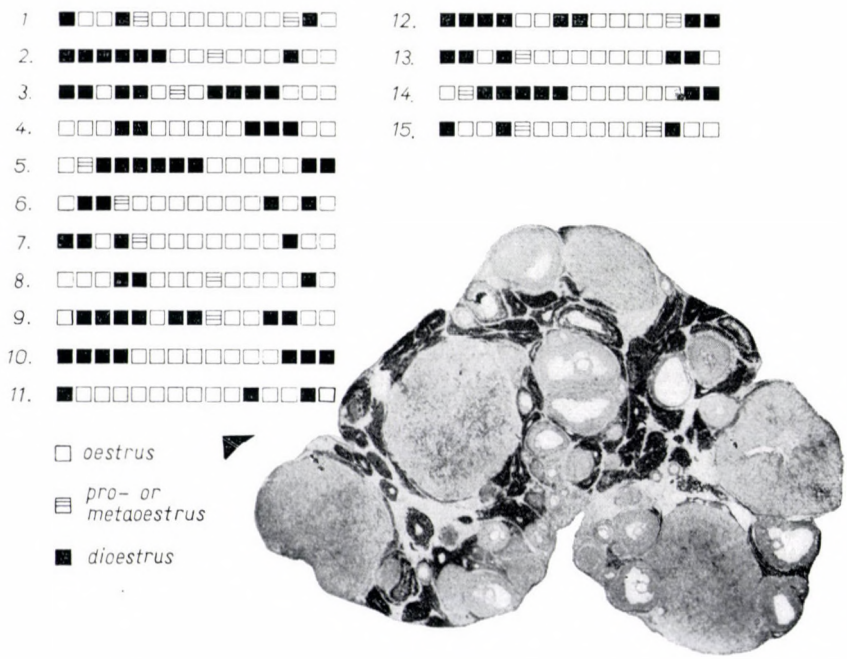

Fig. 2. Daily vaginal smears and the histology of a representative cyclic ovary of a female young the mother of which had been injected with a single dose of 5.0 mg testosterone on the 19th day of pregnancy. — Frozen section stained with haematoxylin-Scharlach R.

Experiment 2. A single dose of TP to mothers

A single dose of 5.0 mg TP was injected intramuscularly into five females on the 19th day of pregnancy. Two or three days later these pregnants delivered altogether fifteen females and lactated successfully.

After opening of the vaginal orifice daily smears of the female young were recorded. They had irregular vaginal cycles with oestrous predominance (Fig.2). At the age of 100 days, when killed, their ovaries were of cyclic character and contained corpora lutea (Fig. 2).

Experiment 3. Repeated doses of TP to mothers

Six pregnant rats in three subgroups received testosterone injections from the 19th day of pregnancy until birth in the following doses:

daily 2×0.5 mg TP 2 rats
daily 2×1.0 mg TP 2 rats
daily 2×2.0 mg TP 2 rats

With these doses three effects were noted: (1) intra-uterine death, (2) delayed parturition (in 5 pregnants out of 6) and (3) failure to lactate. In consequence of the latter merely nine female offsprings survived, only two of those born at term. In the daily vaginal smears they had persistent vaginal cornification irrespective of the amount of androgen their mothers received. At the age of 100 days, when killed, their ovaries were polyfollicular and contained no corpora lutea (Fig. 3).

Experiment 4. Removal of the testes 10 days after birth with or without pre-treatment with ovarian hormones. Transplantation of ovary and vagina in the neck-muscles at the age of 70 days

Fifty newborn male rats were divided into two groups.

(A) The testes of twelve rats were removed 10 days after birth and at 70 days of age one ovary and a piece of vagina was transplanted into the dorsal neck-muscles of each.

(B) Before the same operation as in group A, thirty-eight newborn rats in three sub-groups received a daily treatment of ovarian hormones (see doses in Table 1).

Animals of both groups were killed one month after transplantation.

None of the ovarian grafts in group A contained corpora lutea. They consisted of growing follicles and interstitial tissue (Fig. 4a). The non-cyclic pattern of GTH secretion in this group was clearly shown also by the histological picture of the vaginae transplanted close to the ovarian grafts. The epithe-

lium of the vaginal grafts was filled with concentric layers of cornified cells; no other cellular elements were found in the majority of the grafts (Fig. 4b).

In contrast, the majority of the ovarian grafts in group B contained corpora lutea (Fig. 4c) and a cyclic pattern of GTH secretion was revealed by the

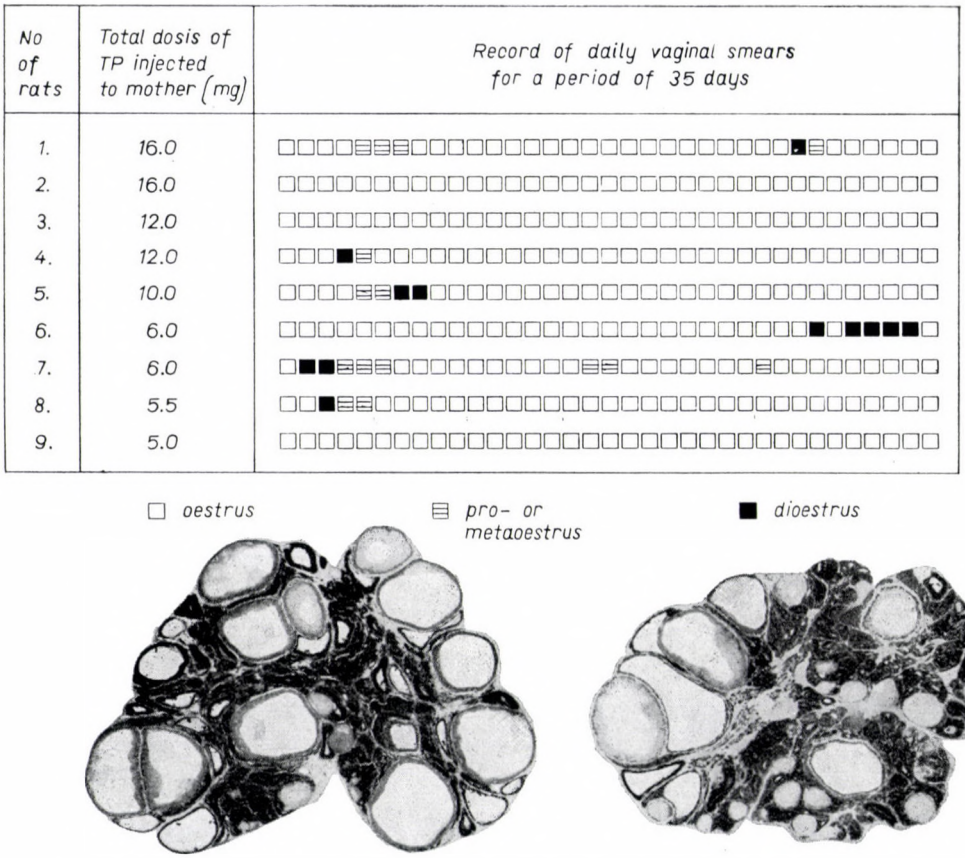

Fig. 3. Daily vaginal smears and the histology of two representative polyfollicular ovaries of female young rats whose mother had been injected repeatedly with different doses of testosterone (see also the text) from the 19th day of pregnancy until birth. — Frozen sections stained with haematoxylin-Scharlach R.

histological studies of the vaginal grafts. The lumen of the grafts was filled with detached cell layers in which cornified layers alternated with layers of nucleated epithelial cells associated with a great number of leukocytes (Fig. 4d). The percentage of the luteinized ovaries in the three different sub-groups increased with the daily dose of ovarian steroids administered before castration (Table 1).

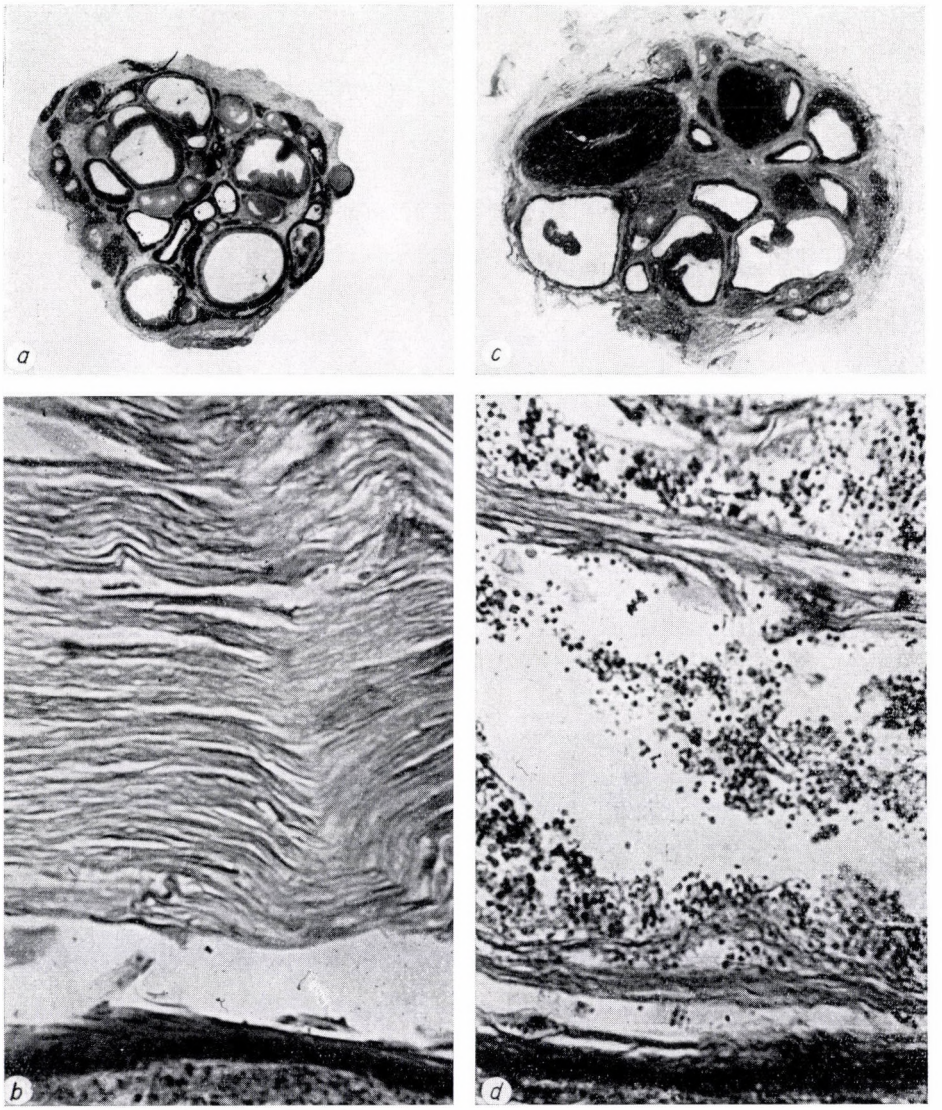

Fig. 4. a and *b.*: Intramuscular ovarian and vaginal grafts of a male orchidectomized 10 days after birth. *a:* polyfollicular ovarian graft without corpus luteum; *b:* vaginal transplant the lumen of which is filled with concentric layers of cornified cells. — *c* and *d:* intramuscular ovarian and vaginal graft of a male treated daily with 0.1 μg oestradiol and 2.5 μg progesterone during the first 10 days of life and then orchidectomized immediately. — *c:* cyclic ovarian graft with corpora lutea. *d:* vaginal transplant containing concentric layers of cornified and non-cornified cells + leukocytes. — *a* and *c*: frozen sections stained with haematoxylin-Scharlach R.; *b* and *d*: paraffin sections stained with haematoxylin-eosin

Table 1

Number and percentage of luteinized ovarian grafts in adult male rats castrated 10 days after birth after pre-treatment with ovarian hormones

Group	Treatment at the age of 1-10 days	No. of rats	Rats with luteinized ovarian graft	
			No.	per cent
A	No treatment	12	0	0
B	Daily 0.1 μg oestradiol benzoate (OB)	13	6	46.1
	Daily 0.1 μg OB + 0.5 μg progesterone	13	8	61.5
	Daily 0.1 μg OB + 2.5 μg progesterone	12	9	75.0

Discussion

Following a single dose of 2.5 mg TP injection into female rats, SWANSON and VAN DER WERFF TEN BOSCH [18] could not observe any alteration in the cyclic pattern of GTH secretion of the female offsprings. They interpreted their finding that the TP given to the mother did not pass, presumably, the placental barrier in such a measure that it could get into the foetuses in an efficient amount. When the paper of the above authors was published, the present experiments were well under way, and we could observe the masculinizing effect of TP injected either directly into the foetuses or into the pregnant rats in a dose of 0.5 mg or 5.0—16.0 mg, respectively. Obviously, the amount of TP injected into the animals in SWANSON and VAN DER WERFF TEN BOSCH's [18] first experiment was insufficient to produce the early-androgen syndrome in the female offsprings. In fact, in their most recent paper SWANSON and VAN DER WERFF TEN BOSCH [19] gave an account of successful treatment: a single dose of 10—25 mg TP given to pregnant rats or injection of 20—100 μg TP into the foetuses did turn the female hormonal pattern into a male pattern.

In accordance with SWANSON and VAN DER WERFF TEN BOSCH's [19] second experiment, two conclusions can be drawn from the present results. (1) Androgen-sterilization can be induced already before birth by administration of sufficiently large doses of TP into the body of the rat foetus or to the pregnant animal. (2) The critical period depends partly on the maturation of the hypothalamic structures mentioned above, and partly on the hormonal environment existing in the body of the foetus and of the mother.

In favour of the latter view convincing evidence has been furnished also by the results of Experiment 4. In the majority of the cases ovarian hormones proved to be capable of preventing the masculinizing effect of endogenous androgens on the pattern of GTH secretion. This was evidenced by the cyclic character of ovaries and vaginae transplanted into males treated with ovarian hormones in the first 10 days of life. Unexplored is the mechanism of this action

of ovarian hormones. One can think here of a counteraction caused by the ovarian steroids which might antagonize the "masculinizing" effect of endogenous androgens. The other possibility is the negative feedback action of ovarian hormones which might inhibit GTH secretion and hence the androgen production in the treated males. KINCL and MAQUEO's [12] most recent findings, however, do not support the latter assumption but give preference to the former one. Their data suggest that progesterone can protect the hypothalamo-hypophysial-gonadal axis of the female rat against the action of early postnatal androgen administration.

GORSKI [8] found that 5 μg oestradiol benzoate given to rats at 5 days of age resulted in permanent sterility and ovaries lacking corpora lutea indicative of an acyclic GTH secretion. The same effect can be evoked also with progesterone [20]. These results and other similar findings indicate that the early post-natal "masculinization" of the hypothalamus may not be androgen-specific: it can be evoked also by the early administration of ovarian steroids. However, relatively larger doses of ovarian hormones are necessary for this effect. In our experiment 0.1 μg oestradiol administered repeatedly failed to "masculinize" the hypothalamus; on the contrary, this amount could prevent the effect of testicular androgens.

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ÜBER DEN MECHANISMUS DER GESCHLECHTLICHEN DIFFERENZIERUNG DES HYPOTHALAMUS; DIE »KRITISCHE PERIODE« BEEINFLUSSENDE FAKTOREN BEI RATTEN

Unmittelbar in den Körper der Foetuse verabreichtes Testosteronpropionat (TP) (0,5 mg) rief äußere Maskulinierung sowie anovulatorische Ovarien hervor. Die Verabfolgung von 5—16 mg TP in geringen Tagesdosen an trächtige Ratten während der letzten 3—7 Tage der Schwangerschaft hatte persistierende Scheidenverhornung und anovulatorische polyfollikuläre Ovarien bei den Nachkommen zur Folge. Diese Ergebnisse zeigen, daß durch die Verabreichung von TP an Rattenfoetuse oder an schwangere Ratten eine Androgen-Sterilisation herbeigeführt werden kann. Werden neugeborenen männlichen Ratten in der kritischen Periode (die ersten 10 Tage des Lebens) Ovarialhormone injiziert, so verhindert dies die Vermännlichung des hypothalamo-pituitären Systems, was bei männlichen Ratten in dieser Lebensperiode normalerweise eintritt.

О МЕХАНИЗМЕ ПОЛОВОЙ ДИФФЕРЕНЦИАЦИИ ГИПОТАЛАМУСА; ФАКТОРЫ, ВЛИЯЮЩИЕ НА «КРИТИЧЕСКИЙ ПЕРИОД» У КРЫС

Введение 0,5 мг проприоната тестостерона (ПТ) непосредственно в тело плода вызывает внешнюю маскулинизацию, а также развитие ановуляторных яичников. После дачи 5—16 мг ПТ беременным крысам небольшими дозами в течение последних 3—7 дней беременности у плодов возникли остаточное ороговение влагалища и ановуляторные полифолликулярные яичники. Полученные результаты показывают, что введением ПТ непосредственно в тело плода или беременным крысам можно вызвать андрогенную стерилизацию. Введение яичниковых гормонов новорожденным самцам в течение критического периода жизни (первые 10 дней жизни) предотвратило маскулинизацию гипоталамо-питуитарной системы, наблюдаемую в норме у мужских крыс в этот период жизни.

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THE CELL BEHAVIOUR OF THE FRESH-WATER SPONGE EPHYDATIA FLUVIATILIS

A TIME-LAPSE MICROCINEMATOGRAPHY STUDY

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Synopsis

Vital observations on the behaviour of sponge cells were carried out by means of time-lapse microfilming, using phase-contrast microscope. The sponges developed from gemmules and lived between slides. The zone of growth, the canal system and the flagellated chambers were filmed, the speed being one still per second, and the microscope power being ob. ×40 oc. 7 or ob. ×60 oc. 7. Considerable variability of shapes and positions of amoebocytes were observed, as well as of their relations with more stable cell groups (flagellated chambers, pinacocyte epithelia). The type of processes and the movement of amoebocytes depends on their location. The so-called lophocytes (cells with a typical "train"-like process, described by ANKEL and WINTERMANN-KILIAN) represent an interim stage of nucleolate amoebocytes. They acquire this shape when they are in the surface parts of the sponge body. They take another shape, described as collencytes or nervous cells, when they are in the middle parts of the sponge.

Introduction

Time-lapse microfilming of animals enables studying the behaviour of their cells and the interrelations among various types of cells.

Many problems connected with the development of sponges from a gemmule in asexual reproduction and the development of new organisms from dissociated cells (this phenomenon, similar to asexual reproduction, was defined by B. P. TOKIN as somatic embryogenesis) are still disputable. For instance, it is not yet clear in what way flagellated chambers appear during a normal development from gemmules and in the course of the experimentally induced somatic embryogenesis. The exact extent of differentiation which takes place in different kinds of cells after their dissociation, the existence or absence of a nervous system in sponges etc. are still unknown.

Recently the question of whether sponges possess elements of a nervous system has been widely discussed [3, 7, 9]. TUZET and co-workers [11] and PAVANS DE CECCATTY [5, 6, 7], suggest that sponges do have cells characteristic of a nervous system. ANKEL and WINTERMANN-KILIAN [2] suppose that the cells which they found in the dermal membrane of *Ephydatia fluviatilis* and called lophocytes are similar to those of a nervous system. These questions being connected with various understandings of the integration and of the integration mechanisms which provide for the integrity of the sponge organism should be

approached, in addition to the morphological investigations, from other aspects as well.

It seems logical that if there are cells in sponges similar to the nerve cells of other multicellular animals, these cells must show some rather stable relations with one another and with all cell systems having vital functions in these animals. Such systems may be, for instance, the canal system, pinacocytes and skeleton elements.

The data given in this paper were obtained in filming various parts of *Ephydatia fluviatilis* during its development from gemmule, the filming being proposed by G. P. KOROTKOVA.

Material and method

Fresh-water sponges *Ephydatia fluviatilis* were investigated on the second and the third days after their hatching from gemmule. For filming the process of development sponges were cultivated between slides [1, 12]. A gemmule was placed on a slide between the edges of two other slides, placed on the first slide at an equal to the diameter of the gemmule. The cells of a developing sponge, on coming out of the gemmule, first filled the space between the edges of the two slides and then spread and filled the whole space between the slides. In this way a relatively thin preparation was obtained.

The gemulles for the investigation were collected in the ponds of Starii Peterhoff in September and were stored in a refrigerator at +4°C.

Before putting the gemmules to hatch they were washed, in their cultural water. To the first washing fluid a few drops of H_2O_2 were added [8]. For hatching the gemmules were put in an aquarium containing tap water which had been strained through a paper filter and let stand for 24 hours. The aquarium was provided with a glass bottom 0.1 mm ×40 mm ×80 mm, and plastic walls 10 mm high, the bottom and the walls being glued together with a vacuum oil not toxic for sponges (Fig. 1). Cellophane strips 40 μ thick, oiled with vacuum oil on both sides, were placed upon the bottom, 15—20 in an aquarium. The slides with the gemmules were placed upon these strips.

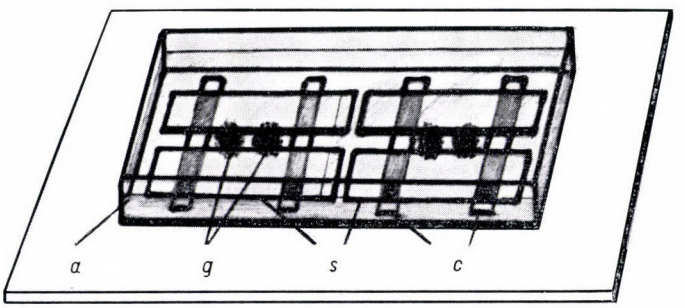


Fig. 1. Scheme of the aquarium for sponge growing: a=aquarium, s=covering slides on cellophane strips (c), g=gemmules

The aquarium thus prepared was placed in an incubator of 23°—24°C. After the sponge cells had crept out of the gemmule shells, the water in the aquarium was changed every 24 hours.

Young sponges *Ephydatia fluviatilis* have a transparent zone at their edges, which has no canal system and almost no flagellated chambers. The cells of the zone of growth lie in three layers, viz., mobile mesenchyme cells between two layers of pinacocytes.

We microfilmed by the phase-contrast microscope some parts of the zone of growth and some areas of the young sponges where a canal system and flagellated chambers had been formed. The filmings lasted from 15 minutes to two hours, the speed being one still per second, and the microscope power being ob. 40 × oc. 7, or ob. 60 × oc. 7.

Results and discussion

The development of a sponge from a gemmule begins on the second or third day of the incubation. In six days the canal system and the oscular tube are formed but at the same time the zone of growth is still seen at the edges.

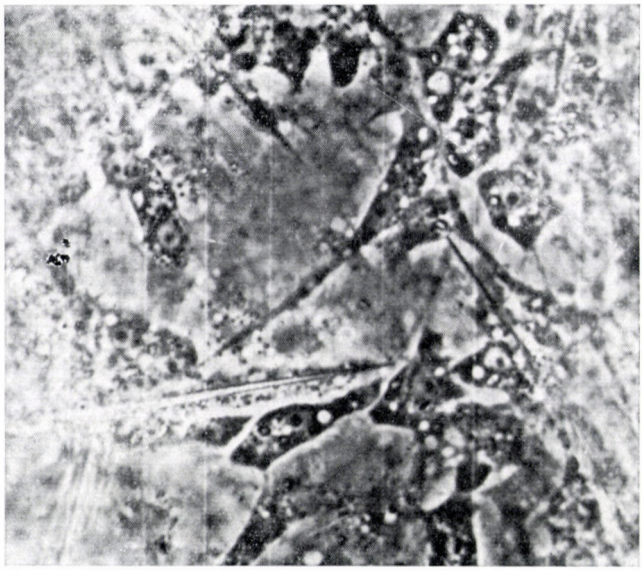


Fig. 2. Mesenchymal part of a sponge: amoebocyte with a long process, scleroblast with a spicule inside its cytoplasm various shapes of amoebocytes (microfilm stills, ocular ×7, objective ×40, phase contrast.)

The film shows a great mobility of the sponge cells, those of the pinacocytes epithelium and the choanocytes being the least active. These hardly moved during the three hours of observation. Amoebocytes wandered among the flagellated chambers, with a mean speed of about 13.4 μ per minute and often came into contact with them. Young scleroblasts with a spicule-forming inside the cells are mobile, too, but their speed is only 2—3 μ per minute.

Wandering amoebocytes can assume various shapes, their variability varying greatly. Some of them remain oval, moving forward by extending short plasmatic pseudopodia. Others form long pseudopodia and "trains" as they move. An amoebocyte with several thin processes can become spindle-shaped, with two long processes, in 10 minutes. The processes of some amoebocytes are two or three times longer than the body of the cell itself (Fig. 2). We did not

observe any contacts between the cells by means of such processes, the contacts being probably established by means of mesogloea and keep changing all the time.

There are cells in the zone of growth which are two or three times larger than amoebocytes. Their nuclei are disguised by a lot of inclusions. These cells move by spreading their pseudopodia. Probably they are binuclear archaeocytes.

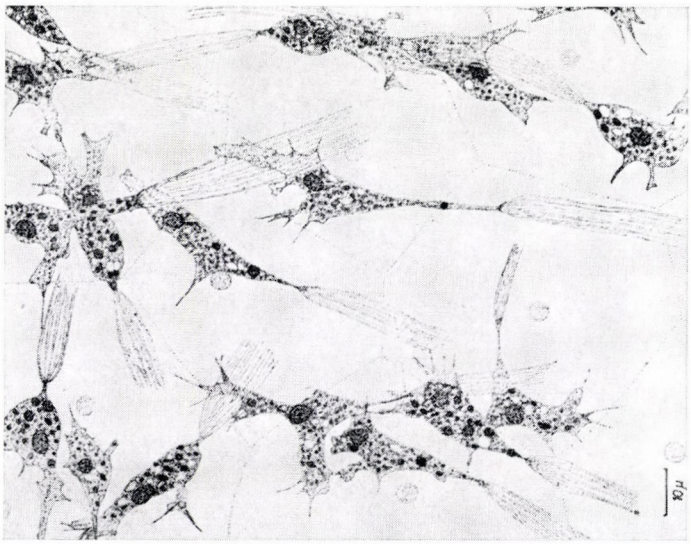


Fig. 3. Lophocytes in atrium epithelium of *Ephydatia fluviatilis* (Photo-micrograph, ×1,000, from ANKEL and WINTERMANN-KILIAN)

When a sponge develops between slides, its canal system arranges itself in a plane, parallel to the substratum. Between the pinacocytes lining the canal bottom and the external layer of pinacocytes, in mesoglea, wandering amoebocytes and scleroblasts are clearly seen. Among them one can clearly distinguish the cells resembling the lophocytes which were first observed by ANKEL and WINTERMANN-KILIAN [2]. According to these authors, lophocytes have a long cytoplasmic process, with a tuft of fibrils on the one end, and some pseudopodia with sharp needles beside the pseudopodia for moving, on the other end (Fig. 3, 4).

We observed amoebocytes with "trains" consisting of a thin layer of cytoplasm, branching at the end, while on the other end of the cell there were numerous threadlike pseudopodia (Figs 5, 6). The nucleus of such cells had a nucleolus, and in the cytoplasm there were inclusions and vacuoles.

We are convinced that the lophocyte-like shape of amoebocytes is transient, it appears only when the cells are near the pinacocyte epithelium of the surface, and changes when the amoebocytes move away. The long, "train"-like

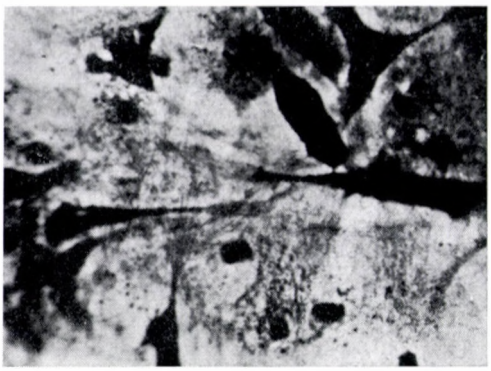


Fig. 4. Lophocytes in atrium epithelium (Plane preparation, from ANKEL and WINTERMANN-KILIAN)

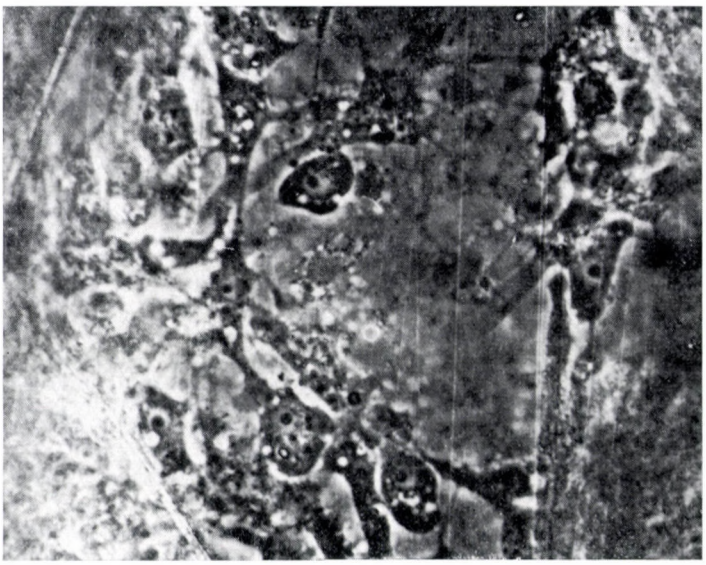


Fig. 5. Mesenchymal cells between two layers of pinacocytes: amoebocytes with a "train" and with thread-like pseudopodia, amoebocyte forming short pseudopodia, various shapes of amoebocytes (microfilm still, ocular ×7, objective ×40, phase contrast)

process, characteristic of these cells usually appears on the side opposite to the direction of their movement. The long "train" may be then drawn in, and the cell may change its shape. The fact that these cells resemble lophocytes suggests that their organization depends on their location, their stability depending on certain conditions. When the lophocyte-like cells change their position, their shape changes, too, and they become collencyte-like or spindle-like.

A new sponge *Ephydatia fluviatilis* especially in the zone of growth, has a well-visible layer of pinacocytes, its boundaries being indistinct in a live sponge. Large vacuoles are situated around the vacuole-like nucleus, which contains a small nucleolus. According to MEEWIS [4], pinacocytes represent a category of cells having no nucleolus. SIMPSON [10] while studying the marine sponge *Microciona prolifera* observed nucleoli in the nuclei of pinacocytes and suggested that pinacocytes originate from archaeocytes and not from collen-

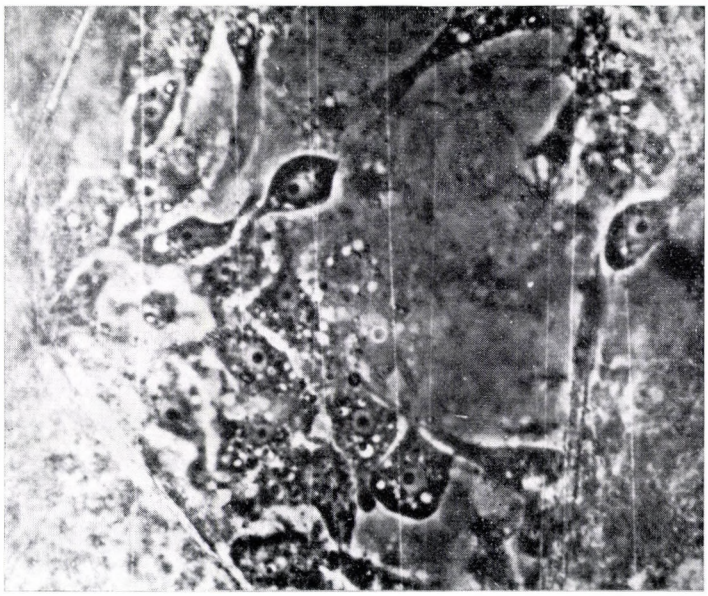


Fig. 6. The same part of mesenchyme 3 minutes later: amoebocyte with a "train", beginning to draw it in, amoebocyte with short pseudopodia, nuclei of pinacocytes (microfilm still, ocular ×7, objective ×40, phase contrast)

cytes, which have no nucleoli. This suggestion has been confirmed by the fact that there are nucleoli in the pinacocytes of sponges developing from gemmules.

Observing the behaviour of cells in the parts of the sponge body where differentiated structures had already been formed, we saw no stable cell elements except choanocytes and pinacocyte epithelium.

We can draw some preliminary conclusions from the above observations on the development of flagellated chambers. WINTERMANN-KILIAN [12] supposed these formations to result from archaeocytes by mitotic division when the daughter-cells did not move apart and form an aggregate of cells increasing in quantity.

In the zone of growth we observed some hardly moving groups of archaeocytes with few inclusions (Fig. 7). While amoebocytes were actively moving,

this group of cells had an almost fixed position. It was probably one of the stages of the formation of a flagellated chamber. Division of an archaeocyte usually takes 3 minutes; in 4—5 minutes the daughter-cells acquire an irregular shape, their nuclei become visible, the cells take an amoeboid shape and move apart (Figs 8, 9, 10). But the group of archaeocytes mentioned above remained motionless during microfilming (one hour's time).

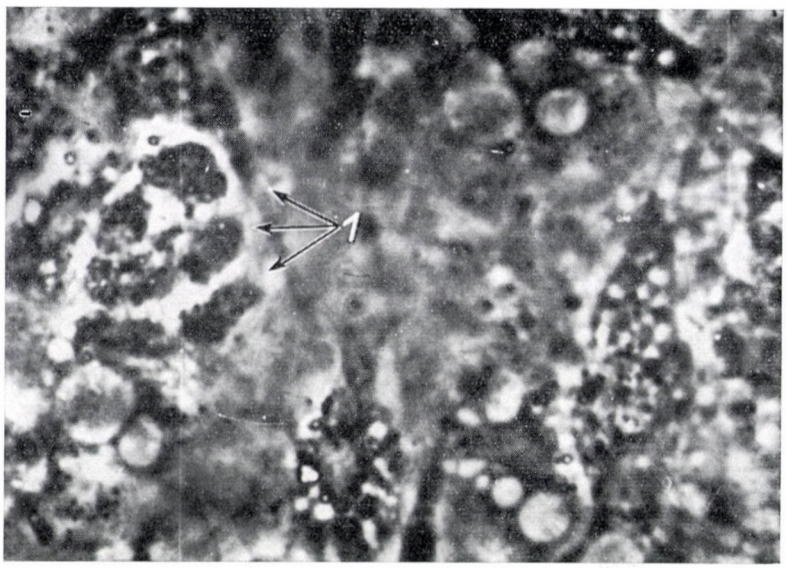


Fig. 7. Part of the zone of growth: (1) a group of motionless archaeocytes (microfilm still, oc. ×7, ob. ×60, phase contrast)

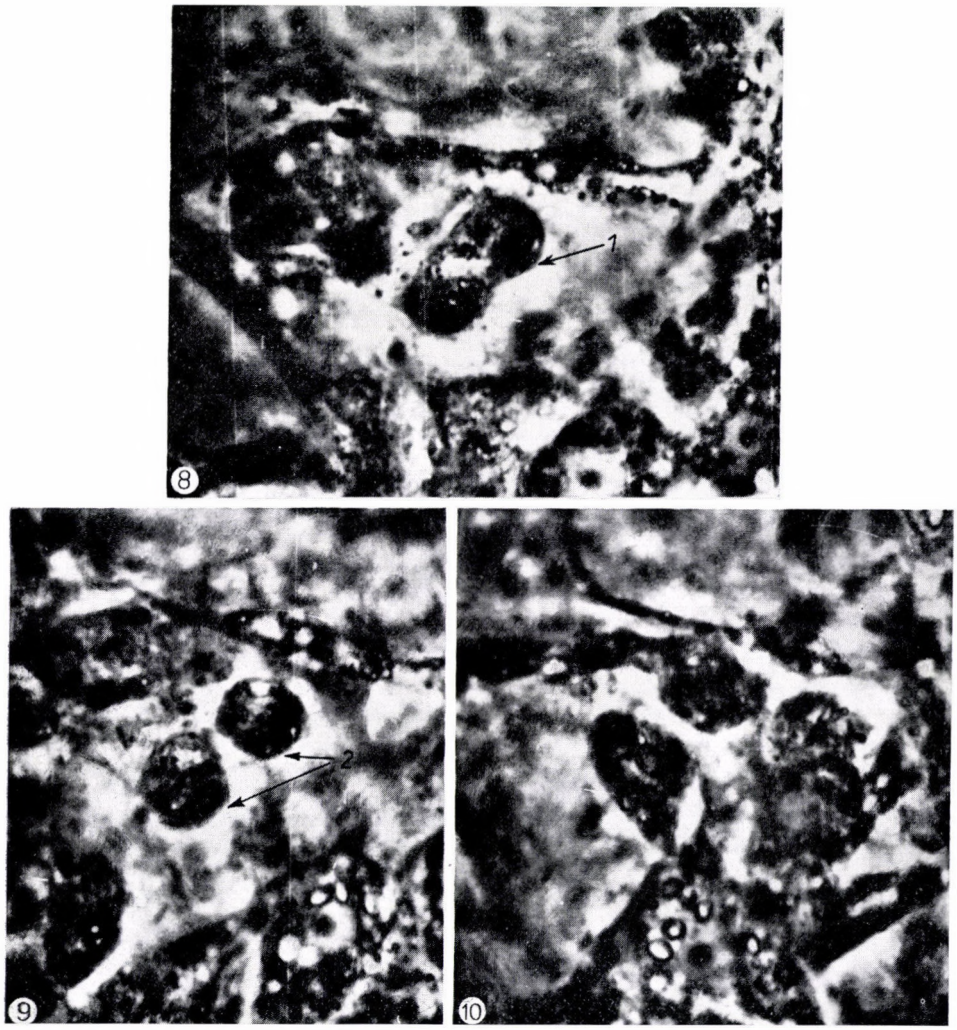
We intend to continue microcinematography and hope to trace the development of flagellated chambers with more precision.

We did not attempt to cultivate sponges for a long time. Deprived of running water and feeding, the sponges perish by the 14th day, their canals beginning to contract on the 10th day.

Vital observations on the behaviour of cell elements of the fresh-water sponge lead us to the following conclusions:

(1) Time-lapse microfilming shows an extreme variability in the shape and location of amoebocytes, in their relations to more stable groups of cells (flagellated chambers, pinacocyte epithelia).

(2) The shape of amoebocyte processes and the type of movement depends on the location of amoebocytes. The so-called lophocytes (cells with a typical "train"-like process, described by ANKEL and WINTERMANN-KILIAN)



Figs 8, 9, 10. Part of the zone of growth: (1) dividing archaeocyte, (2) daughter-cells moving apart (microfilm still, oc. ×7, ob. ×60, phase contrast)

represent some interim stage of nucleolate amoebocytes and appear only when they are near the surface of the sponge body.

(3) Other shapes of cells, described as collencytes or nervous cells are the forms which amoebocytes take when they are in the middle of the sponge body. These are also transient forms.

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DAS VERHALTEN DER ZELLEN DES SÜSSWASSERSCHWAMMES *EPHYDATIA FLUVIATILIS*

Vitale Untersuchung mit etappenweiser (time-lapse) Mikrokinematographie

Das Verhalten der Zellen wurde mit etappenweiser Mikrokinematographie an lebenden Schwämmen untersucht. Die Schwämme entwickelten sich von Gemmulae und lebten zwischen Objektträgern. Verschiedene Teile der Schwämme, die Wachstumszone, das Kanalsystem und die Geißelkammern wurden gefilmt, bei einer Bildfrequenz von einer Aufnahme je Sek. Objektiv: 40×, Okular 7 oder Objektiv: 60×, Okular 7, mit Phasenkontrast. Es ließ sich die erhebliche Veränderlichkeit der Form und Lokalisation der Amoebozyten sowie auch ihrer Beziehungen zu den stabileren Zellgruppen (Geißelkammern, Flächenepithel) feststellen. Der Typus der Fortsätze und die Art der Bewegung der Amoebozyten ändern sich je nach ihrer Lokalisation. Die sog. Lophozyten (Zellen mit einem typischen „schwanzartigen" Fortsatz, wie sie von ANKEL und WINTERMANN-KILIAN beschrieben wurden) sind ein Zwischenstadium der Nukleolen-Amoebozyten. Diese Form gewinnen die Amoebozyten an den Oberflächen des Schwammkörpers, während sie sich in den mittleren Teilen des Schwammkörpers in einer anderen Gestalt ausbilden die als Collenzyte oder Nervenzelle beschrieben wird.

ИЗУЧЕНИЕ ПОВЕДЕНИЯ КЛЕТОК ПРЕСНОВОДНОЙ ГУБКИ *EPHYDATIA FLUVIATILIS* С ПОМОЩЬЮ ЦЕЙТРАФЕРНОЙ МИКРОКИНОСЪЕМКИ

С помощью цейтраферной микрокиносъемки производилось прижизненное наблюдение за поведением клеточных элементов губки *Ephydatia fluviatilis*, развивающейся из геммулы и расположенной между двумя стёклами. Съемка велась в различных участках тела: области зоны роста, канальной системы и жгутиковых камер с интервалом 1 кадр в секунду при увеличении микроскопа об. 40×ок. 7 или об. 60× ок. 7. Показана чрезвычайная вариабильность формы, положения амебоцитов, а также и взаимосвязей этих

клеток с более устойчивыми клеточными группировками (жгутиковыми камерами, пинакоцитными эпителиями). Местоположение амебоцита отражается на характере его отростков, типе движения и т. п. Так называемые лофоциты — клетки с характерным шлейфоподобным отростком, описанные у пресноводных губок *Анкелем* и *Винтерманн—Килиан* — на исследованных стадиях развития являются временным состоянием ядрышковых амебоцитов. Эта форма клеток появляется в связи с пребыванием амебоцитов в поверхностных участках тела губки. Другие формы клеток, описанные как колленциты или нервные клетки, возникают из амебоцитов в связи с пребыванием их в средних участках тела губки и также являются врменными.

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STUDIES ON CARTILAGE FORMATION

X. PHOSPHOGLUCOSE ISOMERASE ACTIVITY OF THE CHONDRIFYING ARTICULAR SURFACE IN VARIOUS STAGES OF REGENERATION

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(Received March 23, 1966)

Synopsis

Experiments were conducted on 19 dogs, removing the articular surface of the distal part of the femur, according to Krompecher's method. The dogs were sacrificed 7, 33 and 70 days later. After isolating the granulation tissue adhering to the articular surface, as well as that adhering to the articular capsule, the phosphoglucose-isomerase activity of these tissues was examined in the stages mentioned above, by Bodansky's method. The activity of the chondrifying granulation tissue adhering to the articular surface was compared with that of the granulation tissue adhering to the capsule showing no signs of chondrification. No essential differences were noted either between these tissues or between the stages of slight chondrification (33 days) and that of increased cartilage formation (70 days) of the granulation tissue adhering to the articular surface. These findings seem to indicate that the Embden-Meyerhof pathway extending to phosphoglucose isomerase does not change essentially in the course of cartilage formation and differentiation.

Introduction

After complete removal of the articular surface of the distal part of the femur from the knee joints of adult dogs, cartilage tissue was found to develop during regeneration [5, 8].

Studying the period of the first 70 days of regeneration, we have demonstrated that in the period preceding chondrification the rate of capillarization [5] and the haemoglobin content [10] of the articular surface gradually decreased, whereas its lactic-acid level [10] and anaerobic and aerobic glycolytic activity [6] after an initial increase, diminished at the appearance of the cartilage tissue. In this period the regenerating articular surface was found to produce hexosamine in the presence of glutamine and glucose, both under aerobic and anaerobic conditions [11].

On the basis of our findings we presumed the Embden-Meyerhof pathway to become first prevailing, then partially blocked, during the process of chondrification, one of its intermediaries being used to produce mucopolysaccharides. Glucose-6-phosphate or fructose-6-phosphate may be such intermediaries which can serve as sources for glucosamin-N-acetyl derivatives [13] and UDP acetyl-

galactosamine sulphate, the essential building stones of mucopolysaccharides [4, 16]. In the present study the phosphoglucose isomerase activity was investigated in connection with the partial block of glycolysis.

Material and method

Nineteen dogs were involved in the experiments. The knee-joint was operated on according to KROMPECHER's method [8]. After removing the articular surface a haematoma developed there, giving rise to granulation tissue. The granulation tissue (Fig. 1) was found to adhere partly to the bone wound ("granulation tissue adhering to the articular surface") and partly to the articular capsule ("granulation tissue adhering to the capsule"). The former underwent chondrification, whereas the latter did not.

Dogs were killed 7 days (5 dogs), 33 days (7 dogs) and 70 days (7 dogs) after operation. The granulation tissue adhering to the articular surface as well as that adhering to the capsule were separately collected in a dish each, cooled with icy water and minced. From the minced tissue an alcohol-ether powder was prepared [2]. Then an extract was made by adding 20 volumes of 5% $NaHCO_3$ and after 15 minutes' shaking the extract was centrifuged [15]. The activity of the resultant extract was measured [1, 15] as follows: 0.1 ml extract was added to 1.0 μM glucose-6-phosphate dissolved in 0.4 ml tris buffer (pH 9.0, 0.05 M). After an incubation of 15 minutes at 30° C, 3—5 ml 8.3 N HCl and 1.0 ml 1% alcoholic resorcin solution were added and the solution was kept at 80° C for 10 minutes. After cooling to room temperature photometry was performed at 540 mμ. Parallel with each determination a blank was run where the enzyme extract was added only after addition of hydrochloric acid. Fructose served as standard (80—200 μg per 0.5 ml). The difference between the values obtained at zero time and those measured after incubation gave the amount of fructose produced.

Results

Experimental results are summarized in Table 1 and Fig. 2. The data represent the amount of substrate (in μmol) converted in 1 minute by the enzyme contained in 1 g dry matter.

Table 1

Time elapsed after operation:	No of animal							Average:
	1.	2.	3.	4.	5.	6.	7.	
Granulation tissue adhering to the articular surface:								
7 days	2.37	7.98	0.37	3.66	3.41	—	—	3.36
33 days	8.22	7.20	9.63	14.70	12.60	9.92	8.66	9.92
70 days	9.36	11.40	13.70	9.63	5.26	12.50	8.15	10.00
Granulation tissue adhering to the articular capsule:								
7 days	0.74	4.30	0.52	—	1.71	—	—	1.82
33 days	8.15	8.06	6.22	12.60	8.95	9.10	10.97	9.17
70 days	10.06	9.63	14.50	—	5.18	9.85	8.59	9.63

From these results it appears that the phosphoglucose isomerase activity both in the granulation tissue adhering to the articular surface and in that adhering to the capsule behaves similarly. In the 7-day-old stage, when histologically an indifferent granulation tissue is found, the activity is relatively

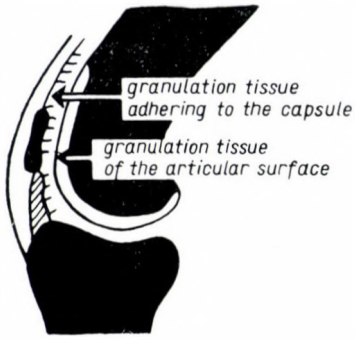

Fig. 1. Scheme of the knee-joint of a dog showing the topographic localization of the granulation tissue examined. One part of the granulation tissue originating from a common source adheres to the bone wound and later differentiates into cartilage. In the granulation tissue adhering to the articular capsule no cartilage formation can be observed

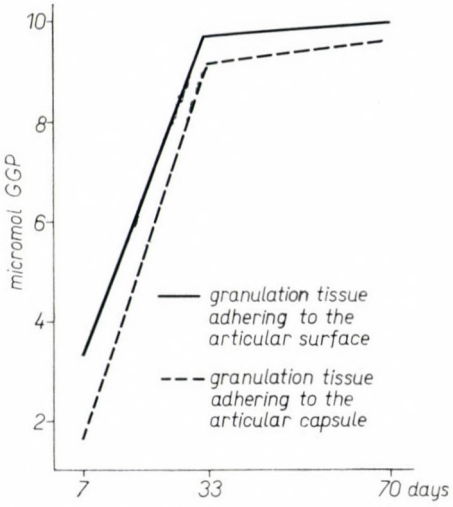

Fig. 2. Phosphoglucose-isomerase activity of granulation tissue. G-6-P (in μM) converted by 1 g dry matter in 1 minute; see Table

low (3.36, resp. 1.82 μmol converted G-6-P per minute per g dry matter). In the 33-day-old stage, when circumscribed cartilage islets appear in the granulation tissue adhering to the bone wound, the activity is markedly increased (9.92 and 9.17, respectively). In the 70-day-old stage when coalesced cartilage islets are found in the poorly vascularized granulation tissue overlying the bone wound, the activity remained unchanged (10.00 and 9.63).

Discussion

The present observations seem to indicate that, though there is a marked change in phosphoglucose isomerase activity between the 7- and 33-day stages, no characteristic change in activity was found during cartilage formation. The two layers (chondrifying and nonchondrifying) displayed about the same activity. The Embden-Meyerhof pathway extending to phosphoglucose isomerase or the preceding steps, do not seem to be blocked. In our case it seems unlikely that glucose-6-phosphate was the source of the amino sugar component. This result directs our attention towards fructose-6-phosphate and phospho-fructokinase. OHKUBO, NOWINSKI and BLOCKER, Jr. [12] found the Embden-Meyerhof pathway blocked with the latter enzyme in skin wounds in healing. Other data [3, 9] seem to support the assumption that phosphofructokinase may play an important role in the regulation of glycolysis.

The phosphoglucose-isomerase activity measured by us was fairly high. According to data in the literature [14] 1 g of fresh skeletal muscle possesses an activity of 0.083 μM.

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UNTERSUCHUNGEN ÜBER DIE KNORPELBILDUNG

X. Die Phosphohexoseisomerase-Aktivität der knorpelbildenden Gelenkfläche in verschiedenen Stadien der Regeneration

Vorliegende Untersuchungen wurden an 19 Hunden durchgeführt. Es wurde die distale Gelenkoberfläche des Femurs nach der Methode von Krompecher entfernt (Gelenkknorpel mit einigen mm Spongiosa-Grundlage). Die Tiere wurden am 7., 33. bzw. 70. Tag nach dem Eingriff getötet und untersucht. Das Granulationsgewebe von der Wunde der Gelenkfläche und das an der Gelenkkapsel haftende Granulationsgewebe wurden isoliert und die Phosphohexoseisomerase-Aktivität mit der Methode von Bodansky bestimmt. Es konnte kein wesentlicher Unterschied zwischen der Aktivität des knorpelbildenden Granulationsgewebes der Gelenkfläche und dem keine Knorpeldifferenzierung zeigendem, der Knorpelkapsel anhaftendem Granulationsgewebe nachgewiesen werden. Auch die Unterschiede zwischen dem Granulationsgeweben der in der Knorpelbildung weniger oder weiter fortgeschrittenen Gelenkoberflächen (33. bzw. 70. Tag) kann vernachlässigt werden. Diese Befunde weisen darauf hin, daß sich der bis zur Phosphohexoseisomerase reichende Abschnitt des Embden-Meyerhoff Weges im Laufe der Knorpelbildung bzw. Knorpeldifferenzierung nicht wesentlich verändert.

ИССЛЕДОВАНИЕ ХРЯЩЕОБРАЗОВАНИЯ

X. Активность фосфоглукоз-изомераза на поверхности кондрификаиионного сустава при различной стадии регенерация

В ходе экспериментов по исследованию хрящеобразования авторы удалили по методу Кромпехера дистальную поверхность коленного сустава (суставной хрящ с несколькими миллиметрами губчатого вещества) бедренной кости 19 собак. Через 7, 33 и 70 дней после вмещательства животные забивались. Грануляционную ткань, прилипающую к суставной поверхности и грануляционную ткань, прилипающую к суставной сумке авторы изолировали и в указанных стадиях проводили по методу Боданского определение фосфоргексо-изомеразной активности. Они установили, что между активностью хрящеобразующей грануляционной ткани суставной поверхности и активностью грануляционной ткани, прилипающей к сумке, нет существенной разницы. Между активностями грануляционной ткани суставной поверхности, определенными в различных стадиях хрящеобразования (33-ий и 70-ый день) также отмечалось лишь пренебрегаемое отклонение. Эти результаты показывают, что в ходе хрящеобразования, вернее дифференциации хряща, участок до фосфоргексоз-изомеразы пути Эмбден—Мейерхофа не изменяется в существенной мере.

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EPENDYMOSECRETION (EPENDYMAL NEUROSECRETION)

VI. LIGHT AND ELECTRON MICROSCOPIC EXAMINATION OF THE SUBCOMMISSURAL ORGAN OF THE GUINEA PIG

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Synopsis

Light and electron microscopic structure of the subcommissural organ of the guinea pig was examined. By light microscopy it has been established that the ependymal cells contain two different Gomori-positive substances: The A_1 granules are moderately staining structures of indistinct borders appearing in a violet colour with aldehyde fuchsin and arranged all over the cytoplasm, while the A_2 substance consists of apically-arranged, intensively staining granules larger in diameter and showing sharp contours; they appear purple in the aldehyde fuchsin stained preparation. By electron microscopy it has been established that the A_1 substance is a secretory vesicle with reticular inner structure. The vesicles are produced in the ergastoplasm. Similar secretory vesicles are also produced in the hypendymal glial cells. The A_2 substance is a corpuscle of dense, granular content, larger than the former, surrounded by a membrane and differing from the A_1 type substance in its electron microscopic structure. The development of these granules could not be observed. Among the ependymal cells two types can be distinguished: In the one type relatively large A_1 vesicles of a dilute content as well as an ergastoplasm with a number of wide cisternae can be found. In the other type there are many small A_1 vesicles of denser content as well as little ergastoplasm with narrow cisternae. The appearance of the two cell types was brought into connection with the different state of equilibrium of the production, storage and discharge of the A_1 vesicle.

Introduction

Ependymosecretion is the process in the course of which the ependymal cells of the central nervous system exercise a special secretory function similarly to glandular cells [29, 30]. The most characteristic such ependymal cell group is found in the form of the subcommissural organ in which the ependymal cells produce a Gomori-positive substance. The secretory material of the subcommissural organ which is discharged partly into the cerebrospinal fluid, partly — probably — into the blood, is involved in a number of physiological processes [10, 11, 18, 20, 22, etc.].

Electron microscopic examination of the subcommissural secretion has been dealt with in connection with the following species: *Myxine glutinosa* [1], petromyzon [16], frog [15, 19], *Gecko japonicus* [13, 14], rat [9, 12, 25, 27, 28, 31, 32], guinea pig [12], *Bos taurus* [2, 7].

In our earlier comparative morphological investigations and histochemical examinations in progress we have found that in the subcommissural cells of some species the Gomori-positive substance, first of all that demonstrable

with aldehyde fuchsin staining, is not homogeneous, it consists of two morphologically readily distinguishable Gomori-positive materials. The two substances differ from each other in arrangement, shape and staining intensity. Since from the aspect of structure and function of the subcommissural organ the exact knowledge of the contained material is very important, in the present work the subcommissural organ was examined with light and electron microscopic methods, with special regard to the electron microscopic structure of the substances contained by the ependymal cells.

For these examinations we chose the guinea pig because in the subcommissural organ of this animal the two substances referred to are readily observable. The light microscopic structure of the subcommissural organ of the guinea pig was studied by BARRY [3, 4], BOSQUE, ARRANZ and ARNAIZ [5], OKSCHE [17], PESONEN [23], WISLOCKI and LEDUC [33]. To its electron microscopic structure data were contributed by LIN and DUNCAN [12].

Material and method

For light microscopic examinations Bouin- or Helly-fixed and paraffin-embedded sections were stained with Gomori's aldehyde fuchsin according to GABE and with chrome haematoxylin as modified by BARGMANN. Acid phosphomonoesterase reaction was carried out according to PÓSALAKY and VADÁSZ [8]. Naphthol AS-BS-phosphate was used as substrate and 5-chloro-2-toluidene employed as unstable diazonium salt. The brains used for the phosphatase reaction were fixed in a mixture of 4 per cent neutral formol and 0.22 *M* sucrose at 0° C for 5 hours. After fixation the material was washed for $^1/_2$ hour with 0.22 *M* sucrose three times and for 15 hours with 0.88 *M* sucrose at 0° C. Subsequently 10 μ thick cryostate sections were prepared. After an incubation in Pósalaky—Vadász' solution at 37° C for $1^1/_2$ to 2 hours the sections were covered with glycerol gelatin. Part of the cryostate sections were stained as a control with aldehyde fuchsin.

For electron microscopic examinations the heads of the animals were perfused with the aid of an apparatus assemble according to PALAY [21]. The perfusion fluid was heated with a water-jacket taken from an ultrathermostate. Omitting artificial respiration, after having opened the thorax, a glass cannula was led through the left heart ventricle into the ascending aorta and there fixed with ligature. Blood was washed off with Palay's solution for 20 to 30 seconds and subsequently fixation was carried out by perfusing 4.5 per cent glutaraldehyde solution (in sugar-free Millonig buffer diluted to the half) for about 20 minutes. After preparing the corresponding brain portion, fixation was continued for about 30 minutes in the same fixative and then the preparations were washed for several days at 5° C in Millonig solution containing 7,5 per cent sucrose. Post-fixation in 1 per cent OsO_4 solution for two hours, embedding in araldite. The ultrathin sections were prepared by Reichert's OMU-2 ultramicrotome and after lead-citrate staining examined in the JEM 6C electron microscope.

Results

Light microscopic structure. The subcommissural organ of the guinea pig consists — similarly to that of other vertebrates — of two parts: the ependymal and hypendymal part. The ependymal part is built up by epithelium-like cells arranged in several rows, whereas the hypendyma by glial and nerve fibres, glial cells and a number of capillaries. The cells constituting the ependymal part are arranged in three rows: in the surface, medium and deep row.

The cells in the surface row are conical, their apical part forms the ventricle surface. The cells of the medium layer are bipolar and they contact the ventricle surface only by a thin process, their basal process extends into the area of the hypendyma. The basal cells are oval, generally unipolar, their process runs toward the hypendyma. Similar ependymal glial cells although entirely separated from the epithelium are found in the hypendyma.

In the cells of the ependymal part various substances can be observed with chrome haematoxylin and aldehyde fuchsin staining. In the cytoplasm of the ependymal cells, first of all in the perinuclear area, faintly staining, small granules with indistinct contours are found. To facilitate discussion, we named these granules "A_1" substance. The A_1 material may fill up the whole cytoplasm, least densely its most apical part. As a contrast, on the apical part of the ependymal cells immediately below the surface a more intensively staining granular substance of distinct contours can be found. The granules of this material (A_2 substance) are larger than those of the A_1 substance. The dark, coarse-granulated A_2 material staining purple with aldehyde fuchsin di tinguishes itself from the paler, finely-granulated A_1 substance, which shows a bluish-violet hue. The A_2 granules appear only apically, first of all in the superficial cell layer. In the cells of the medium layer, too, they appear only in the apical process extending towards the ventricle surface. More seldom it can be found also in the deep cell row and in the cells of the hypendyma. Its amount is frequently less than that of the A_1 substance (Fig. 1).

Electron microscopic structure. The cells of the ependymal part of the subcommissural organ are very long. Their thickness is found to be 3—5 μ, the portion containing the nucleus is wider, 8—9 μ thick; the average length of the cells is 90 μ (Fig. 3). At their apical part the ependymal cells are connected by junctional structures (zonula adhaerens, zonula occludens [6]). The extension of these is varying, but in most cases they extend much deeper than in the surrounding wall ependyma (Fig. 2). *The surface of the cells* protrudes hernia-like among the desmosomes (Fig. 3), folds into the ventricle, on the surface some irregular mikrovilli can be observed and a few cilia, much fewer than in the wall ependyma. From the basal bodies of the cilia, cilial roots with transversal striation originate. No structure corresponding to Reissner's fibre could be demonstrated in the ventricle.

The median, bipolar and basal cells of the ependymal part have an ultrastructure similar to the apical cells. The basal and hypendymal cells are often arranged with their longitudinal axis parallel to the ventricle (Fig. 3, H_1, H_2).

The subcommissural cells differ from the normal wall ependyma in the first place by containing many *ergastoplasmic elements.* Characteristic parallel ergastoplasmic cisternae can be observed first of all next to the nucleus at its apical pole where the cisternae are arranged mainly parallel with the nuclear

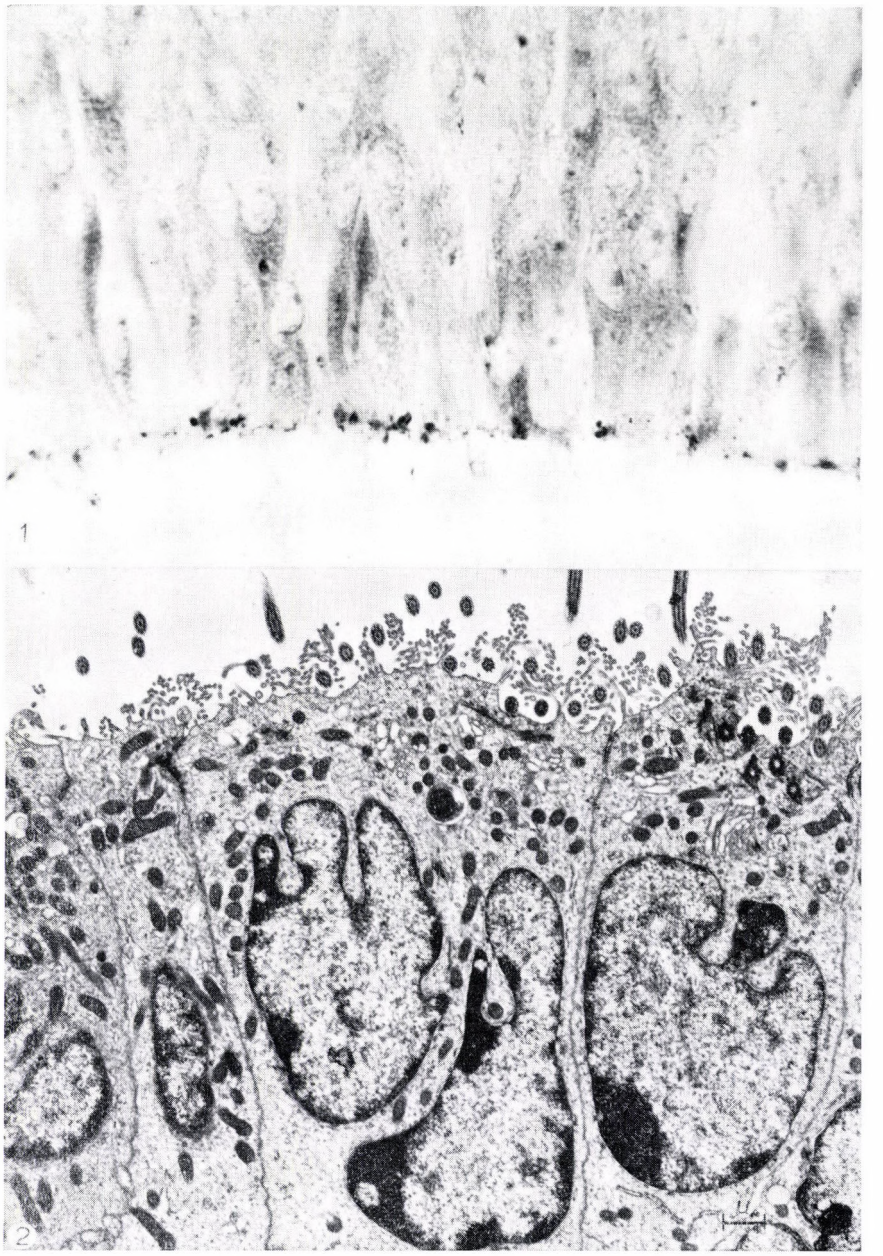

Fig. 1. Light microscopic picture of cells of the subcommissural organ. Note the diffusely arranged A_1 granules in the cytoplasm and the apical darker A_2 granules. Aldehyde fuchsin

Fig. 2. Normal wall ependyma from the environment of the subcommissural organ. On the surface of the ependymal cells of cubic character cilia and microvilli are seen in a comparatively great number. The cytoplasm contains besides mitochondria and Golgi apparatus only 1—2 ergastoplasmic cisternae. The invaginations of the nuclear membrane generally look toward the free surface

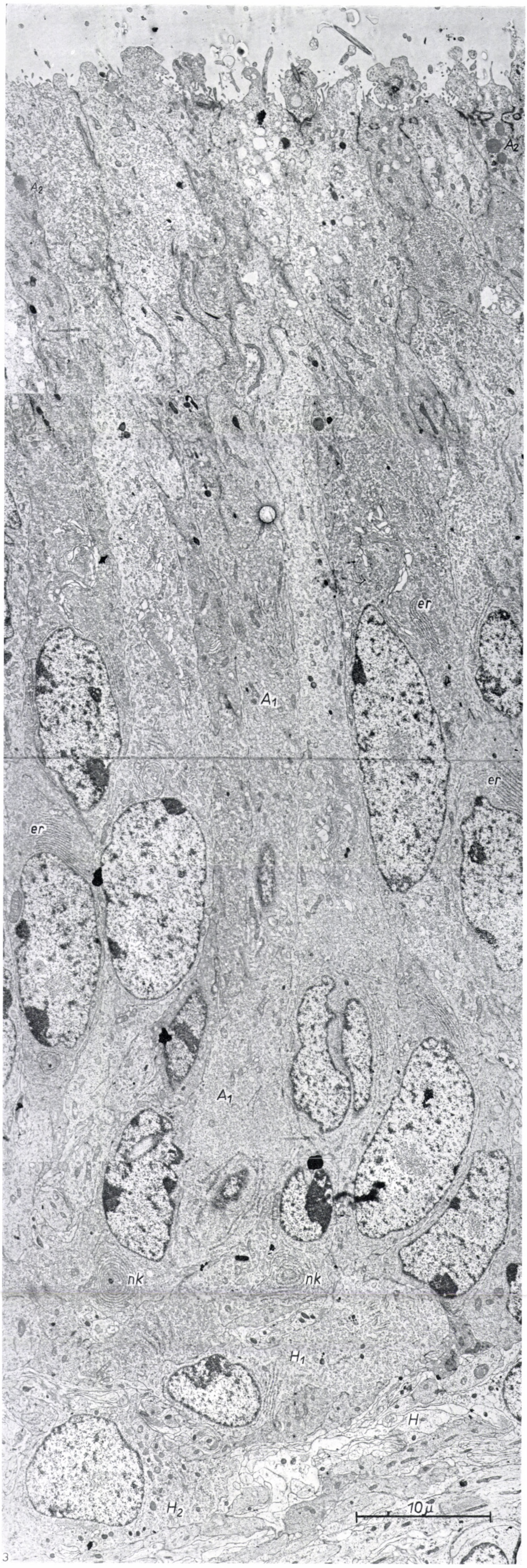

Fig. 3. Low-power electron micrograph of the cells of the subcommissural organ. The free surface of the exceedingly long, fibre-like cells bulges between the junctional structures into the ventricular lumen. The surface is very poor in cilia and microvilli. In the apical cytoplasm of the cells rather frequently dark round corpuscles (A_2) are found while the rest of the cytoplasm contains a great number of smaller secretory vacuoles (A_1). Note the arrangement of ergastoplasmic cisternae (er) around the apical pole of the nucleus: apically from here the Golgi apparatus is found. The rod-shaped mitochondria are scattered in the cytoplasm. In the basal cells the ergastoplasmic cisternae are sometimes concentrically arranged below the basal pole of the nucleus ("Nebenkern", nk). Two hypendymal cells (H_1, H_2) of identical structure with the former ones are well visible. H = hypendyma

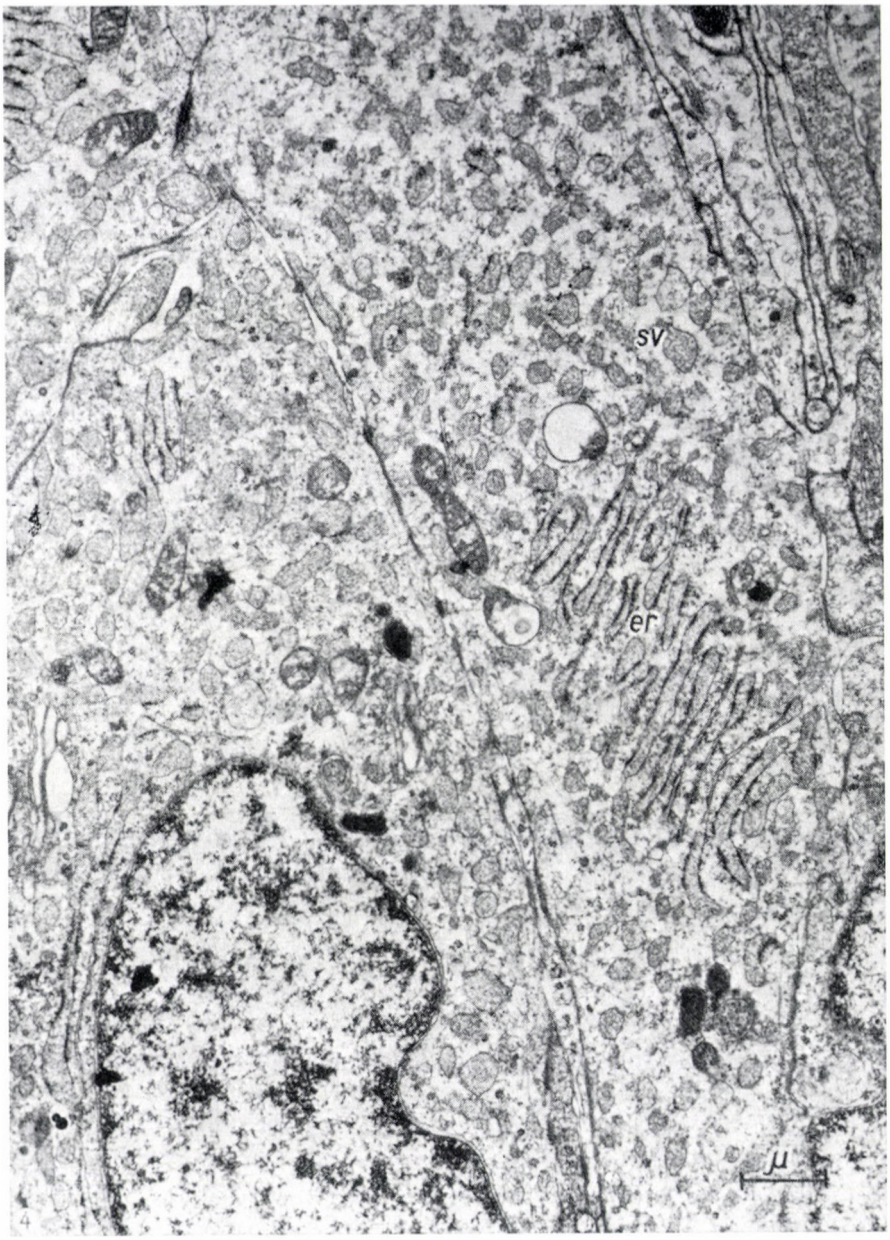

Fig. 4. Perinuclear region of the subcommissural cells. Note the great number of secretory vacuoles (sv) in the cytoplasm and the parallel ergastoplasmic cisternae (er)

membrane (Fig. 3). It is characteristic of these cisternae that the ribosomes adhere to the cisternal membrane only over some sectors while other sites are left free. The ribosomes are missing mainly at the marginal hollow of the cisternae (Fig. 5). Ribosomes are found not only bound to membranes but freely

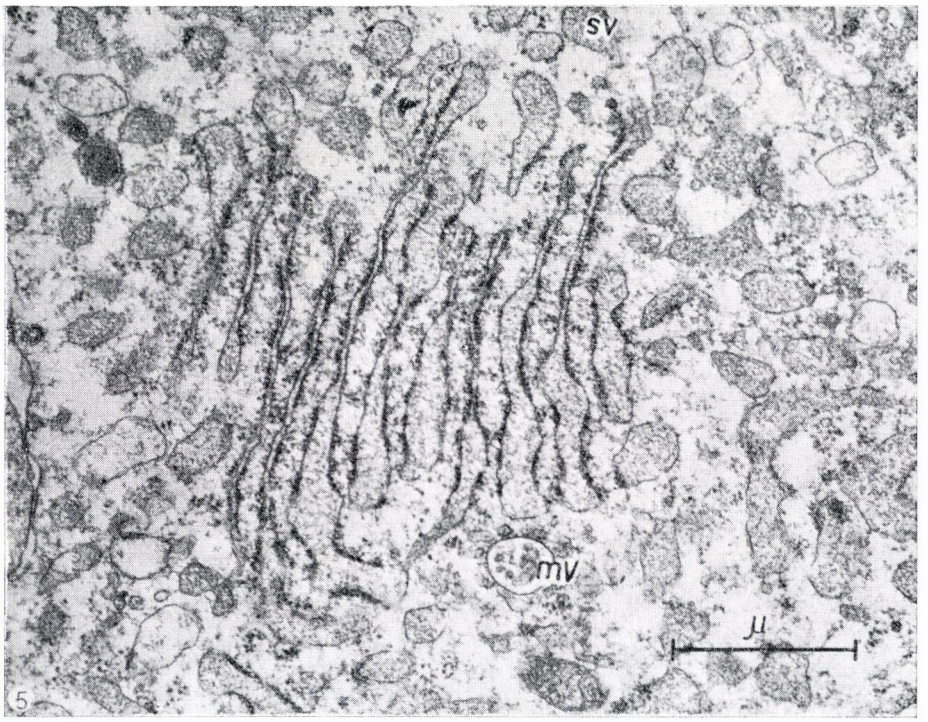

Fig. 5. Ergastoplasmic region consisting of parallel cisternae. In the cisternae the medium dense substance is readily seen which dilates the lateral parts of the cisternae. Ribosomes do not cover the whole surface of the cisternae, at some places they are lacking. sv = secretory vacuole, mv = multivesicular body

scattered in the cytoplasm where most of them form small groups, rosettes. In the interior of most cisternae there is a medium electron-dense, filamentously coagulated material which accumulates particularly at the dilated ends of the cisternae.

The whole cytoplasm of the cells, except for the most apical parts, is filled by *vacuoles* variable in diameter and shape (Figs 3, 4, 5 and 7). In the vacuoles a filamentous substance similar to the content of the ergastoplasmic cisternae is found. Between the dilated ergastoplasmic cisternae and the vacuoles referred to, all stages of transition are found (Fig. 5). Beside the marginal degranulated dilation of the ergastoplasm vacuoles, with several ribosomes attached to their membrane are often seen.

The ergastoplasmic cisternae are often found on the basal pole of the cell in a concentric arrangement and form "Nebenkern"-like structures (Fig. 3).

In some areas — mainly in the apical part of the cytoplasm — apparently empty vacuoles were observed. These were of the same size or larger than the

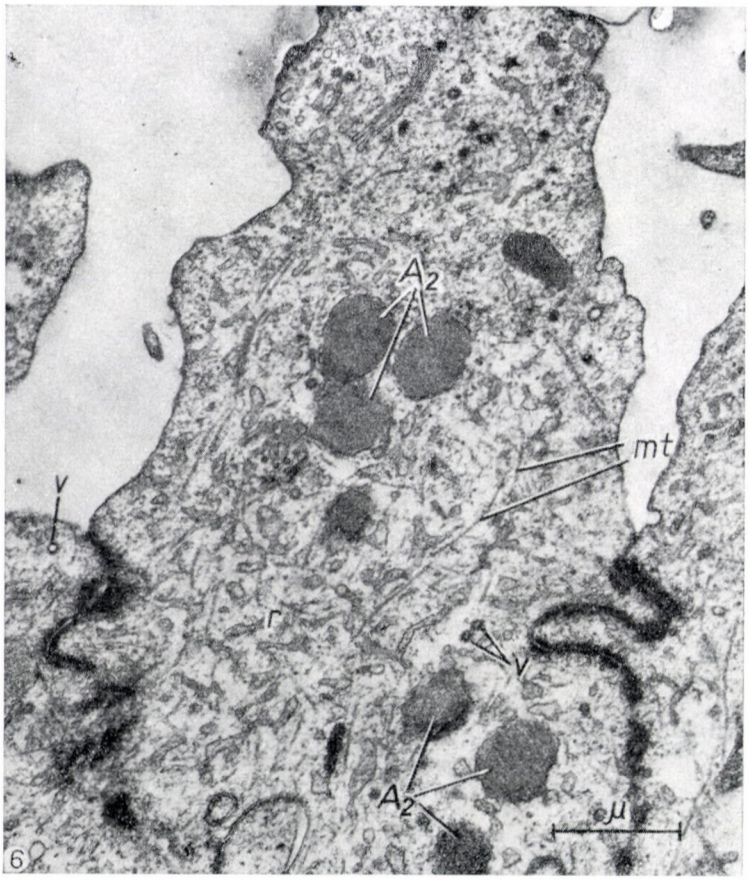

Fig. 6. Apical cytoplasmic portion of a subcommissural cell strongly bulging into the ventricle. A_2 = dark corpuscle, mt = microtubuli, r = endoplasmic reticulum, v = bristle-coated vesicle

vacuoles filled with reticular substance. In some of the larger vacuoles some filamentous substance may be found. Association of the large vacuoles with the cell membrane or opening of the vacuoles toward the ventricular space could never be observed.

The cytoplasm in some of the cells is lighter while that of other cells is darker. The *difference in density* (Fig. 3) depends on size and number of the secretory vacuoles in the cytoplasm and on the density of the substance in the vacuole.

In the apical part of the cells, next to the surface, in the area corresponding to the A_2 granules, round or oval *corpuscles of dense structure*, 0.5 to 1.5 μ in size are frequently found. These corpuscles are granular in structure and provided with a simple limiting membrane. Around the corpuscles — also in the apical part — in most cases some little irregular tubes and vesicles filled with medium dense substance and belonging to the endoplasmic reticulum are arranged. In the apical area occupied by the former structures, secretory vacuoles originating from the ergastoplasm only sporadically occur.

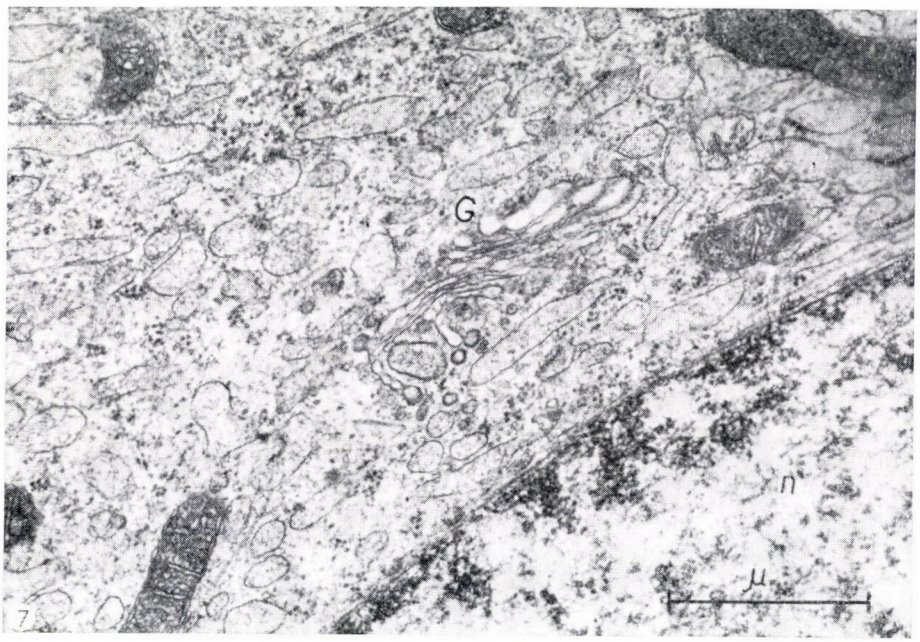

Fig. 7. Golgi apparatus (G) from the environment of the nucleus. n = nucleus

The *Golgi apparatus* (Fig. 7) is arranged in the region above the nucleus, but in one or two cases it was seen in the apical part of the cell. The Golgi-cisternae are as a rule dilated in our material, but this can be ascribed most probably to the effect of glutaraldehyde fixation. In some cases we succeeded in finding a connection between the ergastoplasmic cisternae and the Golgi elements, but we could never observe with certainty secretory vacuoles developing from Golgi-cisternae.

The mitochondria are longitudinal, and assuming often bizarre forms they are found in both the apical part and the basal one of the cells.

Cytoplasmic *microtubuli* (Fig. 6) can be detected in comparatively large numbers in the apical third of the cell; their arrangement is irregular, their main orientation parallel to the longitudinal axis of the cell.

Below the ventricular part of the cell membrane, mostly in the bulging portion of the cytoplasm, dark, mostly *star-shaped* irregular structures of 700—800 Å diameter are lying. In the same area ROTH and PORTER's [24] *bristle-coated vesicles*, described by PALAY and BRIGHTMAN [6] also in the ependyma, are found (Fig. 6). Finally, scattered in the cytoplasm, *large dark structures* are found with a dark, homogeneous content; their form is oval, often polymorphous.

The *nucleus* of the subcommissural cells (Fig. 3) is found in the central or basal part of the cell. It is mostly oval, showing some superficial retractions. The nucleolus mostly attaches to the interior surface of the nucleus' membrane. In the nucleus besides the characteristic chromatin structure some areas can be found where the chromatin particles produce characteristic, lattice-like, well-defined fields.

The hypendymal glial cells below the ependymal part are similar to the ependymal cells. In the glial cells A_1 vacuoles, their development and A_2 granules can be observed (Fig. 8).

Discussion

The main aim of our investigation was to examine the electron microscopic structure of the various Gomori-positive substances found in the cells with light microscope, but not discussed in the literature up to now. In the guinea pig's subcommissural organ the electron microscopic secretory vacuoles arranged in the whole cell body, consist of structures containing a reticular substance surrounded by a membrane. They are similar to the secretory vacuoles of the species examined by others. On the basis of their size and arrangement the vacuoles correspond to the granules named A_1 substance in our light microscopic examinations.

In our material the development of this substance from the ergastoplasm is easily observable. In the rat, according to literary data, the secretory vacuoles — the morphology of which is the same as in the guinea pig — develop in a closer connection with the nucleus; here the secretion appears in the duplication of the nuclear membrane [12, 28, 25]. In the guinea pig, on the other hand, the development of secretory vacuoles is in no such a close connection with the nuclear membrane, although a considerable part of the ergastoplasm lies next to the nucleus here, too. No relation was found between the secretory vacuoles and the Golgi apparatus as observed by MURAKAMI and TANIZAKI [15] in frog. Around the Golgi apparatus as a matter of fact smaller vacuoles appear which are, however, empty and do not show a transition to the secretory vacuoles. In our examinations the secretory substance seems to originate undoubtedly from the ergastoplasm. The ergastoplasm forming the vacuoles was found to be

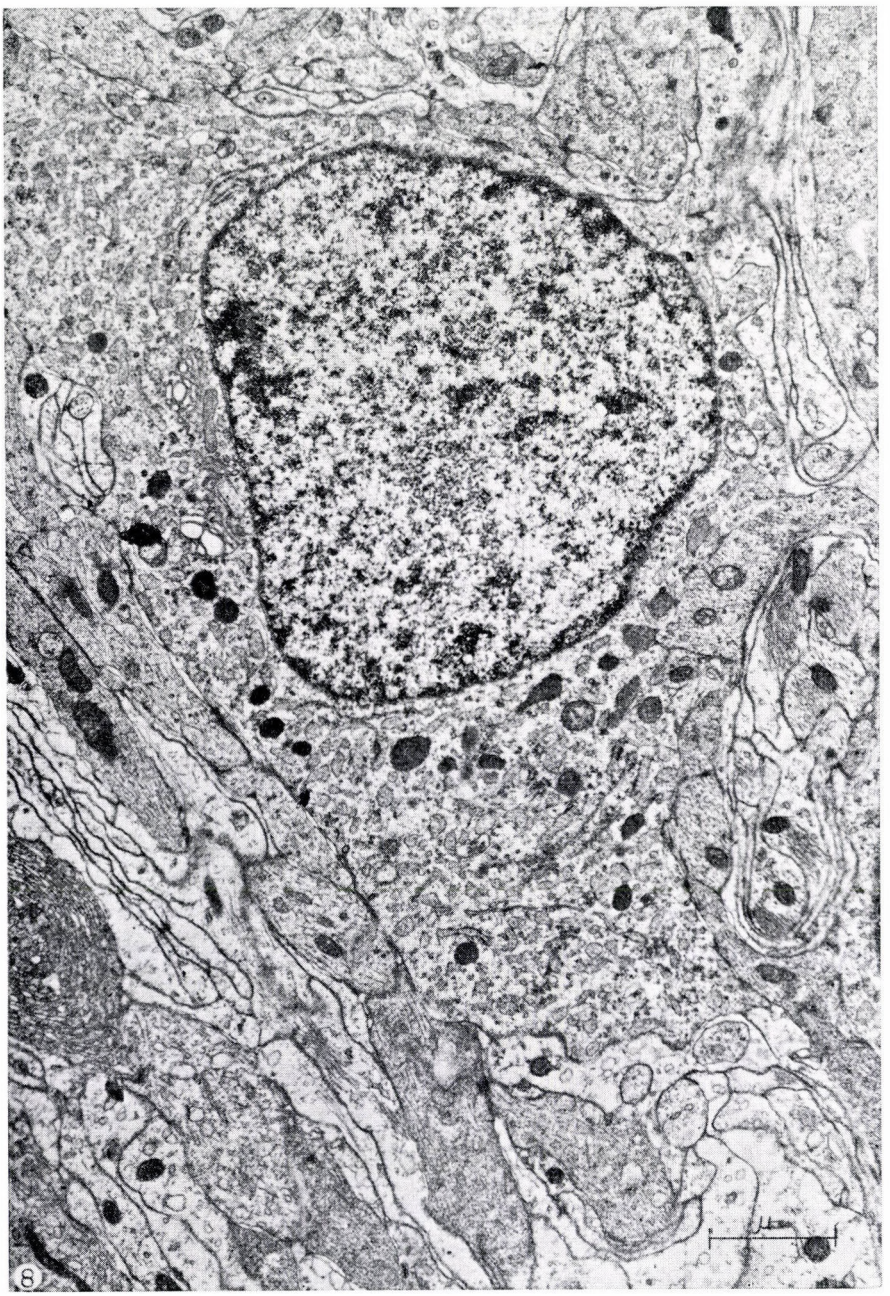

Fig. 8. Secretory glial cell below the ependymal layer

special: On the external surface of the cisternae the ribosomes do not form continuous rows but leave shorter areas free of granules.

In the hypendymal glial cells the secretory phenomena referred to can also be observed pointing to a similar, active secretory function of these cells.

It is remarkable that the cells are not uniform, the size, number and concentration of the content of the vacuoles is variable [s. also in 12]. In some cells the number of the secretory vacuoles is lower, the ergastoplasmic cisternae are wide, the content of the vacuoles is dilute, while in other cells rich in vacuoles with concentrated content there are few, flattened ergastoplasmic cisternae. The two forms can possibly be regarded as different secretory phases which represent different stages of secretion and discharge.

Besides the numerous pictures clearly demonstrating the production of secretory vacuoles no discharge of the vacuoles was observed either in apical or basal direction. This is presumably due to the momentary physiological condition of the organ (discharge, storage, etc.) and probably the substance examined represents first of all a subcommissural organ of storage character. It is probable, however, as described by several authors and as clearly shown by light microscope that the secretory material is discharged in the first place into the cerebrospinal fluid in apo- or merocrine form [12, 15, 16, 27, 28].

Besides the secretory vacuoles found in the whole cytoplasm and identifiable with the light microscopic A_1 substance characteristic structures can be observed with the electron microscope in the apical end of the cell. The number of structures surrounded with a membrane is lower, their size ranges from 0.5 to 1.5 μ, their contents are definitely electron-dense and finely granulated. On the basis of their arrangement, shape, size, contents and number they can be identified with the Gomori-positive granules found with the light microscope in the apical part of the subcommissural cells and named by us type A_2 granules.

The production of these corpuscles cannot be observed by electron microscopy unequivocally as that of the A_1 vacuoles. In this respect attention should be called to the apically arranged elements of the endoplasmic reticulum which partly are filled with a medium dense substance. Between the vesicles and the A_2 granules in some cases we succeeded in observing a communication and so, a possible relationship in genesis cannot be excluded.

Discharge of A_2 granules could not be observed in the present study. Since on this subject no literary data are available the proper place of the granules, their character as secretory or other substance could not be unequivocally determined. Since the light microscopic acid phosphatase reaction showed only evenly distributed granules in the cells (Fig. 9) the lysosomic character of the dark corpuscles (A_2 substance) accumulating at the apical part of the cytoplasm seems to be improbable. A decisive answer to this question can only be obtained by an electron microscopic acid phosphatase reaction or the histochemical

examination of other lysosomic enzymes. It may also be possible that the material of the A_2 granules is similar to that of the A_1 substance, but the content of the A_2 granules is only more concentrated or maturated. In this case the difference in staining would be explainable by the difference in the concentration of maturation. However, the possibility cannot be excluded that the dark apical granules represent a special secretory substance which is not identical with

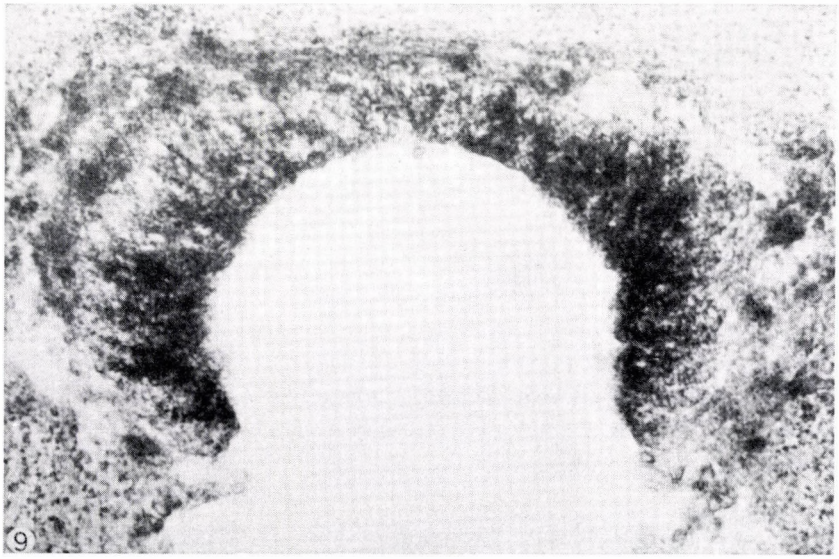

Fig. 9. Subcommissural organ with acid phosphatase reaction. Note the diffuse enzyme activity in the cytoplasm. The hypendyma also shows activity

that present in the A_1 granules. In order to elucidate the character of the A_1 and A_2 granules as well as that of the similarly apical, tiny, star-shaped, probably not Gomori-positive granules light and electron microscopic studies with radioisotopes are in progress.

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EPENDYMOSEKRETION (EPENDYMALE NEUROSEKRETION)

VI. Licht- und elektronenmikroskopische Untersuchung des Subkommissuralorgans vom Meerschweinchen

Die licht- und elektronenmikroskopische Struktur des Subkommissuralorgans des Meerschweinchens wurde untersucht. Mit dem Lichtmikroskop wurde festgestellt, daß die Zellen zweierlei Gomori-positives Material enthalten: Die A_1 Granula, die sich im gesamten Gebiet des Cytoplasmas lokalisieren, sind mäßig färbbare, mit Aldehydfuchsin lila erscheinende Gebilde mit verwaschenen Grenzlinien, während das A_2 Material aus sich apikal lokalisierenden, stark färbbaren, mit Aldehydfuchsin scharlachrot erscheinenden Granula besteht, welche einen größeren Durchmesser und scharfe Konturen haben. Mit dem Elektronenmikroskop wurde festgestellt, daß das A_1 Material ein Sekretvesikulum ist, welches über einen retikulären Inhalt verfügt und welches im Ergastoplasma entsteht. Seine Bildung konnte auch in hypendymalen Gliazellen beobachtet werden. Das A_2 Material besteht aus von einer Membran umgebenen Korpuskeln, die größer sind als das obige Vesikulum und einen dichten, granulären Inhalt haben, der sich in der elektronenmikroskopischen Struktur vom A_1 Material unterscheidet. Die Art der Entstehung der Granula konnte nicht beobachtet werden. Unter den Ependymzellen können zwei Typen unterschieden werden: In der einen Zellart kann man wenige A_1 Vesikel größeren Durchmessers mit dünnem Inhalt und ein Ergastoplasma finden, das über zahlreiche weite Zysternen verfügt. In dem anderen Zelltyp gibt es viele A_1 Vesikel kleineren Durchmessers mit dichterem Inhalt, sowie wenig Ergastoplasma, das enge Zysternen hat. Das Erscheinungsbild der zwei Zelltypen wurde mit einem verschiedenen Gleichgewichtszustand der Produktion, Speicherung bzw. Entleerung der A_1 Vesikel in Zusammenhang gebracht.

ЭПЕНДИМАЛЬНАЯ НЕЙРОСЕКРЕЦИЯ

VI. Исследование субкоммисурального органа морской свинки под световым и электронным микроскопом

Авторы исследовали под световым и электронным микроскопом структуру субкоммисурального органа морской свинки. Под световым микроскопом было установлено, что клетки содержат два различных Гомори-положительных вещества: зернышки A_1, располагающиеся по всей плазме, образования, окрашивающиеся альдегинфуксином в светлый, лиловый цвет, имеющие расплывчатую границу. Вещество A_2 располагается апикально; оно интенсивно окрашивается альдегинфуксином в пурпурокрасный цвет и состоит из зернышек с большим диаметром и резким очертанием. При исследовании под электронным микроскопом было установлено, что вещество A_1 состоит из пузырьков для секрета с содержимым сетчатой структуры, и оно образуется в эргатоплазме. Его образование наблюдается и в гипендимальных глиозных клетках. Вещество A_2 представляет собой тельце с густым зернистым содержимым, окруженное оболочкой; оно крупнее вещества A_1 и его электронномикроскопическая структура различна от структуры вещества типа A_1. Наблюдать способ возникновения зернышек не удалось. Среди эпендимальных клеток можно выделить два типа: в одном типе находятся немногочисленные пузырьки типа A_1 с жидким содержимым и крупным диаметром и эргатоплазма с многочисленными обширными цистернами. В другом типе обнаруживаются многочисленные пузырьки типа A_1 с более густым содержимым и меньшим диаметром, и эргатоплазма с немногими узкими цистернами. Наличие двух типов клеток авторы приводят в связь с различным состоянием равновесия между производством, накоплением и опорожнением пузырьков.

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A STUDY ON THE RESPONSE OF CHROMOSOMES TO ANTIBIOTIC TREATMENT

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Synopsis

Effects of several antibiotics, namely penicillin, streptomycin, chloramphenicol, terramycin and aureomycin on the root tips of different plants, viz., *Vicia faba*, *V. sativa*, *Lens esculenta*, *Trigonella foenum-graecum*, *Lathyrus sativus* and *Nigella sativa* were studied. Different concentrations of antibiotics were used. Two series of experiments — in one series direct treatment up to 168 hours and in the other series recovery treatment up to 168 hours after 24-hour direct treatment — were performed. The most important effect is fragmentation. Other significant effects are erosion, diplochromatids, non-synchronized nature of division, pycnosis and formation of micronuclei. Particularly in relation to fragmentation and division frequency, the effects were found to vary and conspicuous difference was noted in the susceptibility between species with and without high content of heterochromatin. It has been suggested that both fragmentation and division frequency may be affected when nucleo-protein metabolism is hampered. *Vicia sativa*, a plant with low heterochromatin content, shows more resistance in relation to chromosome breakage and less susceptibility to the antibiotics than the other plants tested. As with aureomycin, chromosome breakage has been found principally in the secondary constriction region, the probability of induction of localized chromosome breaks with the help of these antibiotics has been mentioned.

Introduction

The effects of the antibiotics on chromosomes and cell division have been gradually realized in recent years. Marked change in the structure and behaviour of chromosomes has been secured following treatment with a number of antibiotics by WILSON [13]. This study has a great bearing on soil mutagenicity especially where the antibiotics belong to the soil, as several groups of investigators have shown that the mutagenic property of the soil is contributed, to a significant extent, to bacterial decomposition products, antibiotics and decomposed plant pigments [1, 2, 9, 10, 14].

In spite of the exploration of the property of antibiotics in affecting chromosomes, no data is yet available to indicate whether the breakage is preferential or not. By the term "preferential", localization of effects, if any, is implied [5, 7]. It is not certain yet whether the secondary constriction segments, which often show preferential breakage following chemical treatment, have any selective response to antibiotics. In view of the lacuna existing in this aspect of investigation, the present series of experiments were undertaken on species of plants having pronounced secondary constrictions.

Material and method

The names of the plants on which antibiotics were used are given below: (1) *Vicia faba* L. (2) *V. sativa* L. (3) *Lens esculenta* MOENCH. (4) *Lathyrus sativus* L. (5) *Trigonella foenum-graecum* L. (6) *Nigella sativa* L.

The solutions of the antibiotics were prepared as follows: (1) Penicillin solution — Crystapen (1.000,000 units) of Glaxo Laboratories (India) was dissolved in 100 ml of distilled water and was used as 1% or stock. (2) Streptomycin solution — Streptomycin sulphate, equivalent to 1 g. (1 mega unit) streptomycin base, of Glaxo Laboratories (India) Private Ltd. was dissolved in 100 ml of distilled water (1%). (3) Chloramphenicol solution — Chloramphenicol capsule U.S.P. 250 mg of Parke Davies (India) Ltd. was dissolved in 25 ml of distilled water for 1% solution. (4) Terramycin solution — Terramycin, oxytetracycline hydrochloride capsule, 250 mg of Pfizer Ltd., was dissolved in 25 ml distilled water for 1% solution. (5) Aureomycin solution — Aureomycin, chlorotetracycline hydrochloride capsule, 250 mg of Lederle Laboratories Division, Cynamide India Ltd., was dissolved in 25 ml distilled water for 1% solution.

Germinating seeds with 1 to 1.5 cm long roots of *Vicia faba*, *V. sativa*, *Lens esculenta*, *Lathyrus sativus*, *Trigonella foenum-graecum* and *Nigella sativa* were taken for the experiments.

Two series of experiments were performed. In the first series, germinating seeds were placed at the mouths of several small tubes containing the different antibiotics of two different concentrations (1% and 0.5%) so that their roots were completely immersed in the solutions. Root tips from each tube were removed after treatment in the solution for 24 hours, fixed in acetic acid-ethyl alcohol mixture (1 : 1) for 1 hour, heated for a few seconds in a mixture of 2% aceto-orcein and *N* HCl (9 : 1) and finally squashed in 1% aceto-orcein solution. Observations were made up to seven days at intervals of 24 hours.

In the second series, the germinating seeds, after treatment for 24 hours in the two different concentrations of the above antibiotics, were transferred to Knop's solution for recovery. Observations were made at intervals of 24 hours up to seven days.

Observations were made using a Zeiss microscope with an aplanatic condenser of 1.3 N. A., apochromatic objective and a compensating eye-piece ×20 at a table magnification of ×2,900. Microphotographs were taken at a magnification of ×1,850 (approx.).

Observations and discussion

The different antibiotics that have been applied on root tips in the present investigation have shown marked effects on somatic cells, including different chromosomal aberrations and even a generalized effect on cell division. The most important of the effects is chromosome breakage or fragmentation followed by erosion. This behaviour was first noted by LEVAN [6] in his paper on Allium test. Since then it has been found to be a very common feature following chemical treatment; it definitely arises from differences in the stainability of chromosome segments, due to differences in the relative amount of nucleic acid present. It appears that following treatment with the chemicals, certain segments of chromosomes retain nucleic acid whereas others lose it. The latter areas become ultimately devoid of stain. The frequent appearance of non-stainable regions intermittently between stained segments of chromosomes gives the appearance of erosion.

In addition to the above, another peculiarity often noted is the appearance of diplochromatids, in which chromosomes, though split, do not move to two different poles, due to disturbance in the formation of the spindle apparatus. It was originally thought to be a clear manifestation of c-mitotic effect, but recently a number of cases have been seen where the diplochromatid appearance

unlike that induced by colchicine, is not necessarily followed by polyploidy [8, 11].

Another effect of the antibiotics observed so far is non-synchronized nature of division, resulting in different types of appearance in two daughter nuclei. In a number of cases, one of the two daughter nuclei may remain in a resting phase, whereas the other may show chromosome condensation. Such non-synchronization appears as an imbalance between the nucleus and the cytoplasm. The separation of the chromosome to the two poles and the formation of the daughter nuclei rest on a regulated time cycle which is pre-determined. The breakdown of this balance between nuclear and cytoplasmic metabolism results in disharmonious behaviour of the two sets of chromosomes. Evidently, the antibiotics applied here can interfere with the cell metabolism responsible for maintaining the balance. Condensation in prophase is another aspect of non-synchronization, implying that metaphase condensation of chromosomes has been arrived at by the chromosomes while the nucleo-cytoplasmic condition still lies at the prophasic stage. The disruption of metabolism as a result of antibiotic action is undeniable.

Another effect of antibiotics is the pycnosis of nuclei. Such pycnotic condition appears through the application of antibiotics for prolonged period or at a heavy concentration. As with other drugs, this effect results merely from toxicity to the tissue concerned.

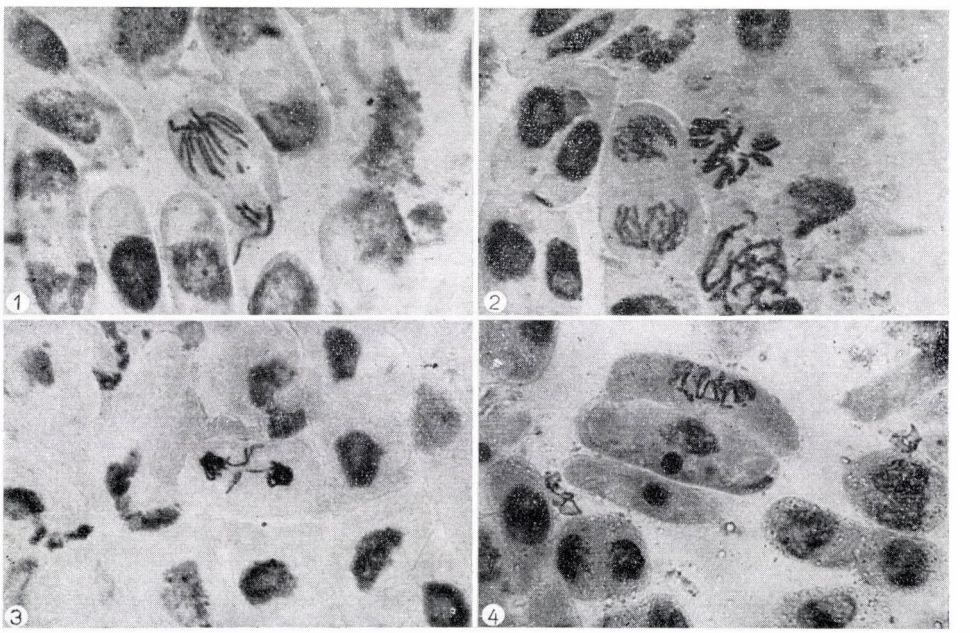
Lastly, micronuclei have often been found to occur in connexion with several antibiotics. Such micronuclei may originate from centric chromosome fragments or lagging chromosomes. It is not possible to state as to which of them have been responsible for the formation of a particular micronucleus. However, the presence of micronucleus principally suggests the occurrence of mitotic abnormalities in the previous division.

The different antibiotics tried in the present investigation were tested on six different species of dicotyledons. Of the different species of plants subjected to treatment, *Lens esculenta* and *Vicia sativa* are characterized by comparatively very low content of heterochromatin as compared to the high heterochromatin content in others. Excepting in *Vicia faba* where high heterochromatin content is already well established [3], the content of heterochromatin has been principally judged in other cases by the number of secondary constrictions.

Effect of penicillin

The principal effects that require special comment are fragmentation and division frequency. Particularly in relation to these two, the effects were found to vary and conspicuous difference was noted in the susceptibility between the species with and without heavy content of heterochromatin. *Trigonella foenum-graecum*, *Lathyrus sativus*, *Nigella sativa* as well as *Lens esculenta* show

chromosome fragments irrespective of the drug's concentration. Such fragments were found to persist, though with gradual recovery the frequency of their occurrence declined. Recovery in Knop's medium evidently results in a gradual restoration of the original condition of the nuclei.

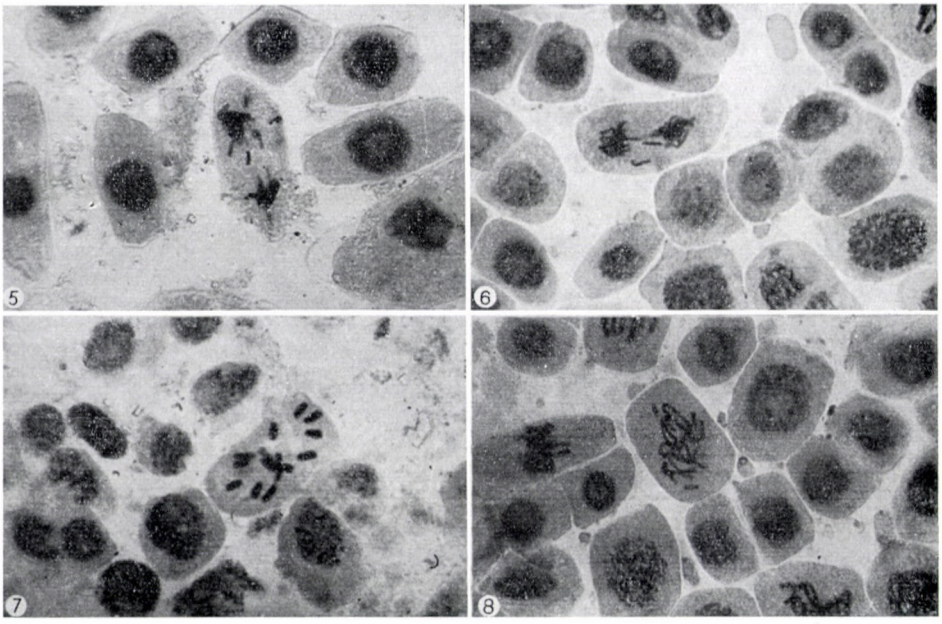


Figs 1—4. Effect of penicillin on plant cells
Fig. 1. Unequal separation and fragmentation of chromosomes in *Vicia faba* (1%, 24 hrs., Knop's, 120 hrs.)
Fig. 2. Diplochromatid structure of chromosomes in *V. faba* (1%, 24 hrs.)
Fig. 3. Lagging chromosome with chromatid bridge in *V. faba* (0.5%, 24 hrs., Knop's, 120 hrs.)
Fig. 4. Micronucleus in *V. faba* (1%, 24 hrs., Knop's, 96 hrs.)

In *Lens esculenta* and *Vicia sativa*, on the other hand, fragmentation was never observed after penicillin treatment. Whether the lack of fragments is due to the absence of sufficient amount of heterochromatin or any other cause cannot be definitely stated. In any case this difference in behaviour between species having heterochromatin and those lacking it is indeed remarkable. In a number of species the exaggerated appearance of telomeric heterochromatin is noteworthy. Under normal conditions, i.e., without any treatment in these species the heterochromatin of the telomeres does not appear to be pronounced. With penicillin treatment the stainability of the telomeric heterochromatin increases in metaphase, indicating heavy nucleic acid charge. Evidently, penicillin treatment affects the nucleic acid cycle in the heterochromatic segment in the sense that it, to some extent, inhibits the discharge of DNA by telomeric

heterochromatin in metaphase stage or may help the synthesis of DNA on these segments.

With regard to division frequency, certain induced changes require consideration. Similar to fragmentation, the effect on division frequency shows



Figs 5—8. Effect of streptomycin on plant cells
Fig. 5. Lagging fragments in *Vicia faba* (1%, 24 hrs., Knop's, 144 hrs.)
Fig. 6. Lagging chromosome with chromatid bridge in *V. faba* (1%, 24 hrs., Knop's, 96 hrs.)
Fig. 7. Contraction and scattering of chromosomes in *Vicia faba* (1%, 24 hrs., Knop's, 24 hrs.)
Fig. 8. Fragmention of chromosome in *V. faba* (1%, 24 hrs.)

marked difference in species with and without heterochromatin. In *Vicia sativa* and *Lens esculenta*, both in high and low concentrations of penicillin treatment, no effect on division frequency was observed. On the other hand, in all the other species the frequency of cell division was markedly influenced as compared to the controls. In certain cases, though in lower concentration the frequency remained the same, yet in higher concentration it became, at least, retarded. When transferred for recovery to Knop's medium, however, the frequency of division showed gradual rise due to the reversible nature of the reaction.

The penicillin treatment, therefore, has revealed two important peculiarities in the effect on plant cells, that is, in relation to division frequency and chromosome breakage. The differential response noted hereby is also correlated with the presence and absence of significant amounts of heterochromatin. At

the present state of our knowledge, it is not possible to work out clearly the way the heterochromatin exerts its effect on these two aspects of chromosome behaviour. It may however be suggested that both these processes may be affected when nucleoprotein metabolism is hampered. That heterochromatin exerts a profound effect on nucleoprotein metabolism has already been claimed by several investigators [4, 12]. In that case the influence of these specialized segments of chromosomes on breakage and division becomes explainable.

Effect of streptomycin

This antibiotic shows certain similarities in its effect with penicillin. Fragmentation was found in practically all the species except in *Vicia sativa* and to some extent in *Lens esculenta*. In *Vicia sativa* fragments were not found at any of the concentrations, whereas in *Lens esculenta* very few fragments could be recorded on direct treatment with 1% solution for 72 to 120 hours as well as on recovery for 48 to 96 hours. Excepting these cases, fragments could not be detected in any of the lower concentrations nor in any period of recovery.

In *Trigonella foenum-graecum* fragments appeared but few in several sets of experiments. The complete absence of fragments in *Lens esculenta* may suggest once more that the absence of significant amount of heterochromatin is the principal factor controlling their response to streptomycin in relation to fragmentation as noted with penicillin as well.

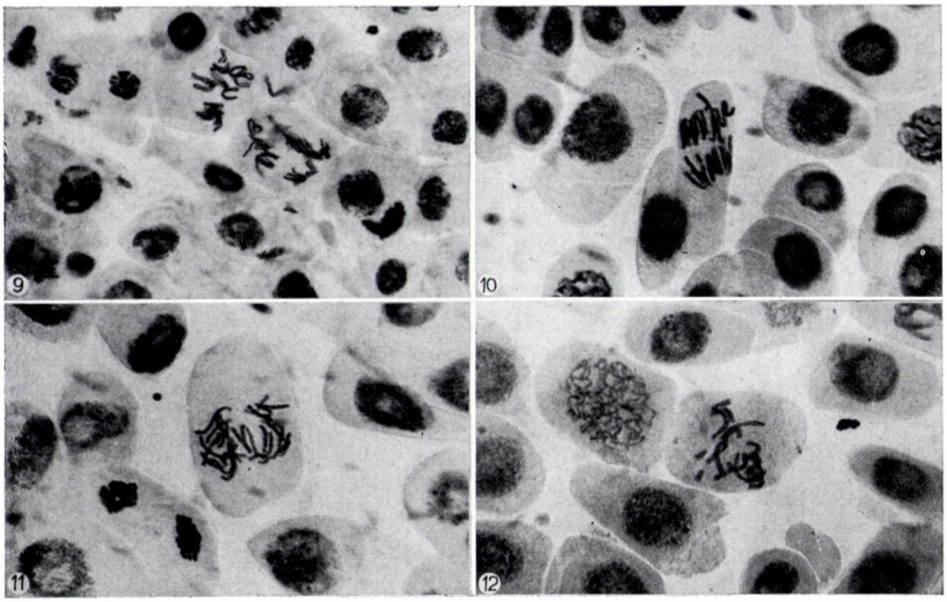
In *Vicia faba*, where the heterochromatin content is heavy, heavy fragmentation was noted in 1% solution both on direct treatment as well as after recovery. In the lower concentration, on the other hand, no chromosome breakage was observed. In this respect, streptomycin seems to be less effective than penicillin, specially as its capacity to induce fragmentation in plant species is concerned. It may be recollected that in *Vicia faba*, penicillin induced fragments at the lower concentration as well.

In addition to other abnormalities in chromosome behaviour noted both in penicillin and streptomycin, division frequency here too seems to be affected. The response with regard to this effect varies from one species to another. This is a reflection of the differentiated chemical setup of the cytoplasm of the different species. The cytoplasmic difference can certainly bring about a differential response so far as induction of mitotic activity and spindle effect is concerned.

Effect of chloramphenicol

Chloramphenicol too exerts a conspicuous effect on the chromosomes and nuclear division in various species. The most conspicuous difference in the response of plant cells to its effect is exhibited by *Lens esculenta*. With other antibiotics, no chromosome breakage could be recorded in *Vicia sativa* and

Lens esculenta. With chloramphenicol, however, at least in certain concentrations of the drug, chromosome breaks were recorded in *Lens esculenta*. *Vicia sativa* on the other hand, does not show any chromosome breakage whatsoever. So far as the effect of this antibiotic is concerned, this differential response cannot



Figs 9—10. Effect of terramycin on plant cells
Fig. 9. Diplochromatid structure of chromosomes in *Vicia faba* (1%, 96 hrs.)
Fig. 10. Fragmentation of chromosomes in anaphase in *V. faba* (0.5%, 24 hrs., Knop's, 144 hrs.)
Figs 11—12. Effect of aureomycin on plant cells
Fig. 11. Diplochromatid structure of chromosomes in *Vicia faba* (1%, 72 hrs.)
Fig. 12. Fragmentation of chromosome in *V. faba* (1%, 96 hrs.)

merely be attributed to the quantitative difference in heterochromatin as both the species are characterized by low heterochromatin content. The importance of this observation becomes more significant in view of the general effect of chloramphenicol on cells of other species of plants. Though chromosome breakage was recorded in the rest of them, in no case was the frequency of fragments comparable to the frequency obtained with other antibiotics. Evidently, the occurrence of chromosome breaks in *Lens esculenta* by chloramphenicol — a comparatively weak chromosome-breaking agent — is difficult to explain. If in general the effect of this antibiotic was strong compared to others, the occurrence of breaks in *Lens esculenta* would have been explainable by assuming that the severity of this effect overpowers the resistance offered by *Lens esculenta*, with low heterochromatin content. In view of the fact that chloramphe-

nicol does not show any such severe effect, it appears that the response of *Lens esculenta* to chloramphenicol is not merely limited by its heterochromatin content.

The frequency of division, however, has shown different data in different species. This is a factor which is conditioned to a significant extent by the physiological condition of the roots, not externally detectable. However, in none of the cases was lethal effect observed as shown by the continued occurrence of cell division as well as the recovery of the tissue in Knop's medium.

Effect of terramycin and aureomycin

The effects of these antibiotics more or less follow the same pattern as those of penicillin and streptomycin. In *Vicia sativa*, similar to all the other antibiotics, no fragment could be recorded in any concentration of the drug or in any period of recovery. In *Lens esculenta*, though fragments have been recorded, these were very few in number as compared to the effects observed with other antibiotics. However, especially with aureomycin treatments, cells containing fragments are very few in number after 24 and 48 hours of recovery. This is possible because cells with heavy fragments either do not divide or those with fewer fragments take a longer period to start further division.

From the series of results obtained with different antibiotics, it may be concluded that *Vicia sativa*, a species with low heterochromatin content, shows more resistance in relation to chromosome breakage and less susceptibility to the antibiotics than others. Especially with aureomycin, chromosome breakage was found to be principally involved in the secondary constriction region. This may also hold true for other cases as well but definite evidences in support of this behaviour with other antibiotics are yet to be obtained. Once an understanding of this issue is secured, then these antibiotics will be classed with a few other rare compounds which can induce localized chromosome breakage.

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UNTERSUCHUNG DER CHROMOSOMEN VERÄNDERUNGEN NACH ANTIBIOTIKUM BEHANDLUNG

Es wurde die Wirkung verschiedener Konzentrationen einiger Antibiotika (Penicillin, Streptomycin, Chloramphenicol, Terramycin und Aureomycin) auf die Wurzelspitzen von *Vicia faba*, *V. sativa*, *Lens esculenta*, *Trigonella foenum-graecum*, *Lathyrus sativus* und *Nigella sativa* untersucht. In der ersten Versuchsreihe wurde eine 168 Stunden lang dauernde direkte Behandlung durchgeführt, in der zweiten Reihe folgte nach 24 stündiger direkten Behandlung eine 168 Stunden lang dauernde Wiederherstellungsbehandlung in Knop-Medium. Die bedeutendste Folge der Behandlung war Fragmentation der Chromosomen. Weitere signifikante Wirkungen waren Erosion, Diplochromatiden, asynchrone Teilungen, Pyknose und Bildung von Mikronuklei. Es ließen sich vor allem in bezug auf Fragmentation und Teilungsfrequenz Unterschiede nachweisen und Spezies mit viel und wenig Heterochromatin waren auffalend verschieden empfindlich. Es darf angenommen werden, daß sowohl die Fragmentation, als auch die Teilungsfrequenz von Störungen des Nukleoproteinstoffwechsels beeinflußt werden. *Vicia faba* — eine Pflanze mit wenig Heterochromatin — ist gegenüber Chromosomenbrüchen resistenter und gegen Antibiotika weniger empfindlich, als die anderen untersuchten Arten. Da mit Aureomycin hauptsächlich in den sekundären Konstriktionen Brüche verursacht werden, wird die Möglichkeit der Induktion lokalisierter Chromosomenbrüche mit diesen Antibiotika erörtert.

ИЗМЕНЕНИЯ ХРОМОСОМ ПОСЛЕ ПРИМЕНЕНИЯ АНТИБИОТИКОВ

Авторы исследовали действие применения различных концентраций некоторых антибиотиков (пенициллина, стрептомицина, хлорамфеникола, террамицина и ауреомицина) на различные виды растений: *Vicia faba. V. sativa*, *Lens esculenta*, *Trigonella foenum-graecum*, *Lathyrus sativus*, *Nigella sativa*. Были поставлены 2 серии опытов: одна серия с дополнительным применением антибиотиков в течение 168 часов, а другая с дополнительным применением антибиотиков в течение 168 часов после их 24 часового прямого применения. Наиболее важным изменением была фрагментация хромосом. Кроме того наблюдались эрозия, диплохроматиды, асинхронное деление, пикноз и образование микронуклеусов. Отклонения наблюдались главным образом в отношении фрагментации и частоты деления и заметная разница определяется и в чувствительности отдельных видов растений с высокой или низкой концентрацией гетерохроматина. Авторы полагают, что в случае препятственности метаболизма нуклеопротеинов поражаются фрагментация и частота деления. Растение *Vicia sativa*, имеющее низкое содержание гетерохроматина, показывает повышенную резистентность в отношении расхождения хромосом, и меньшую чувствительность к антибиотикам, чем остальные исследовавшиеся виды растений. Что же касается ауреомицина, расхождение хромосом было отмечено главным образом в области вторичного перехвата. Обсуждается возможность индукции локализованного расхождения хромосом при помощи вышеуказанных антибиотиков.

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ELECTRON MICROSCOPIC STRUCTURE OF ANTERIOR PITUITARIES TRANSPLANTED INTO THE ANTERIOR CHAMBER OF THE EYE IN RATS

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Synopsis

A homologous anterior pituitary was transplanted into the anterior chamber of the eye of male rats with an intact anterior pituitary. The grafts were examined 50 days later in the electron microscope. In the central parts of larger grafts — probably owing to the insufficient blood supply — the cells were degranulated. In the well-vascularized areas of the grafts all the chromophil cells could be recognized. The proportion of the chromophil cells changed, the prolactin-secreting cells became dominant. These cells showed signs of active granule production as well as of "intracytoplasmic granulolysis". The somatotropic cells were well-granulated. In the thyrotropic cells definite signs of granule production were observed; some of the granules were not round but elongated. The gonadotropic cells have decreased in number and diminished in size.

Introduction

Numerous authors have examined transplanted anterior pituitaries with the light microscope [4, 6, 8, 9, 16, 17, 19, 23, 29, 40, 42]. These investigations have revealed that in the anterior pituitary devoid of its direct hypothalamic connections the chromophil cells degranulate to a great extent.

Few data are available on the electron microscopic structure of transplanted anterior pituitaries. In autografts of anterior pituitaries of female rats Rennels [35] found an increase in the number of prolactin-secreting cells. He also established that occasionally the other types of granulated cells can also be recognized. Petrovic and co-workers [32] found in pituitary homografts transplanted into the anterior chamber of the eye every type of granulated cells; these authors do not mention an accumulation of prolactin-secreting cells. Recently Potvliege [33] described the ultrastructure of the pituitaries grafted under the kidney capsule of female rats thereby confirming Rennels' [35] previous data.

One of us observed with the light microscope in anterior pituitary grafts transplanted in the anterior chamber of the eye necrosis and subsequently fibrosis in the centre of the grafts where the tissue was poorly vascularized [23]. These investigations were continued by means of the electron microscope.

The aim was to study the electron microscopic changes occurring in the anterior pituitary transplanted into the anterior chamber of the eye. Attention

was also extended to the question whether the pituitary of male rats — containing few prolactin-secreting cells [2, 34] — undergoes the same alteration after transplantation as in the experiments of RENNELS [35] the pituitary of female rats, which are rich in prolactin-secreting cells.

It was also examined whether vascularization plays any role in causing structural changes in the grafted anterior pituitary.

Material and method

Anterior pituitaries of male rats of the same strain were transplanted into the anterior chamber of the eyes of 5 male rats whose pituitaries had not been removed. Into each of two animals a whole and into each of three animals a half anterior pituitary was transplanted. The method was described previously [23]. Fifty days after the transplantation the animals were decapitated without any anaesthesia. The transplanted pituitaries of the first two animals were fixed in 1 per cent buffered OsO_4 according to PALADE [30] and those of the latter three animals in 4 per cent glutaraldehyde buffered according to MILLONIG [27]. The pieces fixed in osmium were embedded in methachrylate and those fixed in glutaraldehyde following post-fixation in osmium, in Araldit.

Two untreated male and two untreated female animals of the same strain and age were used as controls. The pituitaries of these rats were also prepared for electron microscopic examination, using the above two methods.

The tissues embedded in Araldit were contrasted with uranyl acetate in 70 per cent alcohol. All the sections were stained with uranyl acetate and lead citrate.

The photos were made with a JEM-6C electron microscope. The photos joined to the paper were made from the substances fixed in glutaraldehyde.

Results

General observations

At the edge of the grafts a structure similar to the normal pituitary can be recognized. Among the branching cell columns dilated capillaries lined with fenestrated endothelium [11] are found. The cells packed in columns are surrounded by a common basal membrane and some collagen fibres (Figs 1, 5, 6). In accordance with the observations of FARQUHAR [10] and RENNELS [36], chromophobe cells occasionally form folliculi and pass microvilli into them. Follicular structures and microvilli also occur among the granulated cells (Fig. 4). Ciliated cells are not rare, their basal bodies being sometimes located in the Golgi region (Fig. 3). At the border of the grafts among the parenchymal cells occasionally unmyelinated nerve fibres could be observed.

Deviations from the structure of the intrasellar pituitary

In one of the grafts (it was a half anterior pituitary) many intercellular cavities varying in shape and width filled with a fine granular substance were present. The largest were 50—70 μ in diameter and reminded one of small cysts. The walls of the cavities are lined with different parenchymal cells. The external surface of the cells is surrounded by basal membrane and collagen fibres (Fig. 2).

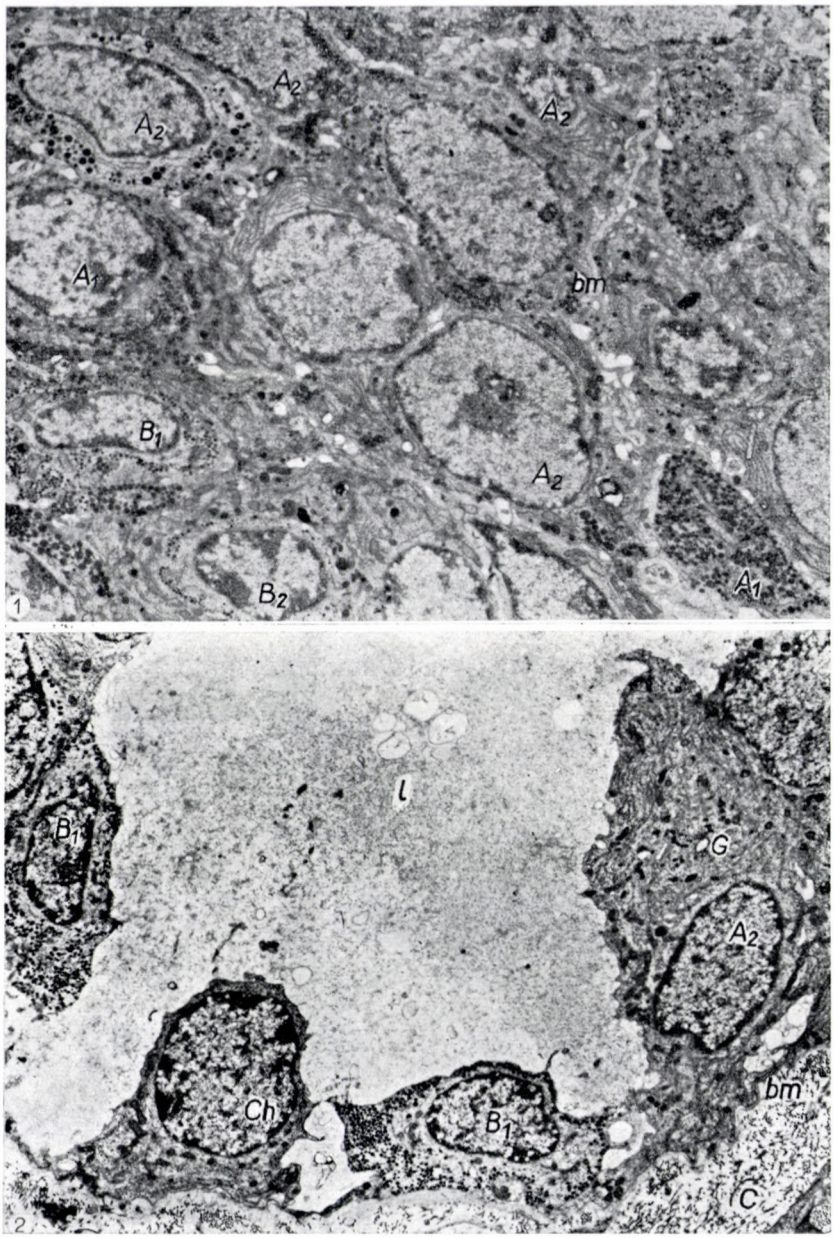

Fig. 1. Various granular cells of the transplanted pituitary. STH cell (A_1), LTH cell (A_2), gonadotropic cell (B_1), TSH cell (B_2). In the obliquely cut cell column a basal membrane (*bm*) is visible. ×3,150

Fig. 2. A portion of a cyst-like structure the epithelial lining of which consists of a regranulating LTH cell (A_2) containing developed ergastoplasm and relatively few secretory granules in the Golgi region (*G*), two gonadotropic cells (B_1) and a degranulated cell (*Ch*). The lumen (*l*) is filled up with a fine granular substance. The wall of the cyst is surrounded by a basal membrane (*bm*) and collagen fibres (*c*). ×3,200

Electron microscopic examination of the transplanted whole anterior pituitary showed that in the vicinity of the central scar a few narrow capillaries and mostly degranulated parenchymal cells were present. In this part the cell columns had disappeared and the degranulated cells were located closely invaginating into one another in a complex manner. In the degranulated cells few ergastoplasmic membranes, many free ribosomes and extensive Golgi apparatus with a few immature atypical granules were seen.

In the transplanted half anterior pituitary the degranulation was moderate and the fibrosis less pronounced; nevertheless, the increased number of collagen fibres sometimes caused deformations in the tissue structure and in the shape of the cells.

In the well-vascularized areas of the graft all the different types of the cells of the intrasellar anterior pituitary were recognizable (Fig. 1).

The fine structure of the different cell types

Prolactin-secreting (mammotropic) cells

In the grafts the prolactin-secreting cells are the most numerous granular cells. The structure of the cytoplasm indicates signs of regranulation and degranulation. The regranulating cells are identical with the prolactin-secreting cells described by HYMER and co-workers [21], RENNELS [35] and PASTEELS [31]. Their diameter is large, their nucleus is located eccentrically, the ergastoplasm and the Golgi apparatus are very well developed, in the zones of the latter there are many immature granules, and in the cytoplasm scattered granules variable in shape and diameter (maximally 750 μ) can be found (Figs 2, 3, 4).

In the degranulating prolactin-secreting cells the ergastoplasm decreased, whereas the Golgi apparatus often remained hypertrophied. As contrasted with the regranulating cells the Golgi systems contain immature granules only occasionally. In the cytoplasm the granules are uniformly distributed, but remarkably variable in diameter and density. The smaller granules have a regular circle shape, they are homogeneous and very dense; the larger ones are oval or circular, the osmiophilic substance is less dense, and is transformed into a finely granulated substance (Fig. 3). The limiting membrane of these larger and less dense granules is better visible and shows here and there smaller or larger ruptures (Fig. 3). These changes of the secreting granules are termed by GIROD and DUBOIS [15] "intracytoplasmatic granulolysis". In the intercellular space granules very rarely occur. "Intracytoplasmatic granulolysis" was never seen in the prolactin-secreting cells of the intrasellar pituitaries of the control animals.

Somatotropic cells

The grafts contain a great number of somatotropic cells as well (Fig. 1). [For their submicroscopic description see 24, 36, 37.] In these cells the number

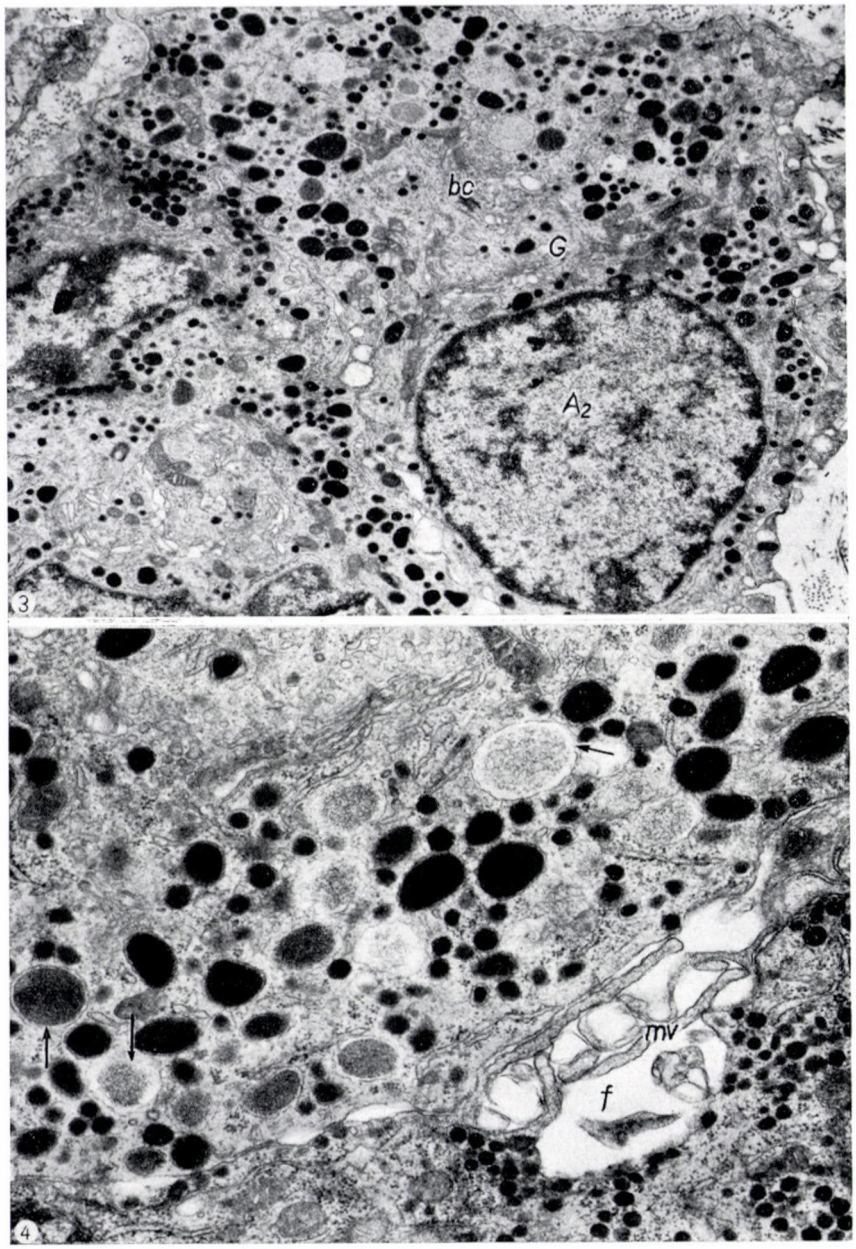

Fig. 3. Degranulating LTH cell (A_2) with some ergastoplasm membrane and numerous secretory granules. The Golgi apparatus (*G*) is relatively well developed, its components contain a few secretory granules. In the Golgi region a basal corpuscle (*bc*) is also visible. The density of the larger secretory granules is variable. ×7,750

Fig. 4. A portion of a degranulating LTH cell. The density of the secretory granules is greatly variable, the membrane limiting the larger granules of low density is ruptured here and there (arrow). The cell below is a TSH cell, forming with the LTH cell a follicle-like structure (*f*); the follicle contains microvilli (*mv*). ×18,900

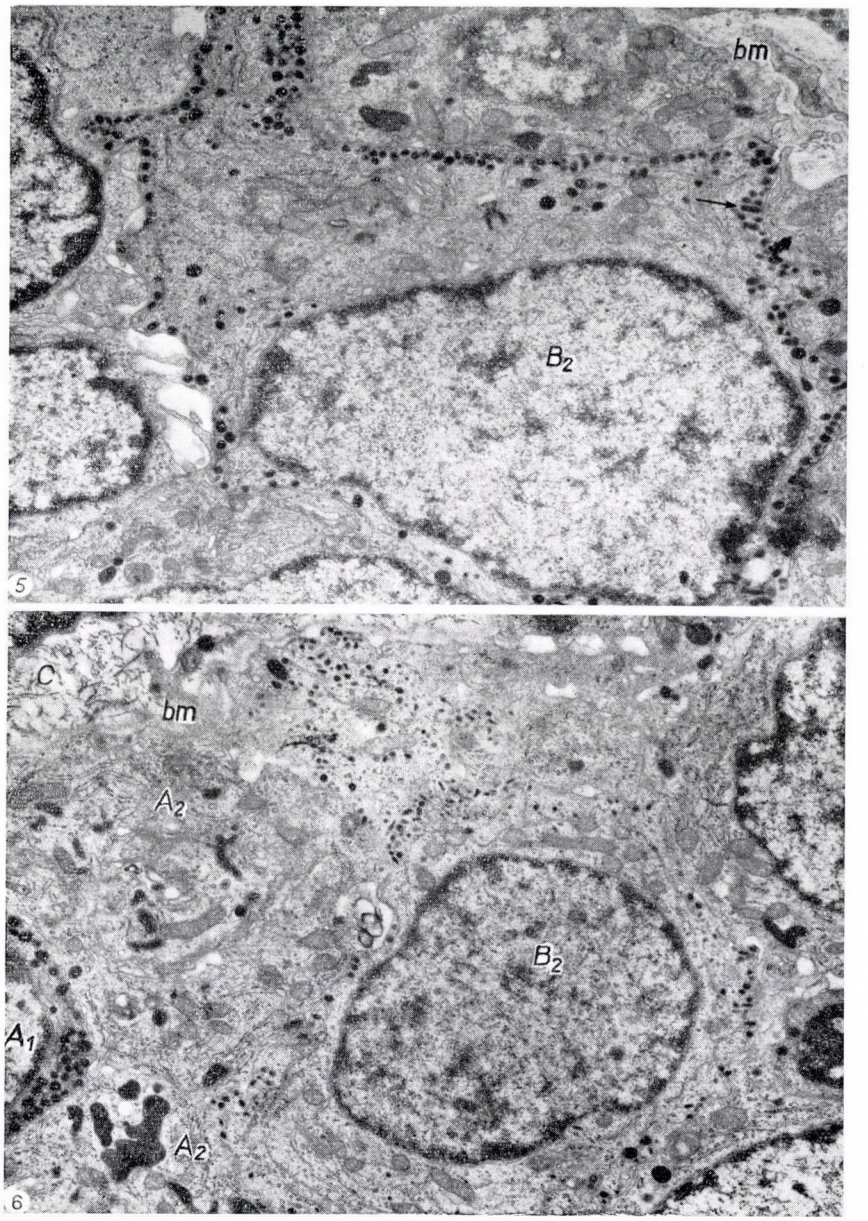

Fig. 5. An angular TSH cell (B_2). Most of the secretory granules are round, but several elongated forms may also be found (arrow). The ergastoplasmic tubules are long, among them many free ribosomes occur; basal membrane (*bm*) on the cell surface. ×9,800

Fig. 6. In this TSH cell (B_2) most of the granules are elongated. Note on the left side the irregular-shaped large secretory granules of the LTH cells (A_2), and the STH cell (A_1). The basal membrane is denoted (*bm*). Collagen fibres (*c*) in the interstitium. ×9,300

of the granules is generally high, the ergastoplasm is only represented by a few tubules, but in some of the cells the Golgi complex is well-developed, sometimes it also contains immature granules.

Thyrotropic cells

Like the intrasellar thyrotropic cells these are angular and usually located at a considerable distance from vessels. Concerning the thyrotropic cells of the graft, the endoplasmic reticulum and the secretory granules seemed worthwhile to consideration. In some of the thyrotropic cells the structure of the ergastoplasm consists of well-developed, long, branching tubuli running parallel, with many free ribosomes among them (Fig. 5). The developed ergastoplasm is associated with a developed Golgi complex; in the latter secreting granules can be observed (Fig. 9).

Two kinds of secretion granules were detected in the thyrotropic cells. The one is the known [12] round granule surrounded by a membrane. Its maximal diameter was 170 mμ in our material. The other type of granule is also surrounded by a membrane, but its shape is elongated, 200—400 mμ long and 100—150 mμ wide (Figs 5, 6 and 7, indicated by arrow). As shown by these figures round and elongated granules may occur within the same cell, however, in some of the cells the long ones dominate (Fig. 6). Usually the elongated granules are arranged vertically to the cell membrane. Some of them show signs of separation in two equal parts (Fig. 7, indicated by arrow). Granules of finger-biscuit and dumb-bell shape are also often found in the Golgi region (Fig. 10).

In the control pituitaries elongated granules could also be seen, but far less frequently than in the transplanted pituitaries.

It should still be mentioned that the thyrotropic cells of the intrasellar and transplanted pituitaries contain several round and oval, very osmiophilic corpuscles, with a maximal diameter of 500 mμ; only a suitable plane of section reveals that these are lysosomes (Figs 8, 9).

Gonadotropic cells

These are rare, and smaller in size in the grafts than in the intrasellar pituitary. [Their fine structure is characterized: 13, 18, 22, 36.] The majority of the gonadotropic cells of the graft contain many granules. The Golgi-apparatus has regressed, the endoplasmic reticulum is represented by a few rough-surfaced vesicles. Relatively slightly granulated gonadotropic cells occur as well. They contain a few rough-surfaced long tubules and more developed Golgi complex. In our material the diameter of the secretion granules of the gonadotropic cells attained 240 mμ. In these cells — though far less frequently than

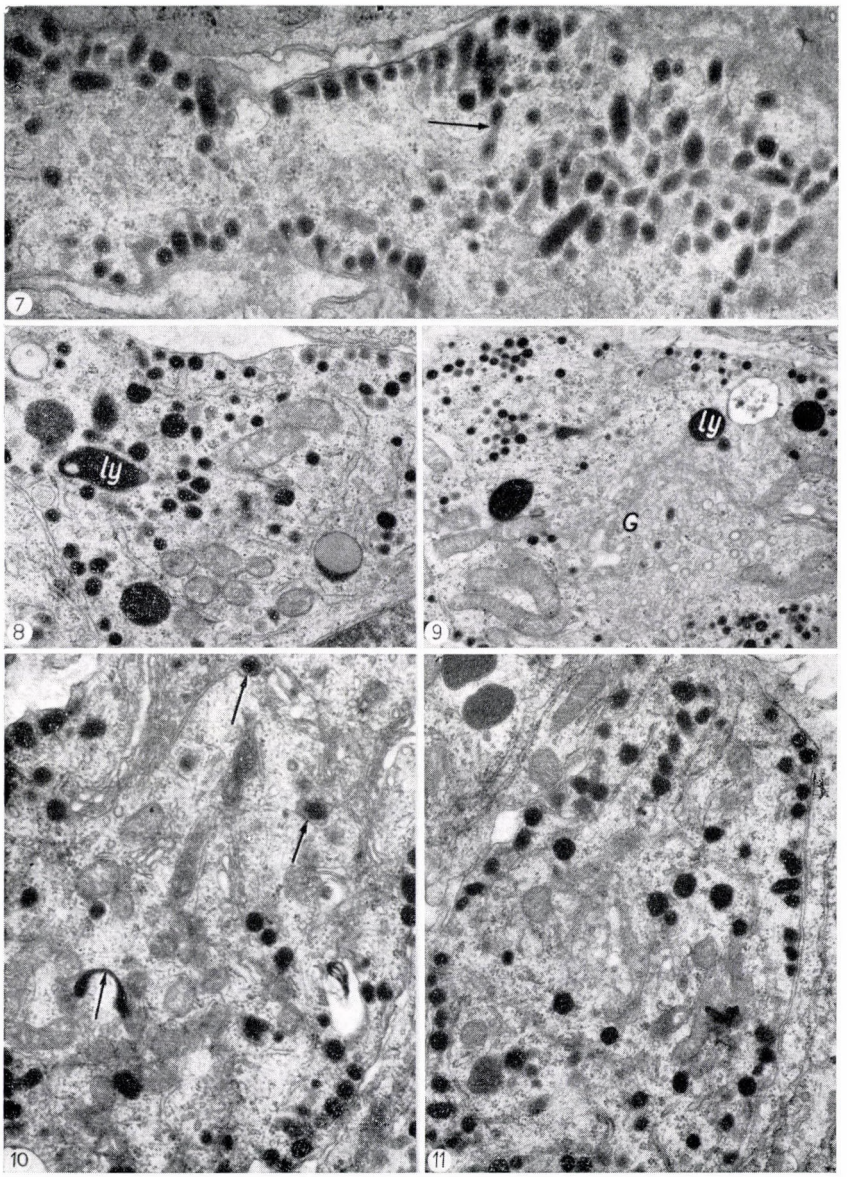

Fig. 7. Elongated granules of TSH cells, most arranged vertically to the cell surface. Note the constriction (arrow) in some elongated granules. ×23,500

Figs 8 and 9. In the TSH cells of both the intrasellar (Fig. 8) and transplanted pituitaries (Fig. 9) besides the typical round granules a few large lysosomes (*ly*) can be seen. The Golgi vesicles (*G*) contain immature granules. Fig. 8. ×15,300, Fig. 9. ×11,100

Fig. 10. Golgi region in a TSH cell. Note the granules in the Golgi elements (arrow) and the elongated horseshoe-shaped granule (double arrow). ×18,000

Fig. 11. Several ergastoplasm tubules and elongated granules in gonadotropic cells containing relatively few granules. The diameter of the granules located in the centre of the picture is 210 mμ. ×17,000

in the thyrotropic cells — several elongated granules could also be found besides the characteristic round granules (Fig. 11). Elongated granules also occur in the gonadotropic cells of the intrasellar pituitary.

We did not succeed in determining the cells responsible for ACTH production. The corresponding literature too, leaves this question open.

Discussion

According to previous examinations the interruption of the direct connection between the pituitary and the hypothalamus decreases the number of the chromophil cells and causes degranulation in the graft. NIKITOVITSCH-WINER and EVERETT [29] have demonstrated a regranulation of the chromophil cells in pituitary grafts retransplanted under the medial eminence as soon as the gland had vascularized with the portal vessels, i.e. the vascular connection between the hypothalamus and pituitary had been reestablished.

Our experiments showed that the blood supply may also play a role in causing structural alterations in the transplanted pituitary. This assumption is based on the fact that in the incompletely vascularized central part of the transplanted anterior pituitary the normal structure had disappeared and the cells only contained few immature granules. In contrast to this, in the well-vascularized edge the structure remained unchanged and well-granulated cells could be found. It may therefore be concluded that if only the interruption of the hypothalamic connections were the cause of the degranulation this would have to be equal in all parts of the graft. This statement is supported by the experiment of PETROVIC and co-workers [32], who grafted pieces of 1 mm^3 into the anterior chamber of the eye and observed no cell necrosis or considerable cell degeneration.

The light microscopic examinations of HALÁSZ and co-workers [16, 17] and FLAMENT-DURAND [14] as well as DESCLIN and FLAMENT-DURAND [6] are very remarkable. These authors found that degranulation of the basophil cells of the anterior pituitary transplanted into a circumscribed region of the hypothalamus, the so-called hypophysiotropic area, did not occur. When, on the other hand, the transplantation was performed in other areas of the brain, complete degranulation of the basophils took place. These authors assume that the degranulation of the basophil cells is due to the lack of the trophic factors produced in the hypothalamus.

The great increase in the number of *prolactin-secreting cells* may be attributed to the fact that the transplanted pituitary is liberated from hypothalamic inhibition [5, 9]. The results of RENNELS [35] and our own experiments suggest that prolactin-secreting cells become equally predominant in the pituitary graft irrespective of the sex of the donor rat, although the pituitary of the female rat is rich, whereas that of the male rat is poor in these cells.

The above morphological data support the results of the biological experiments suggesting that the grafted pituitary produces prolactin [1, 7, 8, 9, 26, 28, 38, 40, 43, 44].

It seems worth-while to mention that the known morphological signs of the granule excretion [11, 25, 36, 41] could only be seen occasionally. On the other hand, large granules of the prolactin-secreting cells often showed "intracytoplasmic granulolysis". This phenomenon was observed in the gonadotropic cells of the rat by HERLANT [19] and in those of the golden hamster by GIROT and DUBOIS [15]. It is still unclear why "intracytoplasmic granulolysis" occurs frequently in the prolactin-secreting cells of the transplanted pituitary and why it is lacking in those of the intrasellar pituitary.

The somatotropic cells also contain many granules in the graft and are, therefore, potentially active. Degranulated or regranulated somatotropic cells were not found in our material.

Thyrotropic cells

The developed ergastoplasm membranes and the many free ribosomes and the secretory granules in the developed Golgi apparatus of some of these cells suggest that they produce granules. HARTLEY and co-workers [18] examining ultracentrifuged granules have proved that the hormones are stored in the secretion granules. The question arises whether the granule production of the thyrotropic cells of the transplanted pituitary also means hormone production. The elongated undivided granules — occurring very frequently in the thyrotropic cells of the grafted pituitary and very rarely in the intrasellar pituitary — seem to infer that the granule production (or possibly hormone production) of the cell is disturbed.

It is interesting that also under normal circumstances the thyrotropic cells of the pituitary of the salamandra contain two kinds of granules: smaller elongated and larger round granules [3]. The size of the elongated granules is the same in the salamandra as in the transplanted pituitary of the rat. In the salamandra the larger granules proved to be secretory granules, whereas in our material they were lysosomes (Figs 8, 9).

Gonadotropic cells

In accordance with earlier light microscopic examinations [29] only very few and small gonadotropic cells were found in the graft. According to DESCLIN's experiments [5] the transplanted pituitary does not produce gonadotropic hormones. But it should be remarked that among the gonadotropic cells of the graft some differences may be observed concerning granulation, the development of the ergastoplasm and the Golgi complex.

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DIE ELEKTRONENMIKROSKOPISCHE STRUKTUR DES HYPOPHYSENVORDERLAPPENS NACH TRANSPLANTATION IN DIE VORDERE AUGENKAMMER VON RATTEN

In die vordere Augenkammer männlicher Ratten mit erhaltener Hypophyse wurden homologe Adenohypophysen transplantiert und 50 Tage später die Transplantate elektronenmikroskopisch untersucht. In den zentralen Teilen der größeren Transplantate waren die Zellen degranuliert, vermutlich infolge der ungenügenden Blutversorgung. In den gut vaskularisierten Gebieten der Transplantate wurden sämtliche chromatophilen Zellen nachgewiesen. Der Anteil der chromatophilen Zellen hatte sich verändert. Das Übergewicht ging an die heterotropen Zellen über. In den letzteren ließen sich die Anzeichen einer lebhaften Granulumproduktion, ferner einer "intraplasmatischen Granulolyse" feststellen. Die somatotropen Zellen waren stark granuliert. In den thyreotropen Zellen konnte man ausgeprägte Zeichen einer Granulumproduktion beobachten. Ein Teil der Körnchen war nicht rund, sondern länglich. Die gonadotropen Zellen zeigten eine zahlenmäßige Abnahme und eine Verringerung ihrer Größenmaße.

ЭЛЕКТРОННОМИКРОСКОПИЧЕСКАЯ СТРУКТУРА ПЕРЕДНЕЙ ДОЛИ ГИПОФИЗА, ПЕРЕСАЖЕННОЙ В ПЕРЕДНЮЮ КАМЕРУ ГЛАЗА У КРЫС

В переднюю камеру глаза крыс-самцов с сохраненным гипофизом авторы производили пересадку гомологичного аденогипофиза. По истечении 50 дней они исследовали трансплантаты в электронном микроскопе. В центральных частях более крупных трансплантатов наблюдалась дегрануляция, предположительно в результате недостаточности кровоснабжения. В удовлетворительно васкуляризованных частях трансплантатов были обнаружены все хромофильные клетки. Изменилось соотношение хромофильных клеток, перевес приобрели лютеотропные клетки. В этих клетках видны признаки, указывающие на интенсивное образование зернышек, а также на «внутрицитоплазматический гранулолиз». Соматотропные клетки оказались сильно гранулированными. В тиреотропных клетках отмечаются выраженные признаки образования зернышек. Одна часть последних не круглой, а продолговатой формы. Гонадотропные клетки меньшие по размерам и их число также уменьшено.

Szabolcs Virágh, Tibor Tiboldi, Kálmán Kovács, Miklós Julesz — Budapest IX., Tűzoltó u. 58, Szeged, Korányi S. rkp. 12, Hungary.

Imre Törő — Budapest IX., Tűzoltó u. 58,

RECENSIO

Preimplantation stages of pregnancy *A Ciba Foundation Symposium*

Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR, Churchill, London (1965) pp. 430

About twenty years ago the books dealing with the evolution of man still taught the morphological and physiological processes of fertilization and segmentation as well as of early gastrulation on the basis of observations gained on ovules of lower animals. Very few human ovules were available. The works of the researchers of the Carnegie Institute made an insight into the morphological changes of the human ovule possible and the time has come to carry out also submicroscopical and fine biochemical examinations mammalian — and not human — ovules. This irruption of the examination methods of molecular biology into the field of early development is demonstrated by this book, a new member of the Ciba Symposium series. Lectures elucidate the events of the period before implantation, that is, of fertilization and segmentation, from different aspects: from the ultrastructural changes during fertilization to the nucleic acid and protein synthesis of the developing embryo.

The second part of the symposium discusses genetic problems, the issues of chromosome damages, heteroploidy and experimental mozaicism. In the third part the actions of the maternal milieu are treated with special reference to the preimplantation period.

On the symposium experts of distinction gave lectures and discussed the questions, among others WADDINGTON, ENDERS, MULNARD, MINTZ, PINCUS. The book like the other members of the Ciba series contains the papers, the whole text of the discussion and the abundant material of illustration. The latter should be particularly stressed because electron micrographs are of higher quality than those published earlier.

G. CSABA (Budapest)

INTERNATIONAL SYMPOSIUM ON PALEOLIMNOLOGY

Tihany, Hungary, August 28—31, 1967

AN INTERNATIONAL SYMPOSIUM ON PALEOLIMNOLOGY will be held at the Biological Research Institute, Tihany, Hungary, on 28—31 August 1967. The meeting is being co-sponsored by the International Association of Theoretical and Applied Limnology (SIL) and the Biology Department of the Hungarian Academy of Sciences, with support of the International Union of Biological Sciences (IUBS) and Section PF of the International Biological Program (IBP). The integrating topics for the three days of papers are: *a)* primary and secondary sedimentation, inorganic and organic chemistry and cycles, diagenesis, coring methods; *b)* morphological remains of plants and animals and their interpretation, species diversity as a function of trophic level, the "new" palynology; *c)* case histories of particular lakes and regions, eutrophication and productivity changes. Each day will comprise invited papers in the morning and contributed papers in the afternoon. The fourth day will be devoted to a field excursion on Lake Balaton and to Hévíz thermal lake and to an exhibit and demonstration of the pioneering work of L. LÓCZY on Lake Balaton and the work of the Biological Research Institute, now in its fortieth year.

THE ORGANIZING COMMITTEE consists of Drs. D. G. FREY, USA; D. A. LIVINGSTONE, USA; WINIFRED TUTIN, England; N. V. KORDE, USSR; and OLGA SEBESTYÉN, Hungary. Interested persons can obtain further information by writing Prof. D. G. FREY, Department of Zoology, Indiana University, Bloomington, Indiana 47401, USA.

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CHANGES IN THE SECRETORY ACTIVITY OF THE CENTRAL NERVOUS SYSTEM OF ANODONTA CYGNEA UPON THE ACTION OF CHEMICAL AGENTS

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(Received January 24, 1966)

Synopsis

(1) Controlled by the central nervous system, the periodical activity of *Anodonta cygnea* L. changes in the same way upon the action of KCl, $CdCl_2$, and oxygen deficiency. (2) Applied at a low concentration (10^{-3} *M* or 10^{-5} g/ml), KCl and $CdCl_2$ cause full discharge of the paraldehyde-fuchsin (Pf) positive substance from the nerve cells within 24 hours. (3) In case the above concentrations of KCl and $CdCl_2$ are applied for 72 hours, the Pf-positive substance is again accumulated in the nerve cells. (4) If the animals are kept for 24 hours in anaerobic environment without oxygen, the Pf-positive substance does not disappear from the nerve cells; instead the secretory substance will disappear by the 72nd hour. (5) The quantitative changes in the Pf-positive substance are presumably due to the direct metabolic action exerted by the applied stimuli on the activity of the nerve cells; this action is not the same as the one causing the changed periodical activity.

Introduction

In both vertebrates and invertebrates the secretory activity of nerve cells is considered a part of the endocrine control [11, 14, 26]. The accumulation and disappearance of Gomori-positive substance as observed in molluscs are also interpreted in these terms. Neurosecretory activity of mussels is correlated with the sexual cycle, according to LUBET [16], and with osmoregulation, according to NAGABUSHANAM [18].

As indicated by several data, neurosecretory activity as part of neurohumoral regulation may be considerably influenced by external stimuli. However, secretory activity is influenced not only by stimuli (e.g. dehydration) acting directly on the endocrine system, but in vertebrates the accumulation and discharge of neurosecretion may be affected also by applying optical [6, 13, 17] and acoustical stimuli [13]. The neurosecretory activity of the nerve cells can be stimulated in a similar way in invertebrates, too, by means of physical actions; among these the actions of heat, exsiccation and light have been examined most thoroughly [1, 2, 12].

Several authors [4, 5, 7, 9, 10, 16, 19, 27] have dealt with the Gomori-positive substance of the central nervous system of Lamellibranchiatae and with its neurosecretory activity, judged on the ground of Gomori's chrome haematoxy-

lin-phloxin staining and paraldehyde-fuchsin (Pf) positivity. However, no evidence has been found so far indicating any alteration caused by external stimuli in the Gomori-positive substance of the central nervous system of mussels, nor are there any data showing the stimuli by means of which this activity of the nerve cells may be affected. The elucidation of this question is all the more reasonable, since this knowledge might permit further and more detailed study of the functional significance of neurosecretory activity.

In close correlation with life functions, the periodical activity of mussels may be changed in a characteristic way by certain chemical stimuli. Namely, the length and the frequency of the periods of activity and rest, lasting for several hours, may be reduced or augmented several times [15, 20, 23]. Controlled by the central nervous system, the actual life rhythm of the animal can be profoundly affected by KCl (10^{-3} *M*), $CdCl_2$ (5—10 mg/liter) and by oxygen shortage. These observations have led to the question whether these chemical actions, affecting vigorously the control activity of the nervous system, do or do not produce any change in the secretory activity of the nerve cells.

Material and method

The tests were made on fully developed specimens of *Anodonta cygnea*. Since no significant accumulation of Gomori-positive substance could be observed in these animals unless in May and June [5], the tests were carried out in these months.

The activity of the animals, which were kept each in 3 litres of Balaton water, was continuously recorded on a mussel-actograph set up for this purpose [25]. The chemical stimuli influencing the periodical activity were applied after 2—3 days of control recording. KCl or $CdCl_2$ was added to the water that was surrounding the animal, or oxygen shortage was brought about by sealing the water surface with liquid paraffin. The tests were made with a total of 35 animals, divided into 7 groups. These were kept for 4—6 days in aerated Balaton water (group 1, i.e. control group); for 24 hours in 5×10^{-3} *M* KCl solution (group 2); for 24 hours in 20 mg/liter $CdCl_2$ solution (group 3); for 72 hours in 5×10^{-3} *M* KCl solution (group 4); for 72 hours in 20 mg/l $CdCl_2$ solution (group 5); for 24 hours in oxygen-deficient water (group 6); for 72 hours in oxygen-deficient water (group 7).

The cerebral, visceral and pedal ganglia, together with the nerve branches, were excised from the mussels, fixed in Bouin's fixative, embedded in paraffin and cut in series of 5 μ thickness. Staining was made mostly with Pf according to GABE [8], but tests were also carried out with chrome haematoxylin-phloxin, which is less demonstrable in the nervous system of mussels.

Results

Resulting from various actions, the alteration of periodical activity is shown on Fig. 1. Presenting so far a continuous activity, the animals can be seen in every case, after a more or less long time, to get into a state of rest, with their shells closed. Subsequently a comparatively frequent alteration of active and inactive phases may be observed, latter being longer than the active phases.

In the case of animals exposed for a longer time to the actions there is no substantial difference as far as periodical activity is concerned.

The results of the histological tests have been evaluated on the basis of comparison with the ganglia of control animals not exposed to any special action. In the animals of the control group the application of the Pf staining technique helped to find a large number of secernent nerve cells characteristic of the early summer season. In the spring the Pf-positive substance begins to appear in every case around the nucleus in the form of purple granules, and in

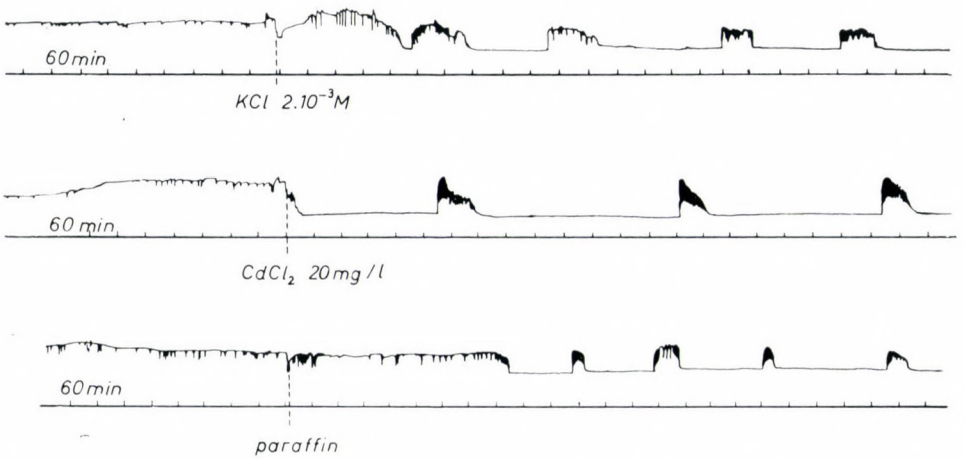

Fig. 1. Alteration in the periodic activity of *Anodonta cygnea* caused by KCl and $CdCl_2$ and by oxygen shortage

the months of May and June it gradually fills up the cytoplasm of the nerve cells (Fig. 2). The nucleus of these cells is poor in chromatin, its structure is vesicular and its shape somewhat polymorphic. The nucleolus is conspicuous.

The following changes were observed in the nerve cells as a result of the experimental actions: After 24 hours of treatment with 5×10^{-3} *M* KCl (group 2) most of the Pf-positive substance had left the cells (Fig. 3). The remaining small amount of Pf-positive substance was sited either scattered all over the cytoplasm or next to the cell-membrane. The nuclei were poor in chromatin and polymorphic in shape. The nucleolus could be readily observed.

After 24-hour treatment with $CdCl_2$ (group 3), most of the Pf-positive granules had left the cytoplasm of the cells (Fig 4). The nucleus was poor in chromatin. The nucleolus was in general observable.

In the nerve cells of animals exposed for 72 hours to 5×10^{-3} *M* KCl (group 4), major cytological changes could be observed. There were vacuoles in the cytoplasm, even the nucleus was vacuolized, and the nucleolus could not

be observed. The cytoplasm of the cells was almost entirely filled up with the Pf-positive substance (Fig. 5). It is striking that this substance had lost its granular character, it was purple and of liquefied consistence. At the border of the vacuoles it occasionally showed a fine granular structure.

After 72-hour treatment with $CdCl_2$ (group 5) the nerve cells were contracted, showing vacuoles in the cytoplasm.

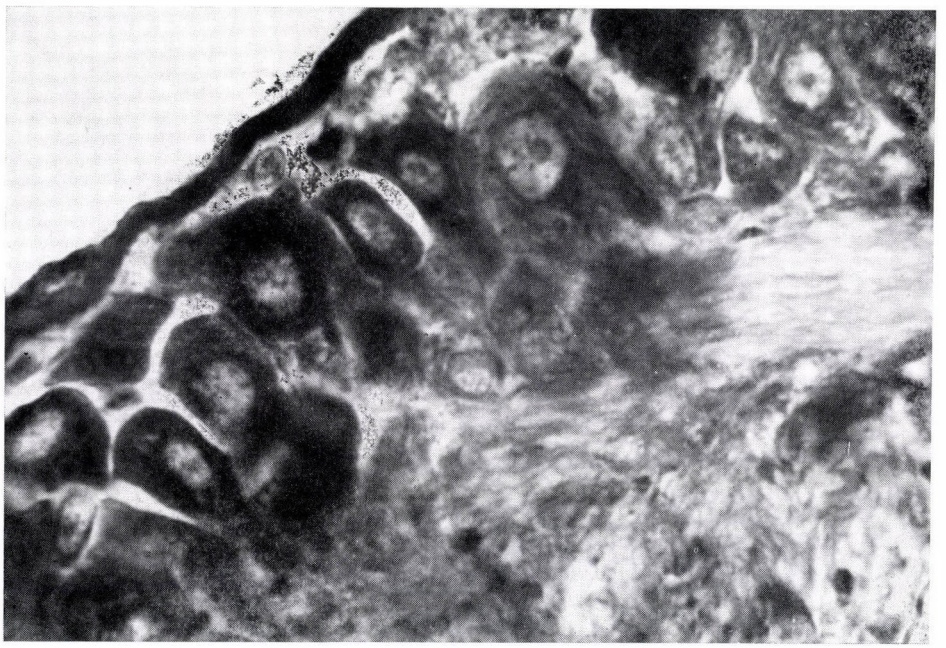

Fig. 2. Cerebral ganglion of *Anodonta cygnea*, cross-section. Nerve cells containing Gomori-positive substance, control picture, Pf staining, ×1,000

At the border of the vacuoles and scattered in the cytoplasm there was a great amount of Pf-positive substance, mostly in form of a liquefied purple-coloured matter (Fig. 6). The nucleus was also vacuolized, poor in chromatin, and the nucleolus was hardly visible or not at all.

No alteration was observed in the nerve cells of animals kept for 24 hours in an environment with oxygen shortage (group 6). In most of the nerve cells a large quantity of Pf-positive substance was seen in the form of purple granules (Fig. 7). There were also cells where the cytoplasm was not entirely filled up with Pf-positive substance. Poor in chromatin, the nucleus was of a vesicular structure; here and there the nucleolus was visible. In the nerve cells of animals kept for 72 hours in water with O_2 shortage (group 7) definite cytological alterations were observed as compared with the control animals. Numerous vacuoles appeared in the cytoplasm, with Pf-positive substance at their border. In

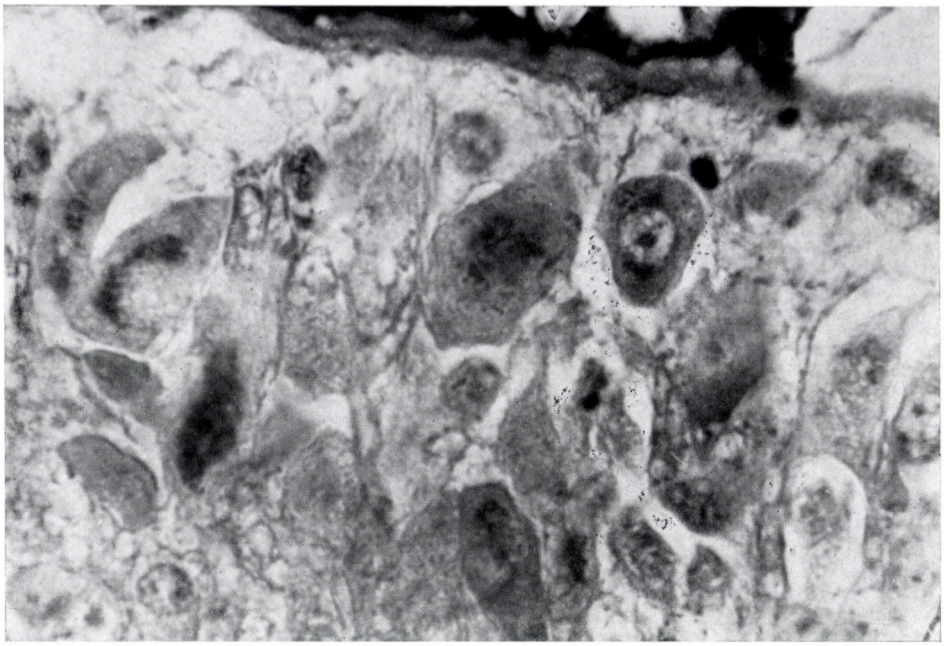

Fig. 3. Cerebral ganglion, cross-section. Effect of 5×10^{-3} *M* KCl after 24 h treatment. Pf staining, × 1,000

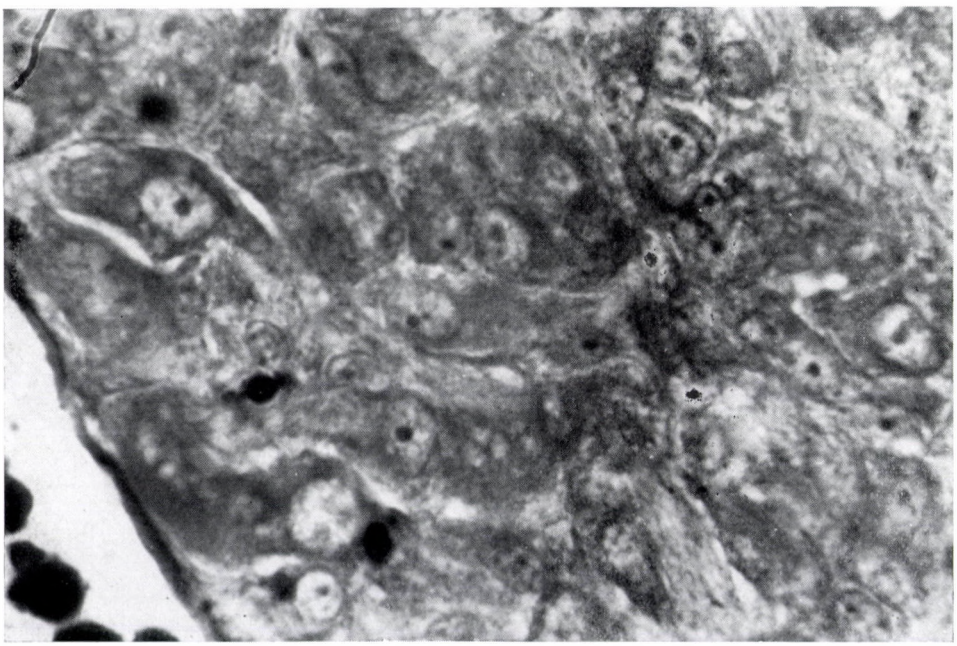

Fig. 4. Cerebral ganglion, cross-section. After 24 h treatment with 20 mg/lit $CdCl_2$. Pf staining ×1,000

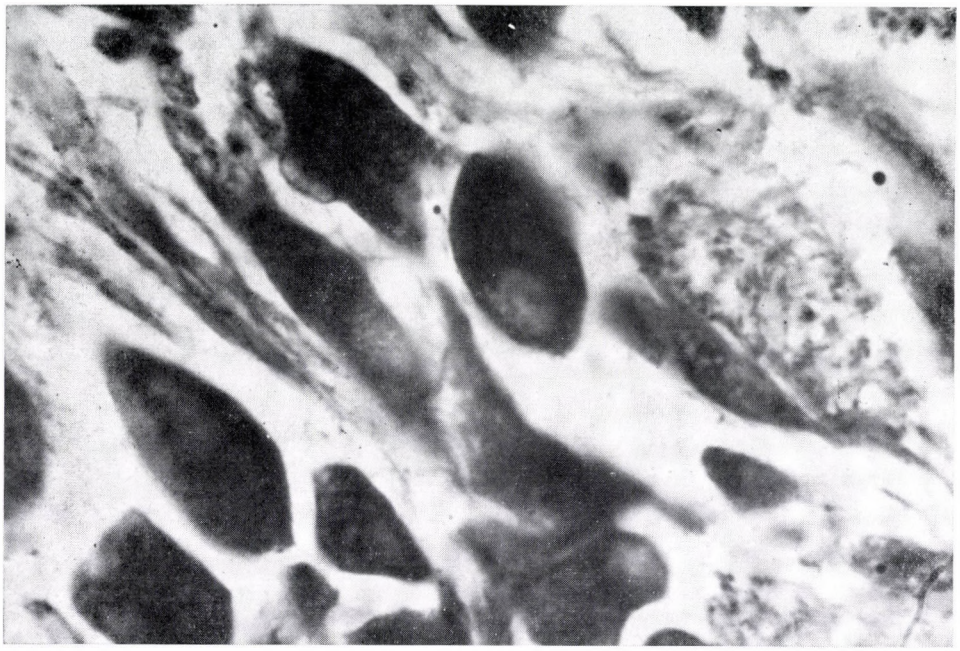

Fig. 5. Cerebral ganglion, cross-section. Effect of 5×10^{-3} *M* KCl after 72 h treatment. Pf staining, ×1,000

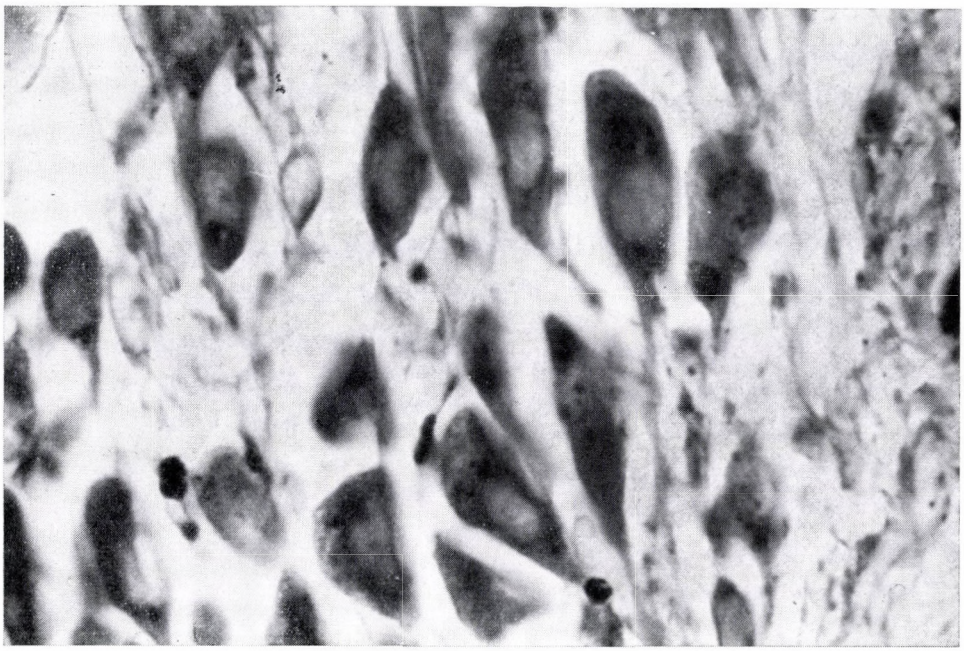

Fig. 6. Cerebral ganglion, cross-section. Effect of 20 mg/lit $CdCl_2$ after 72 h treatment. Pf staining, ×800

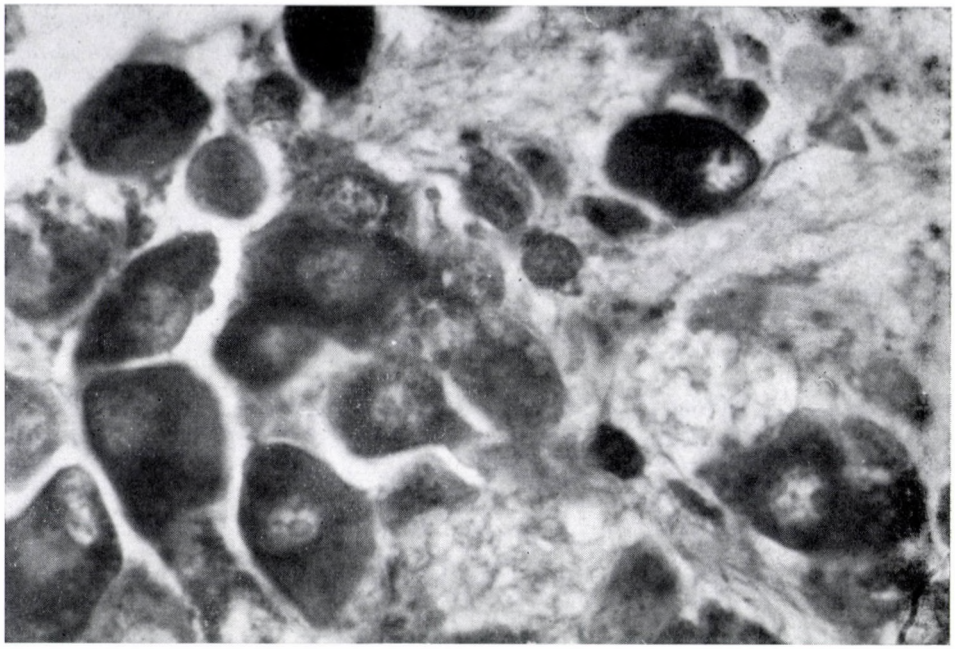

Fig. 7. Cerebral ganglion, cross-section. Effect of oxygen shortage after 24 h treatment. Pf staining, × 1,000

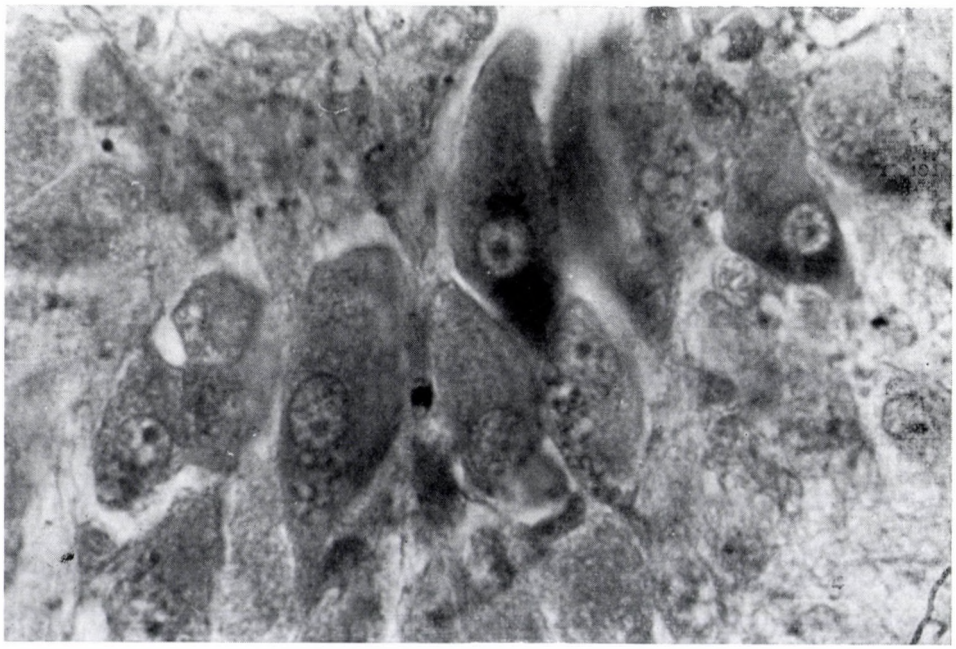

Fig. 8. Cerebral ganglion, cross-section. Effect of oxygen shortage after 72 h treatment. Pf staining, × 1,000

the cytoplasm of some of the nerve cells a significant quantity of Pf-positive substance was seen, while others were quite empty. The cells containing a major quantity of Pf-positive substance had nuclei of vesicular structure, with well visible nucleolus, while the nucleus of the cells without secretion was vacuolized; the nucleolus could be observed in few nuclei (Fig. 8).

Discussion

In agreement with the results of earlier investigations [15, 20, 23], the presence of KCl and $CdCl_2$ and the shortage in O_2 produced characteristic changes in the periodical activity of *Anodonta cygnea* in the present study. Considering that the periodical activity is controlled by the central nervous system, this phenomenon is indicative of substantial changes in the central nervous system, in its activity and in the cyclical processes taking place therein. The present tests seem to show that the chemical stimuli influencing the periodical activity are simultaneously acting upon the secretory activity of the nerve cells too, although our previous studies [25] had shown no correlation between the quantitative changes of Gomori- or Pf-positive substance, on the one hand, and the alteration of active and resting periods, i.e., the central control of periodical activity, on the other.

If the nerve cells of the control animals are compared with those of the observed test animals, it will be noticed that the secretory activity of the nerve cells is affected by low concentrations of KCl and $CdCl_2$. The cells containing Pf-positive substance are evacuated within 24 hours upon the action of KCl and $CdCl_2$. At the same time, the animals kept for 24 hours in an oxygen-deficient environment show no major quantitative difference in Pf-positive substance as compared with the control animals, although their periodical activity undergoes the same changes in all of the three cases as soon as in the first 6 hours. The nerve cells of animals kept for 72 hours in an oxygen-poor environment are similar to those seen after 24-hour treatment with KCl or $CdCl_2$: the appearance of vacuoles in the cytoplasm may be interpreted as due to the discharge of secretion. If the animals are exposed for a longer time (72 hours) to the effect of KCl or $CdCl_2$, the evacuated cells will be refilled. There are again numerous vacuoles in the cytoplasm and Pf-positive substance can be seen in the border of the vacuoles. It is surprising that, instead of the earlier granular form of the secretion, a homogeneous, liquefied purple mass is present.

According to earlier investigations [21, 22], the presence of KCl or $CdCl_2$ or oxygen deficiency brings about the alteration of periodical activity in the same way, i.e. by acting on the peripheral receptors, whereby the effect appears in all of the three cases within a comparatively short time. This parallelism

suggests that the central nervous system, which controls the periodical activity, is influenced by these stimuli by means of approximately the same mechanism. At the same time, the secretory activity of the cells is modified far more intensely by KCl and $CdCl_2$ than by oxygen shortage, since 24-hour treatment with KCl or $CdCl_2$ is as effective as 72-hour oxygen shortage. It may be suggested therefore that the mechanism accounting for the alteration of periodical activity is not the same as that controlling the discharge of Pf-positive substance and the secretory activity of the nerve cells.

It may be assumed that, in the latter case, evacuation is not caused by the afferent stimuli coming from the receptor zones, but that the stimuli damage the metabolism of the cells directly and thus cause the Pf-positive substance to be discharged. The effect of KCl and $CdCl_2$ appears earlier, presumably because the K and Cd ions immediately change the ion equilibrium and the metabolic processes of the nerve cells, as soon as they have reached them. On the other hand, O_2 shortage acts but gradually in the nervous system, probably because the mechanisms, compensating the low O_2 tension that occurs frequently among the life conditions of the animal, are able to compensate even complete absence of O_2 for a rather long time.

The functional significance of the Pf-positive substance and of the secretory activity of cells is not known. Our tests have shown that there is only a temporary trouble caused in the storage of the secretory substance by the stress effects applied. If the life functions of the animal are not irreversibly damaged, a renewed accumulation begins in the evacuated cells, due to an increased production of Pf-positive substance. This suggests that the secretory activity of the cells adapts itself to the unusual chemical stimuli. This possibility of experimentally influencing the discharge and production of secretion not only indicates the lability of secretion storage, but seems to provide proper means for studying the biological role of secretory activity too.

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VERÄNDERUNGEN DER SEKRETIONSAKTIVITÄT DES ZENTRALEN NERVENSYSTEMS DER TEICHMUSCHEL (*ANODONTA CYGNEA* L.) UNTER DER EINWIRKUNG VON CHEMISCHEN AGENTIEN

(1) Die unter dem regelnden Einfluss des zentralen Nervensystems stehende periodische Aktivität der Teichmuschel (*Anodonta cygnea* L.) verändert sich in gleicher Weise unter dem Einfluß von KCl, $CdCl_2$ und bei Oxygenmangel. (2) Die Anwendung von KCl und $CdCl_2$ in niedriger Konzentration (10^{-3} *M* bzw. 10^{-5} g/ml) verursacht binnen 24 Stunden die vollständige Entleerung des Paraldehyd-Fuchsin (Pf)-positiven Stoffes aus den Nervenzellen. (3) Bei 72stündiger Anwendung von KCl und $CdCl_2$ in der angeführten Konzentration häuft sich der Pf-positive Stoff erneut in den Nervenzellen der Tiere an. (4) Wenn man die Tiere 24 Stunden hindurch in einer oxygenarmen Umgebung haltet, erfolgt in den Nervenzellen keine Entleerung des Pf-positiven Stoffes, wohl aber nach Verlauf von 72 Stunden. (5) Bei den Änderungen der Quantität des Pf-positiven Stoffes dürfte die unmittelbare Stoffwechselwirkung der angewandten Reize auf die Funktion der Nervenzellen eine Rolle spielen, diese Wirkung ist aber nicht identisch mit jener, welche zur Änderung der periodischen Aktivität führt.

ИЗМЕНЕНИЯ СЕКРЕТОРНОЙ АКТИВНОСТИ ЦЕНТРАЛЬНОЙ НЕРВНОЙ СИСТЕМЫ *ANODONTA CYGNEA* ПОД ВЛИЯНИЕМ ХИМИЧЕСКИХ АГЕНТОВ

1. При действии окружающей среды с недостатком KCl, $CdCl_2$ и кислорода периодическая активность *Anodonta cygnea*, регулируемая центральной нервной системой, изменяется в одинаковом направлении. 2. При низкой концентрации KCl и $CdCl_2$ (10^{-3} *M*, или 10^{-5} г/мл) паральдегидфуксин-положительное вещество в пределах сутки полностью выделяется из нервных клеток. 3. В случае применения вышеуказанной концентрации KCl и $CdCl_2$ в пределах 72 часов в нервных клетках животных вновь накопляется паральдегидфуксин-положительное вещество. 4. В среде с недостатком кислорода в пределах 24 часов не наблюдается выделения паральдегидфуксин-положительного вещества из нервных клеток, однако, по истечении 72 часов происходит выделение всего количества секреционного вещества. 5. Ответственным за изменения содержания паральдегидфуксин-положительного вещества, по всей вероятности, следует считать непосредственное метаболическое действие, оказанное применявшимися раздражениями на функцию нервных клеток. Это действие не тождественное с эффектом, вызывающим изменения периодической активности.

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CONTRIBUTIONS TO THE MECHANISM OF THE DESTABILIZING EFFECT OF HEPARIN ON THE CELL DIVISION

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Synopsis

The effects of heparin preparations of variable activity on blood coagulation, heparin antagonists and heparin components on regenerating planaria (*Dugesia lugubris*) were studied. It has been established that heparin acts on cell division first of all by influencing the sol—gel condition; this manifests itself in malformation. The blastema-retarding effect of heparin does not run parallel with the malformations; in these supposedly heparin—histon bonds may be involved. From the aspect of the malformation producing effect the most active component of heparin is glucosamine, which when applied alone caused distortions in 100 per cent.

Introduction

In earlier experiments the relationship between heparin and cell proliferation was studied. It was established that cell division requires the presence of heparin or of another acid mucopolysaccharide [8]. In absolute absence of these, cell division comes to stillstand. In the case of "excess" heparin, on the other hand, cell divisions are distorted; in tissue culture atypical mitoses [7], in vivo atypical regeneration processes ensue. Such atypical regeneration process was observed in planaria [8]. Distortions could be encountered also in absence of heparin, thus the limb regeneration of tritons became atypical in about 70 per cent of the cases when small amounts of toluidine blue, heparin-binding substance, were added to the aquarium water [6].

In other experiments the mutagenic effect of heparin was examined and it was established that heparin in itself is non-mutagenic but employed together with another non-mutagenic, irritative factor, it may be co-mutagenic. E.g., the resistance of the *Streptomyces globisporus XJ9* strain to ultra-violet irradiation was changed by heparin. After heparin pre-treatment UV irradiation resulted in a mutation rate equal to that caused by an X-ray dose of 200,000 r on this strain [8]. These experiments suggested the assumption that heparin in the organism is a substance of destabilizing effect, shifting the physiological processes generally in the direction of the sol condition — as in blood coagulation, muscle contraction and lipid clearing — and that the same effect of the substance affects also cell division. Cell division, as established already by Heilbrunn [11, 12], is a process accompanied by changes in the sol—gel condition,

which is shifted in the presence of large quantitaties of heparin towards the sol condition while in the absence of heparin towards the gel condition. This disturbs the normal course of cell division. Alternatively [8], heparin may be, on account of its affinity to protamines or histons, involved in the process of nucleohiston formation and disturbs cell division in this way.

The purpose of the present experiments was (1) to supply further data to the mode of action of heparin and (2) to attempt to identify the component of heparin responsible for the appearance of atypical phenomena. The experiments were conducted on planaria.

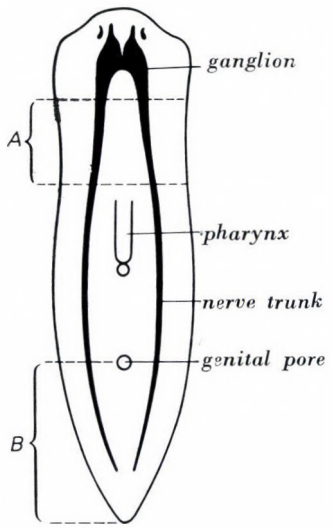

Fig. 1. Scheme of cuttings on planaria

Material and method

For the experiments 640 planaria of the same size were prepared. The animals were cut in three levels (Fig. 1), immediately behind the head ganglion, at the anterior margin of the pharynx and across the genital pore. The piece between the first two cuts was named fragment "A" while that caudal from the third cut, fragment "B". Twenty fragments were placed in a Petri dish and 40 fragments constituted an experimental group. A total of 1,280 fragments were examined.

The control fragments were kept in tap-water while the experimental fragments in tap-water containing test substance in the proper concentration. The following substances were tested: Heparin preparation 3 I.U./mg (United Work of Pharmaceutical Products, Budapest; the substance was prepared from the commercial preparation of 111 I.U./mg by changing its stereoconfiguration; as a result of this its effect on blood coagulation diminished, while its molecular weight was hardly, and the number of its sulphate groups not at all influenced); heparin 111 I.U./mg (Richter); $CaCl_2$; protamine sulphate (Roche); dextrane; glucuronic acid (Fluka); glucosamine HCl (BDH); glucose; galactose (Riedel).

The extent and form of regeneration were regularly controlled under the stereomicroscope for 30 days. After the evaluation of the effect of the substance examined — on the 30th day — all experimental animals were placed in tap-water and their behaviour was observed for a further week.

Results

The results are presented in two tables. Table 1 shows the effects of the two heparin preparations, heparin antagonists and dextrane. In Table 2 the effect of heparin components is recorded as compared with some monosaccharides.

From Table 1 it appears that distortions were observed only when the commercial heparin preparation of 111 I.U./mg was used and even this acted only on the regeneration of the "B" fragment of the animals. The same applies to the effect retarding the development of blastema. In fragment "A" the commercial heparin preparation produced the development of a considerable formation of supernumerary eyes.

Of the heparin antagonists protamine sulphate caused significant distortion in fragment "A". $CaCl_2$ slightly reduced the blastema-retarding effect of the commercial heparin and completely inhibited its effect causing distortion.

When the animals were placed in tap-water after treatment, injuries tended to grow milder although part of the changes seems to be irreversible. In the group which had been kept in protamine sulphate solution of 100 μg/ml concentration the development of the supernumerary eyes continued after the end of the treatment.

From Table 2 it is clear that of the heparin components glucuronic acid and glucosamine were also able to produce distortions in both fragments while

Table 1

The effects of heparin preparations, heparin antagonists and dextrane on the regeneration of planaria

Group	Supernumerary eyes fragment A*	Distortions fragment A*	Without blastema fragment B*	Distortions fragment B*	Supernumerary eyes fragment A**	Distortions fragment A**	Without blastema fragment B**	Distortions fragment B**
Control	17.5	0	9.4	0	15.0	0	9.4	0
Heparin 3 I.U. 1 mg/ml	21.0	0	10.5	0	18.0	0	7.9	0
Heparin 3 I.U. 2 mg/ml	18.0	0	18.4	0	18.0	0	18.4	0
Heparin 111 I.U. 1 mg/ml	38.5	0	28.0	2.7	28.2	2.7	25.0	2.7
Heparin 111 I.U. 2 mg/ml	26.0	0	80.0	65.0	28.5	0	35.5	50.0
$CaCl_2$ 0.5 mg/ml	20.0	0	21.5	3.8	20.0	0	11.5	3.8
$CaCl_2$ + heparin 111 I.U. 2 mg/ml	29.6	2.7	50.0	2.7	33.3	0	64.0	4.6
Protamine 50 μg/ml	26.5	35.0	died	died	31.0	12.1	died	died
Protamine 100 μg/ml	23.0	39.0	died	died	45.5	36.5	died	died
Dextrane 2 mg/ml	5.2	0	21.0	3.5	5.4	0	13.0	0

* at the period of the highest value (as expressed in per cent)
** a week after the end of the treatment (as expressed in per cent)

supernumerary eyes were only obtained in fragment "A". The action intensity of glucosamine, however, is considerably higher. Glucose and galactose caused no distortions and supernumerary eyes rarely appeared upon their action.

Fig. 2. Fragment "A" of a control planaria at the 11th day of regeneration

Table 2

The effects of heparin components on the regeneration of planaria as compared with some monosaccharides

Group	Supernumerary eyes	Distortions	Without blastema	Distortions	Supernumerary eyes	Distortions	Without blastema	Distortions
	fragment A*	fragment A*	fragment B*	fragment B*	fragment A**	fragment A**	fragment B**	fragment B**
Control	0	0	0	0	9.3	0	0	0
Glucuronic acid 2 mg/ml	22.5	65.1	8.3	30.0	died	died	died	died
Glucosamine 2 mg/ml	39.2	100.0	16.8	100.0	16.6	100.0	42.6	100.0
Glucose 2 mg/ml	16.2	0	0	0	16.2	0	0	0
Galactose	17.5	0	0	0	12.8	0	11.0	0
Glucuronic acid—glucosamine 1 mg/ml—1 mg/ml	35.0	16.7	13.5	24.0	28.5	11.2	15.2	22.8

* at the period of the highest value (as expressed in per cent)
** a week after the end of the treatment (as expressed in per cent)

Fig. 3. Fragment "B" treated with heparin (111 I. U., 1 mg/ml) at the 25th day of regeneration. Blastema did not develop

Fig. 4. Fragment "B" treated with heparin (111 I.U., 1 mg/ml) at the 37th day of regeneration. Double head and three eyes

Fig. 5. Fragment "B" treated with heparin (111 I.U., 2 mg/ml) at the 37th day of regeneration. Thinning head and four eyes

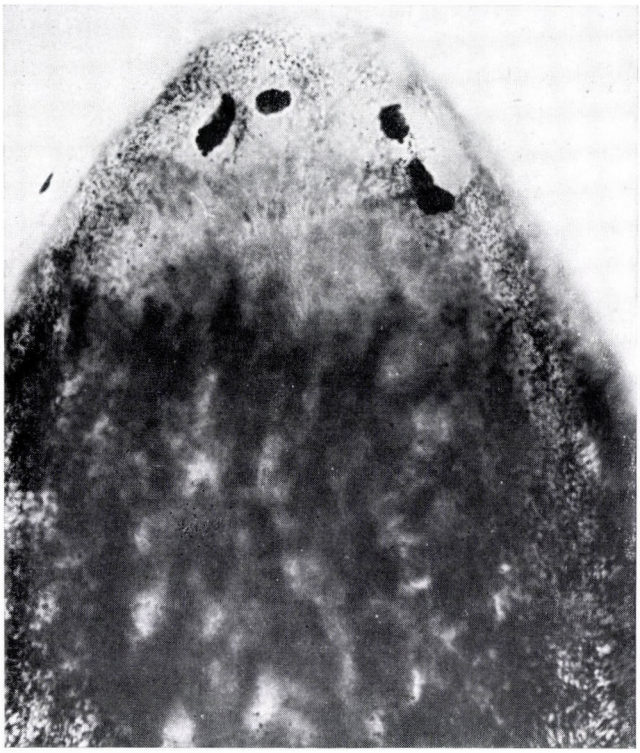

Fig. 6. Fragment "A" treated with protamine sulphate (100 μg/ml) at the 37th day of regeneration. Supernumerary eyes on both sides

Fig. 7. Fragment "A" treated with protamine sulphate (100 μg/ml) at the 13th day of regeneration. Fork-like tail and supernumerary eye

Generally, after the end of treatment the supernumerary eyes normalized but the distortions remained irreversible.

After withdrawal of glucosamine the animals perished.

Control animals and some types of the distortions are presented in Figs 2—7.

Discussion

As referred to in the introduction, in our earlier experiments the heparin effect on cell division was demonstrated. The object of the present experiments was to reveal the mechanism of this effect. This examination seems to be justified because, though a number of authors [1, 2, 3, 4, 5, 13, 14] have dealt with the action of heparin on cell division, hardly any attempts have been made to approach the mechanism. HEILBRUNN [12] was the only author to explain the heparin effect, assuming that heparin being a factor promoting the development of the sol condition is involved in the changes in sol—gel condition during cell division.

Also in our earlier experiments [9] — when examining the heparin effect on the frog's cardiac muscle — we found that the effect of heparin causing diastolic heart-stop can be induced with the use of glucuronic acid alone. Therefore it was attempted in the present experiments to approach the mode of action by examining (a) the effect of heparin preparations different in activity on the sol—gel transformation though hardly differing in chemical structure, (b) the effect of heparin antagonists. At the same time the effect of various components of heparin was investigated.

Experiments conducted on planaria are not suited for a direct investigation of the action on cell division of heparin. The disturbed regeneration is, properly speaking, a disorganization phenomenon — the inhibition or slip of normal organization. In all experiments up to now it has been found, however, that appearance of distortions and of atypical mitoses run parallel. Thus, we are faced by two forms of realization of the same destabilization effect. We relied on this observation when we have drawn conclusions from these experiments on the cell division effect of heparin.

The present experiments have confirmed our previous assumption that heparin preparations of high number of I.U./mg are of destabilizing effect. In these experiments heparin produced distortions and the appearance of supernumerary eyes, respectively. At the same time it appeared that the above phenomena arise exclusively on the action of the heparin preparations of high blood coagulation-inhibiting activity, the preparation of low activity on the blood coagulation was practically ineffective. This means that loss of the blood coagulation-inhibiting capacity — even when SO_4 groups and the molecular weight are retained — is accompanied by the loss of the action on cell division.

If we accept the blood coagulation-inhibiting activity expressed in I.U. of the action on the sol—gel condition, we may assume that the heparin action manifests itself through the sol—gel condition change.

The problem, however, seems to be more complex. The commercial heparin preparation besides producing distortions has the capacity to inhibit the development of blastema, as observable also under the stereomicroscope in 80 per cent of the cases. This agrees with earlier observations of others [3, 11, 14] inasmuch as a cell division-inhibiting action was also attributed to heparin. It is a problem, however, that while the effect of heparin causing malformation can be perfectly prevented with $CaCl_2$, the same substance hardly influences the blastema-retarding effect. This raises the possibility that not all effects of heparin are manifested through influencing sol—gel condition. It can be imagined that heparin bound to histons inhibits normal nucleohiston production and thus retards blastema development.

This duplicity raises further problems. The experiments have shown that in the animals placed from the solution of commercial heparin under normal conditions the development of blastema started, whereas the distortions were repaired only to a very small extent. Thus blastema inhibition can be considered a reversible while the formation of distortions an irreversible process. This duplicity cannot be observed, however, in the group treated simultaneously with heparin and $CaCl_2$. In this case after placing in normal medium the blastema inhibition still increased, more precisely, some individuals dropped the blastema already in development and no new blastema originated. This can only be explained by assuming that from the aspect of blastema inhibition $CaCl_2$ is no antagonist of heparin. This may also point out that blastema inhibition is not based on the sol—gel mechanism. At the same time, presumably, $CaCl_2$ itself — to a lesser extent — has also a blastema-inhibiting effect.

In earlier experiments it was observed that both heparin excess and heparin deficiency (chemical binding of heparin) may lead to distortions [7, 8]. From this we have concluded that heparin as a general destabilizing substance is necessary for cell division in a given — though not exactly known — quantity, but both its excess and deficiency injuriously affect cell division. The present experiments seem to support this assumption: distortions appeared in a great number not only on the action of heparin but also on the action of protamine sulphate as a heparin-binding substance.

The question of fragments "A" and "B" needs to be dealt with separately. It has been observed that on the application of heparin distortions were found only in fragment "B" while in fragment "A" at most the more frequent appearance of supernumerary eyes was established. As pointed out already on the basis of earlier experiments [8], this may be explained with the fact that in fragment "A" the influence of the nervous system (head ganglion) on morphogenesis is very intensive and this inhibits the appearance of more serious

distortions [16]. In fragment "B" on the other hand, where the head ganglion is not present and thus its organizing action is not effective either, there is a free scope for the appearance of distortions. Protamine sulphate, as a heparin-binding substance, caused considerable distortions in fragment "A" while it was of a lethal effect on fragment "B". This points to the fact that the withdrawal of heparin, and of acid mucopolysaccharides generally, is of much more intensive effect that the excess of heparin.

The influence of dextrane was insignificant. In the distortion-producing and blastema-inhibiting effect — as assumed previously — the *acid* mucopolysaccharides are involved.

As regards the components participating in the mechanism, it seems that for the destabilizing effect of heparin — in the case of malformations — first of all glucosamine is responsible which led to distortions practically in all cases. This is consistent with literary data. The investigations of RIZKI [15] have shown that glucosamine is a phenocopying substance and GARBER [10] demonstrated that re-aggregation of dissociated embryonic cells can be inhibited with glucosamine. For blastema-retardation, on the other hand, the whole heparin molecule seems to be responsible since this phenomenon could not be induced to a significant extent either with glucuronic acid or with glucosamine. Anyway, the fact that glucosamine could produce malformation similarly to, or even to a greater extent than, heparin does not facilitate the solution of the problem, since it is not known whether glucosamine is involved in the sol—gel mechanism. This issue requires further examinations.

The fact, that with the application of glucuronic acid and glucosamine together but to a lower dosage rate the number of distortions diminished, seems to point out that the distortion-causing effect of the various components is also a question of dosage.

Summarizing the results, it seems likely that the destabilizing action of heparin on cell division in the first place becomes effective through influencing the sol—gel condition and that binding to histons perhaps — not demonstrably — is only a possible mechanism of the inhibition of cell division, particularly of the inhibition of blastema. Of the components of heparin it is glucosamine which is presumably most responsible for the malformation-causing effect of heparin, but it is not known whether it acts in the same way in itself as the heparin molecule.

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BEITRÄGE ZUM MECHANISMUS DER ZELLTEILUNG-DESTABILISIERENDEN WIRKUNG DES HEPARINS

Es wurde die Wirkung von verschiedenen Heparin-Präparaten (unterschiedliche I. E. in Bezug auf Blutgerinnungshemmung), Heparin-Antagonisten und Heparin-Komponenten auf regenerierende Planarien (*Dugesia lugubris*) untersucht. Dabei wurde festgestellt, daß das Heparin vor allem über die Beeinflussung des Sol—Gel Gleichgewichtes auf die Zellteilungen wirkt, was zu Mißbildungen führt. Es besteht keine Parallelität zwischen der blastem-retardierenden Wirkung und den Mißbildungen, deshalb wird es für möglich gehalten, dass bei letzteren auch Heparin—Histon Bindungen eine Rolle spielen. In der teratogenen Wirkung ist das Glucosamin die wirksamste Komponente des Heparins. In gegebener Versuchsanordnung verursachte das Glucosamin auch allein in sämtlichen Fällen Mißbildungen.

ДАННЫЕ К МЕХАНИЗМУ ДЕЙСТВИЯ ГЕПАРИНА, ДЕСТАБИЛИЗИРУЮЩЕГО ДЕЛЕНИЕ КЛЕТОК

Авторы исследовали на регенерирующихся планариях (*Dugesia lugubris*) действие гепарина, антагонистов гепарина и составных частей гепарина с различным числом свертывания крови. Было установлено, что гепарин изменяет деление клеток в первую очередь путем воздействия на изменения состояния золь-гель. Это действие сказывается в возникновении уродств. Вызванное гепарином ретардация развития бластем не происходит параллельно возникновению уродств, возможно, что в последнем участвует связывание гепарина к гистону. В отношении возникновения уродств наиболее активной составной частью гепарина оказался гликозамин, который и сам по себе способен вызвать уродства в 100% случаев.

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THE EFFECT OF KCl, NaCl, HYDRATION AND DEHYDRATION ON THE SUBCOMMISSURAL ORGAN OF THE DOMESTIC PIGEON

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Synopsis

The effect of hydration, dehydration, and KCl and NaCl load was examined on the subcommissural organ (SCO) of the domestic pigeon. Hydration was followed by no significant morphological changes in the SCO. Dehydration slightly increased the nuclear volume and decreased the secretion content in the SCO. Histological changes of identical nature were observed as a result of NaCl and KCl treatment: increase of the nuclear volume and substantial accumulation of secretion. The results suggest a functional correlation to exist in birds, similar to that existing in mammals, between the SCO and the aldosterone-producing zone of the adrenals.

Introduction

An organ corresponding to the subcommissural organ (SCO) can be found in every vertebrate group, beginning with the amphioxus. The organ is located under or around the commissura posterior [40]. According to its location it is named subcommissural, pericommissural or supracommissural organ.

In the SCO an ependymal and a hypendymal part can be distinguished. In inferior vertebrates the ependyma, in the superior ones the hypendyma is its more developed part. In the primitive vertebrates the organ is rich in secretion [37]. Some authors suppose a correlation between the SCO and the epiphysis. This correlation seems to be confirmed by the fact that in the epiphysis of *Petromyson* and many other vertebrate species there are ependymal cells and fibres which are thought to originate from the SCO. By reason of these findings, a functional correlation is supposed to exist between the SCO and the epiphysis, whereby the active substances produced by the SCO would be stored in the epiphysis, the correlation being similar to that in the hypothalamus—hypophysis system [21].

Besides the morphological research, several investigations have been conducted to reveal the function of the SCO. The role of the SCO in the control of water-balance was demonstrated by GILBERT [8, 9]. The effect of K and Na stress on the SCO of amphibians was examined by LEGAIT [16], while ARVY, FONTAINE and GABE [1] have undertaken similar research on fishes. PALKOVITS and co-workers [5, 6, 7, 26, 27, 29, 30, 31, 32, 33] have found in mammals

that the SCO is responsible for the control of aldosterone production of the zona glomerulosa. Lesions of the SCO caused the atrophy of that zone and a reduced aldosterone production, while SCO extract resulted in the hypertrophy of the zona glomerulosa and in aldosterone overproduction. Further evidence of the role played by the SCO in the control of aldosterone production was obtained by examining the effect of Na- and K-free food. DE LONG and BALOGH [19] came to opposite results, indicating that the glucose-6-phosphate dehydrogenase activity of the SCO is changed neither by Na treatment nor by dehydration. In his comprehensive work LENYS [18] comes to the conclusion that there is a functional correlation between the Na level of the blood-plasma and the secretory state of the SCO. In analyzing the consequences of spirolactone, metopyrone and aldosterone treatment, as well as of bilateral adrenalectomy and SCO lesions, he points out — in opposition to PALKOVITS — that the SCO does not play an important role in the regulation of aldosterone secretion. However, he failed to support the histological evaluation with a direct assay of aldosterone production; his working hypothesis is based on the supposition of a simple feed-back system, which means an excessive simplification of the problem in case of a complex regulation mechanism. Other authors have found the photoreceptor function of the SCO in amphibians [22, 16].

The ontogenesis of the SCO was studied on birds by WINGSTRAND [39], GRIGNON and GRIGNON [10] and PALKOVITS and WETZIG [28]. They found that the organ appears on the 5th or 6th day and contains secretion from the 7th incubation day on. On the 10th incubation day the Reissner fibre is also observable. The hypendyma reaches its final development in postembryonic state only. Degeneration of the ependymal layer of the SCO is not known in birds. This phenomenon has been described by PALKOVITS and co-workers [29] in man.

Earlier literature on birds, dealing whith the histology of the SCO and with its correlation to the Reissner fibre, was summed up by PALKOVITS [33].

KOLMER [15] described the SCO of sparrow, duck, goose and Paleornis. REICHOLD [36] treated the histological structure of the SCO of sparrow and finch. EBERL-ROTH [3] studied the correlation between the Reissner fibre and the SCO in hen, goose, *Cypselus aper*, *Corvus corone*, *Coleus monedula*, *Fringilla coelebs* and *Passer domesticus*. LEGAIT [17] described the structure of the organ in the hen. Examining the SCO of domestic pigeon and the sparrow, VIGH and co-workers [37] have found that the organ contains mainly apical secretion and that fine secretion granules are present in the ependymal cell rows.

OKSCHE [25] examined the SCO of hen, domestic pigeon, Zonotrichia and Taenopyga with regard to secretory activity. According to this author, the SCO contains not only apical, but basal secretion as well.

According to WETZIG and KÄMMERER [38] the hypendyma of Lamellirostres and Phalacrocorax is more or less developed and contains secretion,

while it is underdeveloped in the sparrow and the finch. In singing-birds the hypendymal layer is supposedly not present but in young birds. This seems to be confirmed by the finding of OKSCHE [25], viz. the hypendyma and its secretory contents are well developed in downy goldfinches and by the time of sexual maturity the hypendymal layer disappears.

NOMOKONOVA and SAPIRO [20] examined the SCO of birds from the functional viewpoint. They have found that hydration and dehydration cause accumulation of secretion and appearance of vacuoles in the cytoplasm of ependymal cells. According to these authors this phenomenon suggests intensified secretion and transport. Photogenic catalepsy caused a substantial accumulation of secretion in the SCO of the hen, while the same action resulted in the discharge of secretion in the guinea pig.

According to literary data there are great differences in the SCO among the vertebrate groups, and significant differences may be expected even among various orders of birds. These findings may be still more true in the functional respect.

The functional examination of the SCO of the bird was justified by the results showing that the interrenal substance of the bird's adrenal does not react as a homogeneous organ on different functional actions, viz. this substance is divided into a peripheral part producing aldosterone and another, more deeply sited one, producing glucocorticoid [11, 12, 13, 14]. Relying upon our earlier studies, we have presumed that the regulation of the adrenal through the median eminence extends mainly on the more deeply sited interrenal bundles [34, 35], so that another factor is supposed to be responsible for the regulation of the peripheral zone.

Material and method

The experiments were made in March—May, 1965, on domestic pigeons of which 9 served as control, 6 were hydrated, 6 dehydrated, 8 treated with NaCl and 7 with KCl.

Hydration was applied for 10 days with the help of a gastric tube. Dehydration lastedfor 8 days. NaCl and KCl treatment was made with 4% solution for 21 days. With the help of a gastric tube, salt solution was introduced into the organism of the birds twice a day, 5 ml each time. The birds were exposed to light for 11 hours a day. They were killed with decapitation. The brains were fixed in Bouin fixative. After double embedding according to Péterffi, 5 μ sections were made and stained with PAS and Gomori-type aldehyde-fuchsin, both being combined with haematoxylin nuclear staining. Twohundred nuclei were taken from the SCO and 200 from the ependyma of the third cerebral ventricle, and measured, using 2,500-fold magnification. The nuclear volume was determined by means of the FISCHER—INKE nomogram with the help of the formula $\pi/6\ L\ B^2$. The frequency distribution of the logarithm of the nuclear volumetric values was determined with reference to the control group and the treated groups. The arithmetic mean (m) and the standard deviation (σ) of the frequency distributions were calculated. We have furthermore determined the frequency of the values falling within the range $m \pm \sigma$ so as to be able to compare the empirical frequency functions thus obtained to the frequency function of the normal distribution.

We also made the "t" test in order to check the significance of the arithmetic means. In our case the difference is significant if $t \geq 1.96$. Even beyond this limit because of the error sources inherent to histological methods, only those significant cases were considered as biologically appreciable, where $(m_1 - m_2) > 0.05$.

Results

Evaluation was made partly on the basis of the histological appearance and partly by comparing the nuclear volumetric values of the SCO with those of the ependyma of the corresponding group and with those of the control SCO.

In the control group the histological aspect of the SCO is generally characteristic of pigeons. The apical secretion is readily discernible in the ependymal layer. The cytoplasm of the ependymal cells contains evenly distributed dust-like secretion staining well with PAS and aldehyde-fuchsin. In the hypendymal layer there is comparatively not much basal secretion around the capillaries. In a few birds we found a larger amount of basal secretion, probably due to differences in age (Figs 1, 2).

For the hydrated group the histological aspect did not present any substantial difference as compared with the control. Apical secretory contents were observed here too. In the cytoplasm there was an evenly distributed and faintly stained PAS- and aldehyde-fuchsin-positive secretion. The quantity of basal secretion is unchanged (Fig. 3). The aldehyde-fuchsin-positive secretion of homogeneous distribution decreased in amount. There was no essential change in the quality of either basal or apical secretion (Fig. 4).

In dehydrated animals, the staining of ependymal cells had slightly changed as compared with the control.

In the group treated with NaCl the fine granulation increased in the cytoplasm of ependymal cells and stained more vividly by aldehyde-fuchsin. Vacuoles of sharp outlines appeared in the ependymal cells, with smaller or larger aldehyde-fuchsin-positive droplets. The apical secretory contents slightly increased, while the basal secretion showed the most striking accumulation (Fig. 5).

The group treated with KCl presents exactly the same histological changes as that treated with NaCl. A fine dust-like aldehyde-fuchsin-positive secretion was visible in the cytoplasm of the ependymal cells. The vacuoles with aldehyde-fuchsin-positive droplets, characteristic of the NaCl-group were also present and an increase of the apical and, above all, of the basal secretion could be observed (Fig. 6).

According to statistical-mathematical evaluation of the values of nuclear volume, the curve of the control group can be characterized by an extended and comparatively low maximum. The curve of the hydrated group almost coincides with that of the control group, the maximum of both curves being in the nuclear-volumetric class 1.6. There is no biologically appreciable difference between the control and the hydrated group. In the dehydrated group the curve presents a double maximum and, as compared with the control, is shifted to the right by one unit. There is no significant and biologically appreciable difference between the control and the dehydrated group. In the group treated

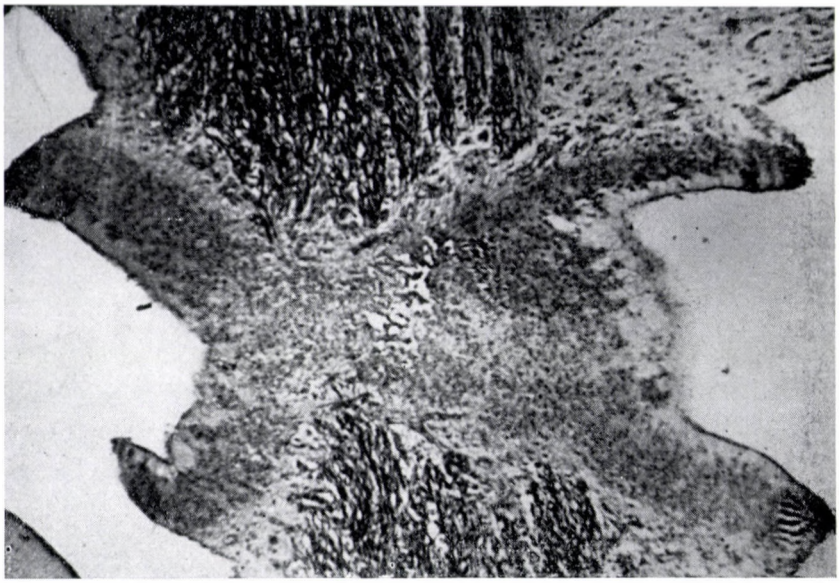

Fig. 1. Control pigeon SCO. Bouin, aldehyde-fuchsin, ×70

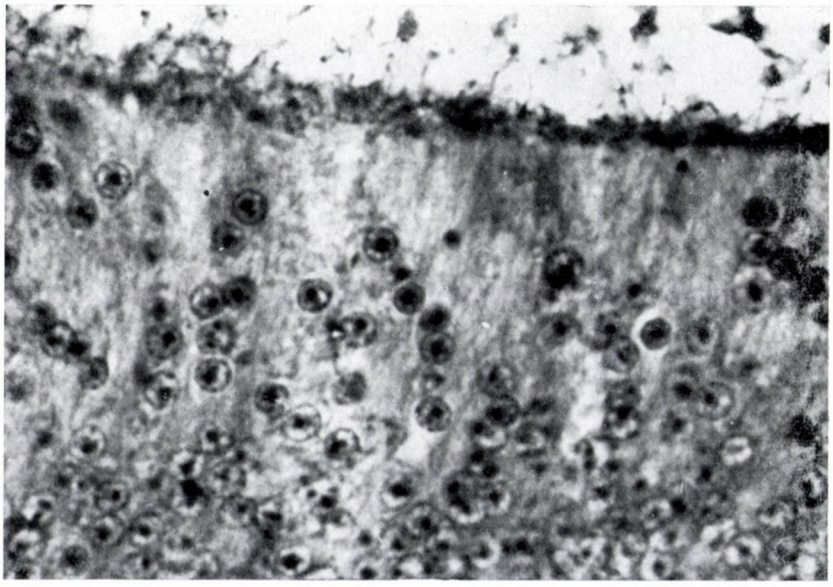

Fig. 2. Control pigeon SCO. Bouin, aldehyde-fuchsin, ×2,016

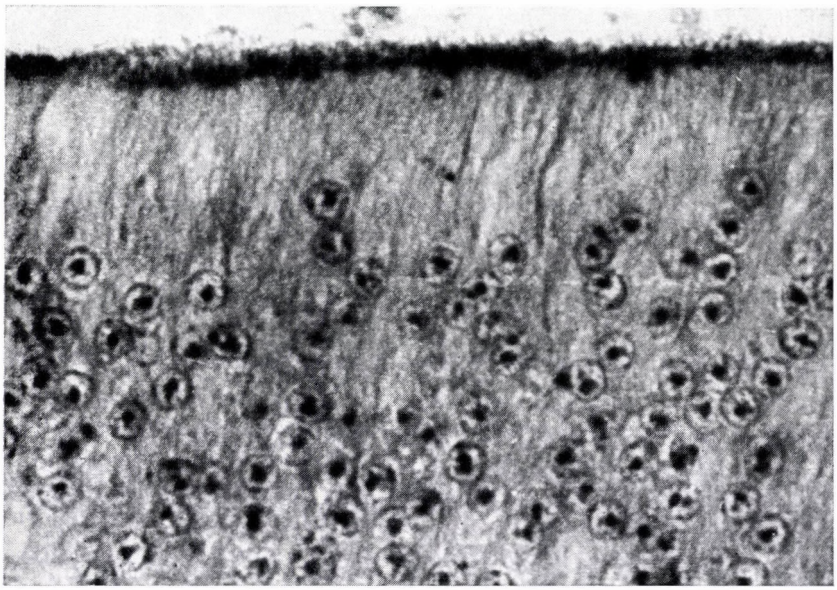

Fig. 3. Hydrated pigeon SCO. Bouin, aldehyde-fuchsin, ×2,016

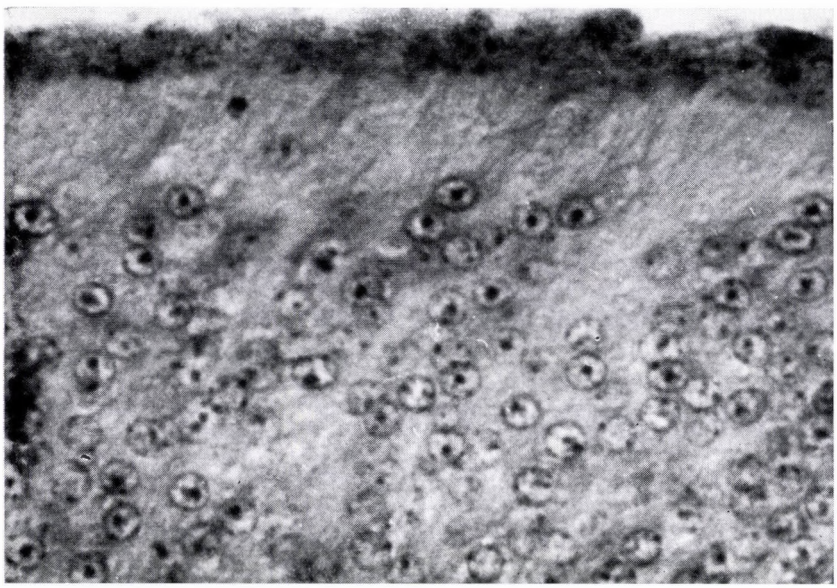

Fig. 4. Dehydrated pigeon SCO. Bouin, aldehyde-fuchsin, ×2,016

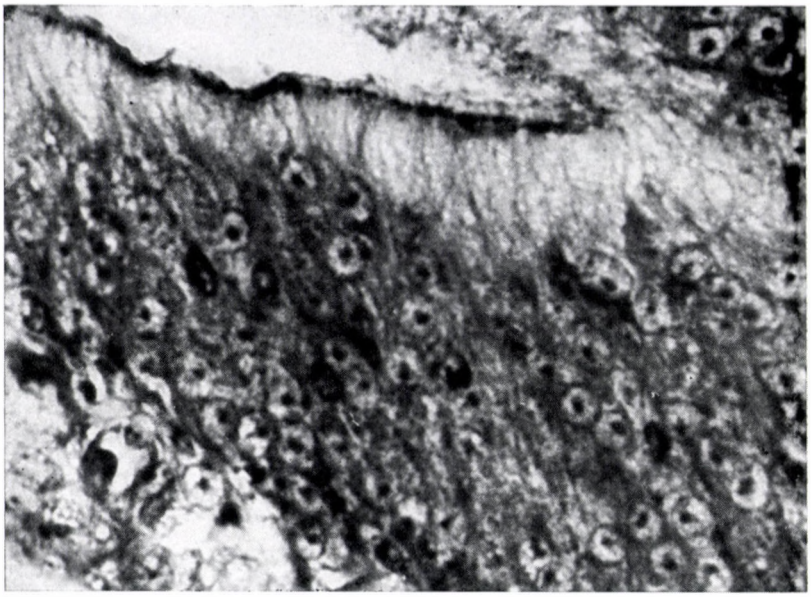

Fig. 5. NaCl-treated pigeon SCO. Bouin, aldehyde-fuchsin, ×2,016

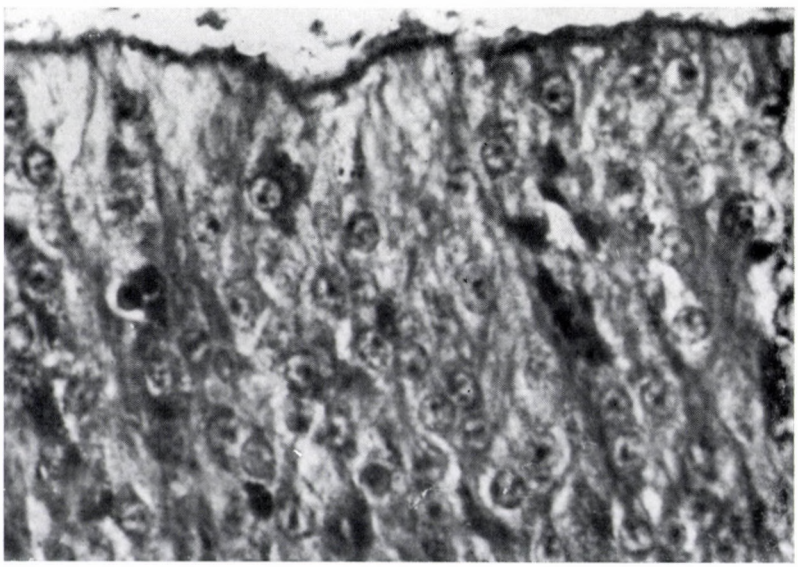

Fig. 6. KCl-treated pigeon SCO. Bouin, aldehyde-fuchsin, ×2,016

with KCl a slight shift to the right can be observed in the curve, while the maximum presents a platform. There is a significant difference between the control group and the one treated with KCl, but it is not appreciable biologically $[(m_1 - m_2) < 0.05]$. In the group treated with NaCl the maximum of the curve is shifted to the right by two units, as compared with the control, the difference is highly significant and biologically appreciable (Tables 1, 2; Figs 7, 8).

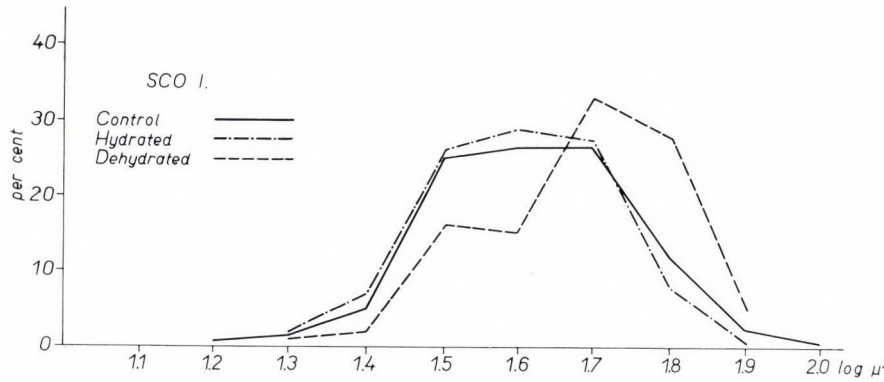

Fig. 7. Values of the nuclear volume of SCO

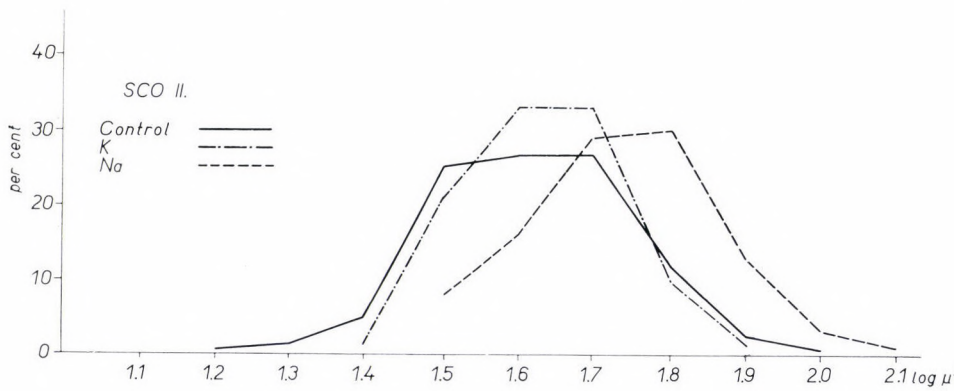

Fig. 8. Values of the nuclear volume of SCO

The nuclear-volumetric values of the ependymal cells were also determined. The logarithmic curves of the nuclear volume show no substantial difference, the maxima appearing in the same class. The group treated with NaCl is the only exception: the corresponding curve is shifted to the right, the maximum being displaced to the right by one unit. This group presents a significantly higher value than the control group and, being $(m_1 - m_2) > 0.05$, this difference is biologically appreciable (Tables 1, 2; Figs 9, 10).

Table 1

Values of "m" and "σ" for ependyma and SCO groups

Group	Ependyma			SCO		
	m	σ	μ	m	σ	μ
Control	1.52	0.140	1800	1.61	0.125	1800
Hydrated	1.49	0.124	1200	1.60	0.126	1200
Dehydrated	1.55	0.132	1200	1.67	0.130	1200
K	1.54	0.144	1400	1.65	0.104	1000
Na	1.62	0.130	1600	1.74	0.132	1600

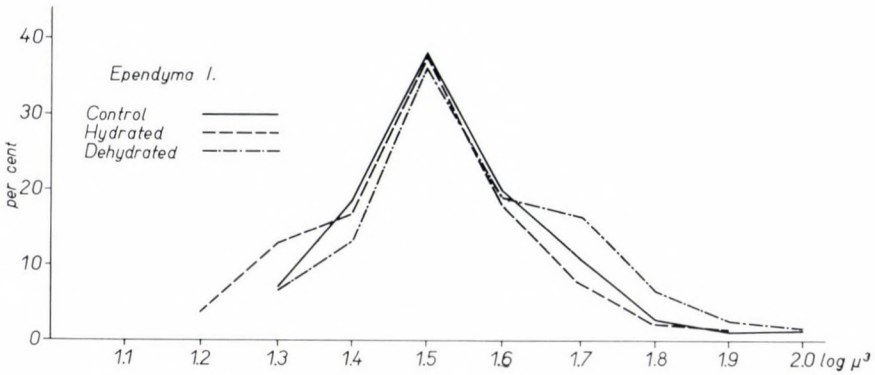

Fig. 9. Values of nuclear volume of ependyma

Table 2

Values of "t" for ependyma and SCO groups

Group	Ependyma	SCO
Control-hydrated	6.0	2.1
Control-dehydrated	5.8	12.8
Control-K	3.8	9.5
Control-Na	21.3*	29.5

* = appreciable difference

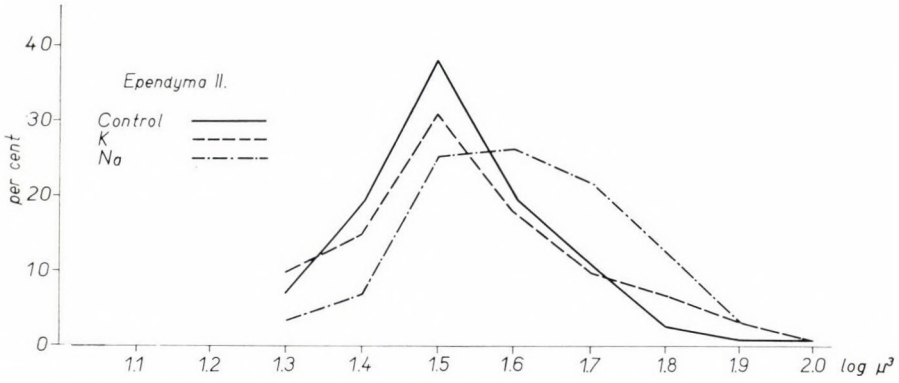

Fig. 10. Values of nuclear volume of ependyma

Discussion

The present studies included experimental interventions producing definite changes in the interrenal substance of the adrenals.

The SCO of the control animal contains less basal and more apical secretion. In the cytoplasm of more apically located cells there are fine aldehyde-fuchsin and PAS-positive granules. These findings agree with the results of VIGH and co-workers [37] and of LEGAIT [17].

In hydrated animals a definite atrophy manifests itself in the more deeply located interrenal bundles of the adrenals. At the same time there is neither a histochemical nor a morphological alteration detectable in the peripheral part [14]. The SCO is like that of the control group, neither the nuclear-volumetric value nor the histological findings presents any substantial difference. Secretion is accumulated in the palisade zone of the median eminence [35]. The morphological aspect of the epi- and hypothalamic structures and the structural change in the adrenals suggest an inhibition to be brought about by hydration in the CRF-ACTH system. This action manifests itself in the atrophy of the glucocorticoid-producing zone. At the same time, there is probably no substantial change in the function of the centres regulating the aldosterone-producing zone.

As a result of this, a definite hypertrophy developed in the interrenal bundles of the adrenals and, particularly, in the deeper zone [12]. In the hypothalamic neurosecretory system, the evacuation of the neurohypophysis is conspicuous while the secretion contents of the median eminence do not change considerably [35]. In the SCO, dehydration causes a slight increase in the nuclear volume and the discharge of the secretion. Our observation confirms the result of NOMOKONOVA and SAPIRO [20], suggesting that dehydration intensifies the secretion of the SCO. The hypertrophy of the deeply located

interrenal zone is caused here probably by the ACTH-releasing action of the neurohumour bound to the large quantity of Gomori-positive secretion which is released from the neurohypophysis. The slight hypertrophly of the peripheral zone can be interpreted as follows it may result from a very strong ACTH action: this assumption is supposed by the fact that, as a result of treatment with large doses of ACTH, a hypertrophy combined with lipid discharge can be observed in the pripheral zone too [13]. It can be assumed that, in addition to the large amount of ACTH, a specific "glomerulotropic" factor may play a role. In terms of this conception we may point to the similarity of the alteration in the adrenal peripheral zone in the SCO. As a result of NaCl treatment a strong atrophy develops in the peripheral part of the adrenal interrenal substance, and a slight hypertrophy in the more deeply located zone [11]. Treatment with hypertonic salt solution produces functional changes in the neurosecretory system, similar to those caused by dehydration. The neurohypophysis is evacuated, while the secretory contents of the median eminence remain essentially unchanged [4, 23, 24]. NaCl treatment causes striking histological changes in the SCO. The value of the nuclear volume substantially increases, a large amount of basal secretion accumulates in the hypendymal layer, and vacuoles containing aldehyde-fuchsin-positive droplets appear in the ependymal cells. The changes in the nuclear volume are opposite to those expected. The changes can be interpreted in the following way: NaCl activates the secretion in the SCO, which, in turn is accompanied by an increase of the nuclear volume. At the same time, however, the discharge of the secretion is inhibited, showing that the synthesis of the secretion in this case does not run parallel with its secretion. FÖLDVÁRI and PALKOVITS [7] examined the effect of Na and K shortage in the SCO of rat. They observed a change in the nuclear volume, opposite in sense to the expected change. These results remind us of interpreting most carefully the changes of the nuclear volume in the SCO. Treatment with NaCl causes thus a definite storage of secretion in the SCO of the pigeon, while the peripheral aldosterone-producing zone of the adrenal becomes atrophied. PALKOVITS and LUKÁCS [30] have found that the extract of the SCO evacuated with adrenalin has no tropic effect on the zona glomerulosa. Thus, the glomerulotropic effect seemed ascribable to the aldehyde-fuchsin-positive secretion stored in the SCO. It seems thus to be probable that in our experiment the atrophy of the peripheral zone of the adrenal was in some correlation with the accumulation of secretion in the SCO.

KCl treatment causes in the peripheral interrenal substance of the pigeon's adrenal a slight atrophy [13]. BROWN and co-workers [2] obtained strong Na and slight K retention with DOCA treatment on sexually mature cocks. These data suggest that the mineralocorticoids may cause in birds — unlike in mammals — not only a significant Na retention, but also a slight K retention. In the SCO, K treatment causes exactly the same histological

changes as NaCl treatment. Strong accumulation of the secretion can be observed both in the hypendymal layer and in the ependymal cells. The value of nuclear volume, on the other hand, does not increase to as a great extent as in birds treated with NaCl. Strikingly, the treatment with NaCl and KCl causes changes of identical character in both the SCO and in the peripheral interrenal substance of the adrenal. For comparison's sake we have measured in each test animal the nuclei of the ependymal lining of the third cerebral ventricle, to show whether the effect on the SCO is specific or not. We have found that hydration, dehydration and KCl treatment do not cause any biologically appreciable change in the nuclear volume of the ependymal cells, whereas NaCl treatment increased the nuclear volume in the ependyma. In birds the sensitivity of SCO to NaCl is thus not as specific as in mammals, in which according to FÖLDVÁRI and PALKOVITS the nuclear volume of ependymal cells did not change upon the action of Na and K deficiency [7].

On the ground of the present results it can be concluded that the changes caused by different actions in the SCO of the domestic pigeon are of the same nature as those caused by the same experimental actions in the peripheral interrenal substance of the adrenal. In other words: it may be supposed that the SCO of birds, similar to that of mammals, exerts some direct or indirect regulating action on the aldosterone-producing zone of the adrenal. We intend to confirm our hypothesis with further experiments.

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WIRKUNG VON KCl UND NaCl, SOWIE VON WASSERBELASTUNG UND DURST AUF DAS SUBCOMMISSURALORGAN DER HAUSTAUBE

Die Wirkung von Belastung durch Hydration, Dehydration, KCl und NaCl wurde am SCO der Haustaube untersucht. Die Wasserbelastung verursachte im SCO keine signifikante morphologische Veränderung. Der Durst führte im SCO zu einer geringen Zunahme des Kernvolumens und zu einer Sekretverminderung. Durch die Belastung mit NaCl und KCl wurden histologische Veränderungen gleichen Charakters beobachtet: Zunahme des Kernvolumens und kräftige Sekretanhäufung. Auf Grund der Ergebnisse ist es anzunehmen, daß auch bei den Vögeln ähnlich wie bei den Säugetieren ein funktioneller Zusammenhang zwischen dem SCO und der aldosteronbildenden Zone der Nebenniere besteht.

ДЕЙСТВИЕ НАГРУЗКИ KCl И NaCl, ВОДНОЙ ПРОБЫ И ЖАЖДЫ НА СУБКОММИСУРАЛЬНЫЙ ОРГАН ДОМАШНЕГО ГОЛУБЯ

Действие гидратации, дегидратации, нагрузки KCl и NaCl исследовалось на субкоммисуральном органе домашнего голубя. После гидратации в субкоммисуральном органе не наблюдалось значительных морфологических изменений. При дегидратации в субкоммисуральном органе было отмечено незначительное увеличение объема ядер и уменьшение секрета. В результате нагрузки KCl и NaCl наблюдались одинаковые гистологические изменения: нарастание ядерного объема и значиртельное накопление секрета. На основе полученных результатов кажется очень вероятным, что у птиц подобно млекопитающим, также существует функциональная связь между субкоммисуральным органом и альдостероносинтезирующей зоной надпочечников.

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THE THYROID-REGULATING HABENULAR MECHANISM AND THE THYROTROPHIC AREA OF THE ANTERIOR HYPOTHALAMUS

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Synopsis

It has been reported that heat-adapted rats bearing bilateral lesions of the habenular nuclei respond to cold exposure with a significantly higher increase of thyroidal ^{131}I-accumulation than the intact controls. In this study, bilateral lesions of the thyrotrophic area of the anterior hypothalamus caused a marked decrease in thyroid activity, and the thyroids of the injured animals did not respond to cold exposure. Rats bearing simultaneous lesions both in the habenular nuclei and in the hypothalamic thyrotrophic area displayed the same thyroidal ^{131}I-accumulation as animals bearing simple hypothalamic lesions. The conclusion is drawn that the thyrotrophic area of the anterior hypothalamus is indispensable in the neural mechanism by which habenular structures exert their influence on the TSH-thyroid system.

Introduction

Heat-adapted (28° C) intact rats transferred into a low (14° C) environmental temperature show a marked increase in thyroidal activity (T/S ratio). This increase is significantly higher in animals bearing bilateral lesions in the habenular nuclei than in normal rats under similar conditions [7]. YASUMURA and KNIGGE [10] found a significantly greater increase in T/S ratio following thiouracil treatment in animals bearing habenular lesions than in the thiouracil-treated controls. The compensatory hyperactivity in ^{131}I-trapping capacity after hemithyroidectomy was also significantly increased in cats bearing habenular lesions when compared to hemithyroidectomized animals without habenular lesions [4]. According to the interpretation of YASUMURA and KNIGGE [10] and KNIGGE [4], this enhanced thyroid reaction is due to the impairment of the TSH-mobilization regulating mechanism situated in the habenular nuclei.

The "overshooting" reaction of the thyroid gland following different challenges (thiouracil treatment, hemithyroidectomy, cold exposure) of the TSH-thyroid system seems to be a characteristic effect of the destruction of the nuclei habenulae. This effect was applied to the investigation of the question, whether the thyrotrophic area of the anterior hypothalamus is indispensable in the neural mechanism by which habenular structures exert their influence on the TSH-thyroidal system, or the habenular mechanism is more or less independent of the anterior hypothalamus-TSH-thyroid feedback loop.

Material and method

Experiments were performed on 113 adult male albino rats of 150—200 g body weight, from our inbred strain (originally Wistar). Before beginning the experiment all animals were adapted to a constant environmental temperature of 28° C for at least 2 weeks. All were kept on a standard diet containing 3 mg KI/kg food during the entire course of investigation. The rats were then divided into 4 groups:

Group A: intact controls (25 animals)
Group B: rats bearing bilateral lesions of the habenular nuclei (31 animals)
Group C: rats bearing bilateral lesions of the thyrotrophic area of the anterior hypothalamus ("hypothalamic lesions" in Table 1) (24 animals)
Group D: rats bearing simultaneous lesions both in the habenular and hypothalamic region ("combined lesions" in Table 1) (33 animals).

Electrolytic lesions were performed using a stereotaxic instrument. Each group was further divided into two subgroups. One subgroup of each type was transferred into a cold room of 14° C temperature seven days following the operation and was kept there for two weeks. The animals of the other subgroup were kept at 28° C environmental temperature for the entire experimental period. Three weeks after the lesions all the animals were injected with 6 mg propylthiouracil and 1 hour later with 10 μC ^{131}I subcutaneously as suggested by VANDERLAAN and GREER [9]. Animals were exsanguinated by puncture of the abdominal aorta one hour after the injection of radioiodine. T/S ratio was determined by the method originally described by VANDERLAAN and GREER [9]. Endocrine organs were fixed in Susa solution, brains in 10% formalin and, after embedding in celloidin-paraffin, stained with haematoxylin-eosin for histological investigations. The site and extent of the different lesions were determined by histological examination of serial sections of the diencephalon.

Results

The results are summarized in Table 1. The control rats showed a 38% increase (from 25.7 to 35.5) in the T/S ratio following cold exposure in contrast to a 79% increase (from 25.6 to 46.0) observed in the animals with bilateral habenular lesions. This difference in the T/S ratio between the rats kept in a warm and those kept in a cold environment is statistically significant in both of these groups (A and B). The group with lesions of the thyrothophic area showed a 22% decrease of T/S ratio in warm environment as compared to the intact controls in a similar environment ($0.02 < p < 0.05$). The subgroup of these animals kept in a low environmental temperature also showed a decrease in the T/S ratio. In fact, the difference between the T/S ratios of animals bearing anterior hypothalamic lesions kept in warm and in cold environment is not significant ($0.20 < p < 0.50$). The T/S ratios for Group D are in all details similar to those for Group C. In Group D there was no difference in the T/S ratios between the rats kept in a warm and those kept in a cold environment, which is also characteristic of anterior-basal hypothalamic lesions [3]. The statistical evaluation also shows clearly that there is no difference between the T/S ratios for Groups C and D ($p > 0.50$) both for warm and cold environments.

The weights of the endocrine organs showed no statistically significant differences.

The histological character of the thyroid glands is in accordance with the T/S ratios. Thyroids from Groups C and D showed, independent of the environ-

Table 1

The T/S ratios for warm- and cold-exposed rats bearing different diencephalic lesions

Group A Controls		Group B Habenular lesion		Group C Hypothalamic lesion		Group D Combined lesions	
Warm	Cold	Warm	Cold	Warm	Cold	Warm	Cold
18.9	17.6	10.1	23.3	12.4	10.9	6.7*	7.2*
19.4	26.6	10.5	25.1	13.9	12.9	8.1*	8.4*
23.7	28.3	12.5	34.8	14.5	13.2	8.6*	9.1*
24.6	28.8	14.3	37.4	14.8	13.6	11.4	9.6
25.8	30.2	24.1	37.9	16.2	15.9	13.3	9.8
25.8	33.5	24.4	38.7	17.8	17.4	16.4	12.3
25.8	35.4	24.8	40.0	19.9	20.5	16.6	15.6
27.0	36.0	25.2	44.5	21.6	24.4	18.1	17.4
27.2	39.1	26.1	46.5	22.9	24.7	18.6	17.5
30.3	41.1	27.8	48.9	27.6	28.6	21.2	21.5
34.5	41.8	28.0	53.4	39.5	31.6	22.5	22.4
	41.8	33.6	59.9		48.1	23.3	25.8
	48.2	35.8	64.1		49.6	24.5	27.9
	48.8	40.6	67.5			33.2	36.3
		41.5	68.1			34.8	36.8
		44.9				39.4	40.9
							46.8
25.7 ± 1.3	*35.5* ± 2.3	*25.6* ± 2.7	*46.0* ± 3.7	*20.1* ± 2.4	*23.9* ± 3.5	*19.8* ± 2.4	*21.4* ± 3.0

t = 3.25, $p < 0.01$ (Group A)

t = 3.96, $p < 0.01$ (Group B)

t = 0.86, $0.20 < p < 0.50$ (Group C)

t = 0.43, $p > 0.50$ (Group D)

t = 2.25, $0.02 < p < 0.05$ (Group A – Group B)

t = 0.08, $p > 0.50$ (Group C – Group D)

t = 0.54, $p > 0.50$ (Group D – Group C)

* Total or partial lesion of the pituitary stalk.

mental temperature, the well-known signs of inactivity (large follicles filled with colloid, flattened epithelia with small nuclei), while thyroids from Group B, especially in animals kept at 14° C, were moderately hyperactive (small follicles, cylindrical epithelia, large nuclei).

The characteristic location of the lesions in the three different experimental groups is illustrated in Fig. 1. The location of the habenular lesions (Fig. 1a) was the same as described in previous papers by the author [5, 6], or by Szentágothai [8], Bogdanove [2], Knigge [4] and Yasumura and Knigge [10]. The hypothalamic lesion was situated in the so-called "thyrotrophic area"

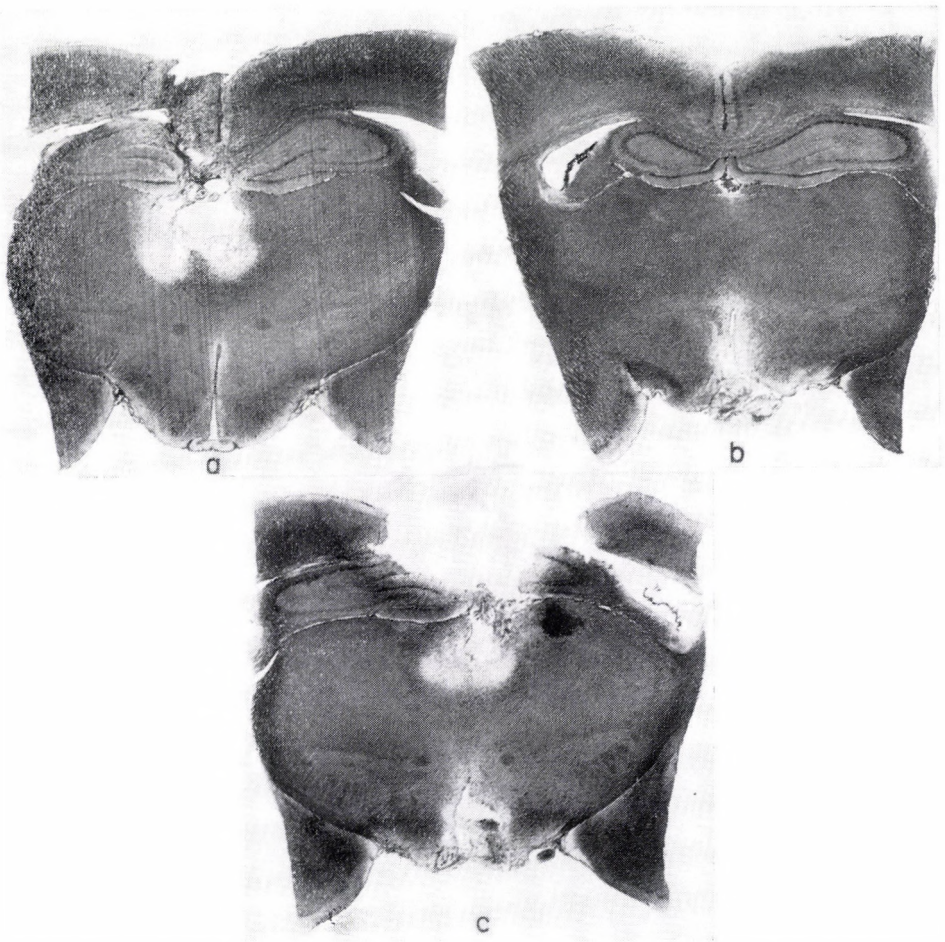

Fig. 1. The characteristic localization of the diencephalic lesions. (a) lesion of the nuclei habenulae; (b) lesion of the thyrotrophic area of the anterior hypothalamus; (c) "combined lesions" both in the habenular region and in the thyrotrophic area

of the hypothalamus (Fig. 1b), i.e., between the optic chiasma, paraventricular nuclei and anterior median eminence. Fig. 1c illustrates the so-called "combined lesions". Since the thyrotrophic area of the hypothalamus is located 1—1.5 mm, and the habenular nuclei 3—3.5 mm, behind the bregma, only the posterior part of the hypothalamic injury and the anterior part of the habenular lesion are shown in Fig. 1c.

Discussion

The results presented in Table 1 as well as the histological picture of the thyroids of animals from the different experimental groups have shown that the "overshooting" effect of habenular lesions was completely abolished in rats bearing simultaneous lesions of the thyrotrophic area of the hypothalamus. This suggests that the thyrotrophic area of the anterior hypothalamus is indispensable in the neural mechanism by which habenular structures exert their influence on the TSH-thyroid system.

One objection that can be raised against this theory is that mentioned by VAN BEUGEN and VAN DER WERFF TEN BOSCH [1]. They assume that in the case of animals with lesions of the thyrotrophic area, the thyroids have a lower basal level of activity. Therefore, superimposition of any influences increasing thyroid activity cannot produce an increase of the same absolute magnitude as will be observed in the intact control animals.

If this were the case in our experiment, the T/S ratios for Group D should be located somewhere between the levels of Groups B and C. Since there was no significant difference between Groups D ("combined lesions") and C ("hypothalamic lesions") in the T/S ratio, the hypothesis of VAN BEUGEN and VAN DER WERFF TEN BOSCH is not valid for this experiment.

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DER HABENULÄRE MECHANISMUS ZUR STEUERUNG DER SCHILDDRÜSEN-AKTIVITÄT UND DIE THYREOTROPE AREA DES VORDEREN HYPOTHALAMUS

Es wurde mitgeteilt, daß wärmeadaptierte Ratten nach bilateraler Läsion der Nuclei habenulae auf Kältewirkung mit einem signifikant höheren Anstieg der ^{131}J-Akkumulation in der Schilddrüse reagieren, als die intakten Kontrolltiere. In den gegenwärtigen Experimenten führte die bilaterale Läsion der thyreotropen Area im vorderen Hypothalamus zu einer deutlichen Abnahme der Schilddrüsenaktivität und die Schilddrüsen der lädierten Tiere reagierten nicht auf Kälte. Ratten mit simultanen Läsionen sowohl der Nuclei habenulae als auch des hypothalamischen thyreotropen Bezirks zeigten die gleiche ^{131}J-Akkumulation, wie die Tiere, bei denen ausschließlich der Hypothalamus lädiert wurde. Es wird angenommen, daß im neuralen Mechanismus, durch den die habenulären Strukturen ihre regelnde Wirkung auf das TSH-Schilddrüsen-System ausüben, die thyreotrope Area des vorderen Hypothalamus unentbehrlich ist.

НЕВРАЛЬНЫЙ МЕХАНИЗМ NUCLEI HABENULARES ДЛЯ РЕГУЛИРОВАНИЯ ФУНКЦИИ ЩИТОВИДНОЙ ЖЕЛЕЗЫ И ТИРЕОПРОПНАЯ ОБЛАСТЬ ПЕРЕДНЕЙ ЧАСТИ ГИПОТАЛАМУСА

Было сообщено, что крысы, адаптированные к высокой температуре, после нанесения билатерального повреждения на nuclei habenulares реагируют на действие холода с гораздо более значительным повышением поглошения ^{131}J щитовидной железой, чем интактные контрольные животные. В настоящих экспериментах билатеральные повреждения, нанесенные на тиреотропную область передней части гипоталамуса, вызвали значительное снижение активности щитовидной железы, причем щитовидные железы этых живодных не реагировали на действие холода. Крысы с одновременными повреждениями nuclei habenulares и тиреотропной области гипоталамуса показали такое же поглощение ^{131}J щитовидной железой, как и животные с повреждением лишь гипоталамуса. Авторы сделают вывод, что тиреотропная область переднего гипоталамуса непременно нужна для неврального механизма, посредством которого nuclei habenulares вызывают свое действие на систему тиреотропный гормон-щитовидные железы.

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STUDIES ON THE BLOOD—THYMUS BARRIER

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Synopsis

The thymus of the guinea pig and of the rat was examined by electron microscopy for the blood—thymus barrier. No stationary barrier was found which would inhibit the penetration of an antigen. Though the tissue elements forming a barrier, such as basal membrane of the endothelial and epithelial cells, adventitia consisting of collagen fibre layers, are occasionally present around some vessels, they are in most cases deficient and dynamically changing in composition. It is assumed that thymocytes may migrate among the cells of the vascular wall and even through the cells.

Introduction

One of the topical problems of experimental histology and tissue-immunity is the function of the thymus. The way to the elucidation of this function was opened by the observation that thymectomy carried out in embryonic or newborn age [7] causes a serious immunbiological defect in the developing organism. This observation has attracted attention to the role of the thymus in the defense of the organism. It appeared as a great contradiction that in the thymus there are no plasma cells [5, 11] and no antibodies are produced [2, 4, 6, 7], indicating that the thymus is different from the lymphoid organs also in its function. These discoveries have changed the recent concepts on the thymus function. MARSHALL and WHITE [6] tried to explain the lack of antibody production by assuming a blood—thymus barrier, similar to the blood—brain barrier, which does not allow the antigen to penetrate the thymus parenchyma. They tried to verify this assumption by injecting antigen directly into the thymus substance [10]. As a result of this, in the thymus otherwise missing reaction centres, plasma cells appeared. Furthermore the thymus planted into the eye chamber proved to be able to produce antibodies [2, 4, 7].

According to the present concept the thymus is our most important lymphopoetical organ where, mainly in the cortex, the mitosis rate is very high. Repopulation of the thymus after emigration of cells upon the action of various stresses takes place from the blood-stream as verified by administering bone-marrow and lymph node cells or labelled thymus cells intravenously. These cells got into the thymus and settled there [1, 9]. Accordingly, it is thought that through the thymus a continuous circulation of lymphocytes takes place.

In the thymus the lymphocytes begin to divide, and obtain an information rendering them capable of transforming into antibody-producing cells when having settled in other lymphoid organs [3]. The mechanism of this streaming in and out of the thymocytes has not been elucidated, neither has the composition of function of the standard cells of the thymus.

Material and method

The thymuses of 19 days old rat embryos and 4—5 days old guinea pigs were fixed with 5 per cent glutaraldehyde perfusion [12] and washed out in Millonig-buffer. Postfixation was carried out in 1 per cent buffered OsO_4. The thymuses from the rat embryos were fixed in 1 per cent buffered OsO_4. The materials were dehydrated in ethanol and embedded in araldit. The ultrathin sections were cut with glass knives on Reichert OMU-2 ultramicrotome. The sections were stained with uranyl-acetate, followed by lead citrate and examined in the JEM 6C electron microscope. The guinea pig organs were perfused with buffered 5 per cent glutaraldehyde; the details of this procedure were described elsewhere [12].

Results

We observed the structure of the small veins and capillaries of the thymus cortex from the viewpoint of the barrier to reveal how the thymocytes would penetrate through the vascular wall if there were such a barrier. In guinea pig it could be established that thymocytes are able to penetrate the blood through the vascular wall and the body of endothelial cells [12]. The question was especially dealt with by R. Murray [8] and Weiss [14].

Our observations are demonstrated in the Figures. In the capillaries of the thymus cortex of the rat embryo (Fig. 1) the lumen is filled up by a thymocyte while the endothelium is missing at places (signed by *). Outside the basal membrane collagen fibrils (CF) are arranged which however intertwined with the membrane infiltrate the latter and in a thinner or thicker layer separate the capillary from the cells constituting the adventitia.

The wall of another capillary is seen in Fig. 2. The capillary lumen being filled up by erythrocytes is lined with a continuous broad endothelium containing desmosomes, rough-surfaced endoplasmic reticulum, mitochondrium and dense bodies. The basal membrane is poorly definite, connective tissue fibrils are not seen and the wall of the capillary is completed by epithelial cells adhering to the endothelium. Around the capillary a pericapillary gap is found.

The capillary wall of the guinea pig (Fig. 3) is lined by a unique, continuous endothelium expanding in ring-form. The membrana basalis is readily discernible and in the surrounding collagen fibre layer the processes of adventitial cells are inserted. The adventitia is constituted by the reticulum epithelial cells and by processes of these laminately connected with desmosomes. In the processes intraplasmic fibres can be observed.

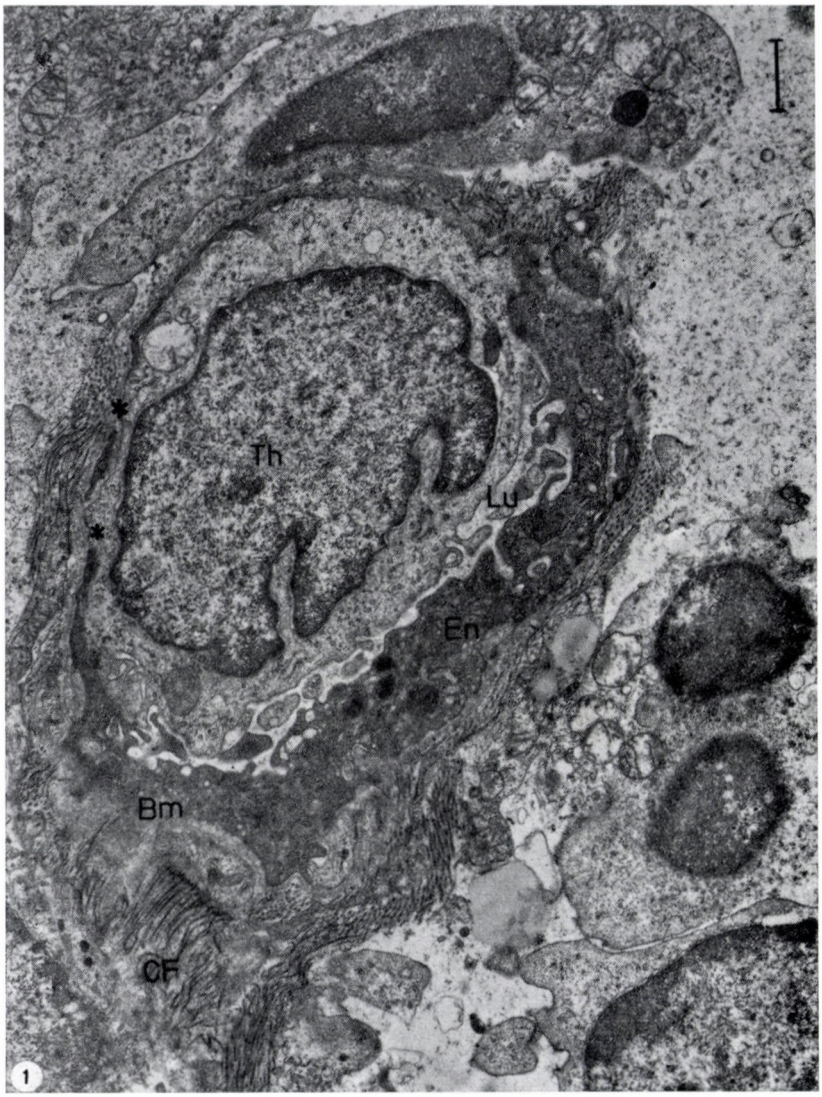

Fig. 1. Rat embryo, thymus cortex ×9,000
Bm — basal membrane; CF — collagen filaments; En — endothelial cell; Lu — lumen; Th — thymocyte; * — endothelial gap

Fig. 2. Rat embryo, thymus cortex ×9,000
BM — basal membrane; D — desmosome; En — endothelial cell; DB — dense body; ER — endoplasmic reticulum; Er — erythrocyte; G — Golgi apparatus; N — nucleus; M — mitochondrium; Pr — pericapillary space; Lu — lumen; Th — thymocyte

Fig. 4. shows a capillary lined with two endothelial cells which are interlinked by desmosomes. The basal membrane is very thin, at some places it seems to be lacking. The vascular wall is surrounded beyond the endothelium with light and dark epithelial cells.

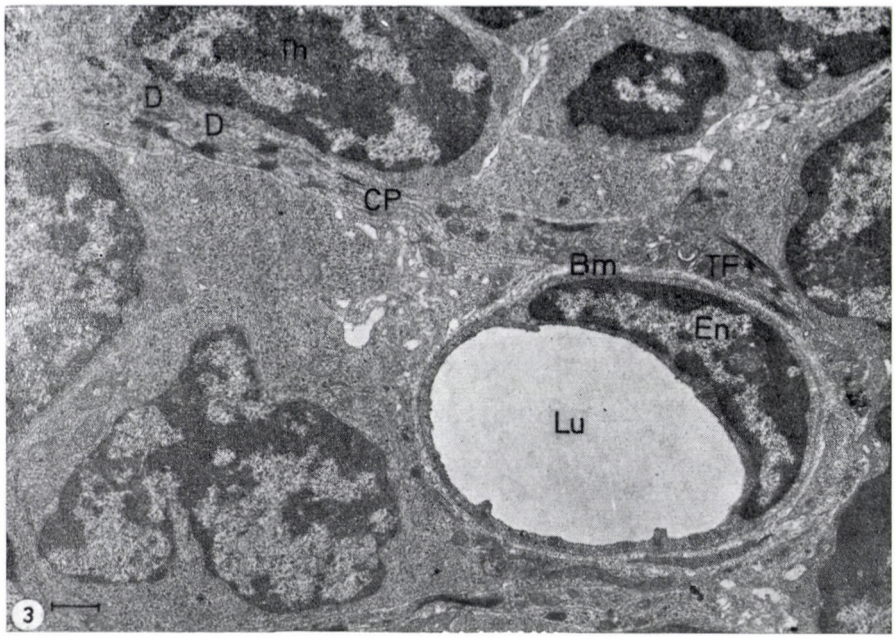

Fig. 3. Guinea pig, cortical capillary. ×6,000
Bm—basal membrane; D—desmosome; CP—cell process; En—endothelial cell; Lu—lumen; TF—tonofilaments; Th—thymocyte

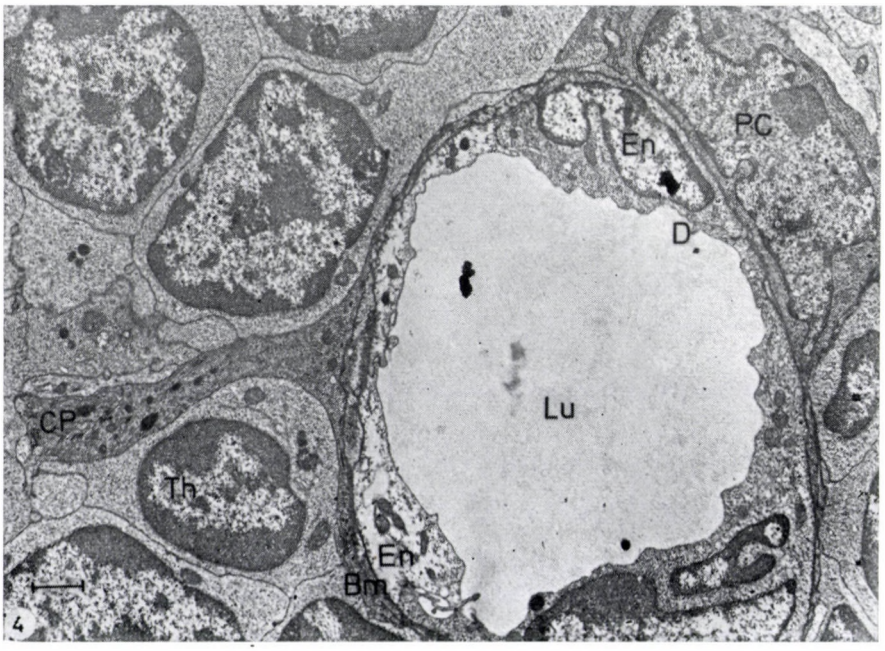

Fig. 4. Guinea pig, capillary. ×4,600
Bm—basal membrane; D—desmosome; En—endothelial cell; Lu—lumen; CP—cell process; PC—pericyte

In Fig. 5 a portion of the vascular wall is seen. It is lined with a flattened endothelium fitting together with a desmosome. Below the endothelium, in the upper left corner the verified basal membrane and outside the latter a collagen layer of indistinct structure is seen. In the latter the processes of the endothelial cell are arranged. To the verified basal membrane the thin processes of an adventitial cell (CP) adhere; where this rises, a wide pericapillary tissue gap is seen. In the vascular wall the point-like cross-section of connective tissue fibrils are found. Beside the capillary a myeloid cell form is visible.

Fig. 6 shows a longitudinally sectioned capillary containing erythrocytes; two flattened endothelial cells are seen with nuclei opposite to each other. The

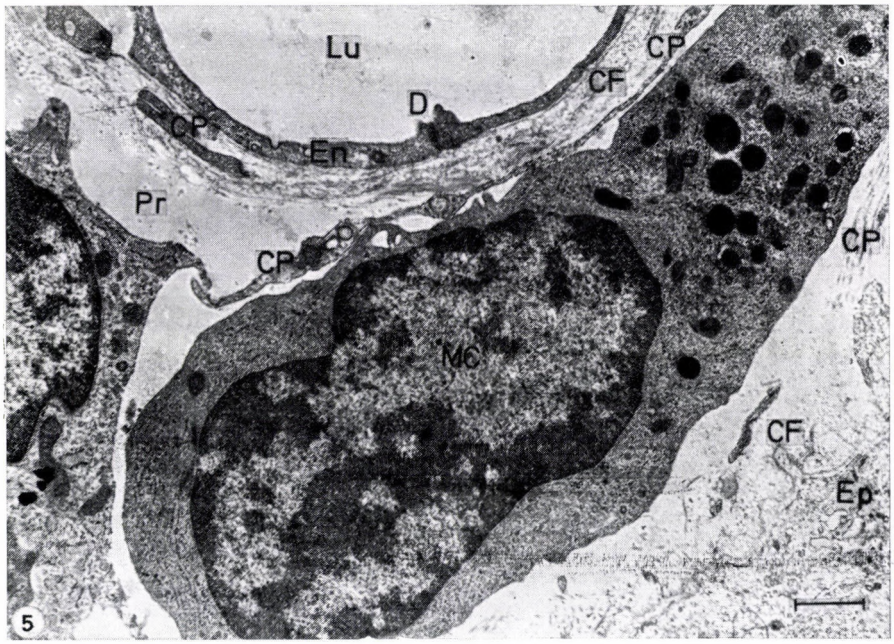

Fig. 5. Guinea pig, portion of a vascular wall in the thymus cortex, ×8,500
Ep—epithelium; D—desmosome; En—endothelial cell; CP—cell process; CF—collagen filaments; Lu—lumen; Pr—pericapillary space

vascular wall consists of a thin layer showing longitudinal fibrillation with, at places, indistinct structure. As adventitia a large adventitial cell adheres to the latter layer.

Fig. 7. High-power magnification of the vascular wall, where the connection of two endothelial cells is seen. At some places the basal membrane is thickened by the longitudinal section of fibril bundles, at other places by the stratification of plates consisting of cytoplasmic processes of epithelial cells and fibril. In the adventitial epithelial cells a number of lysosomes and a concentrically arranged endoplasmic reticulum suggestive of a "Nebenkern" are seen.

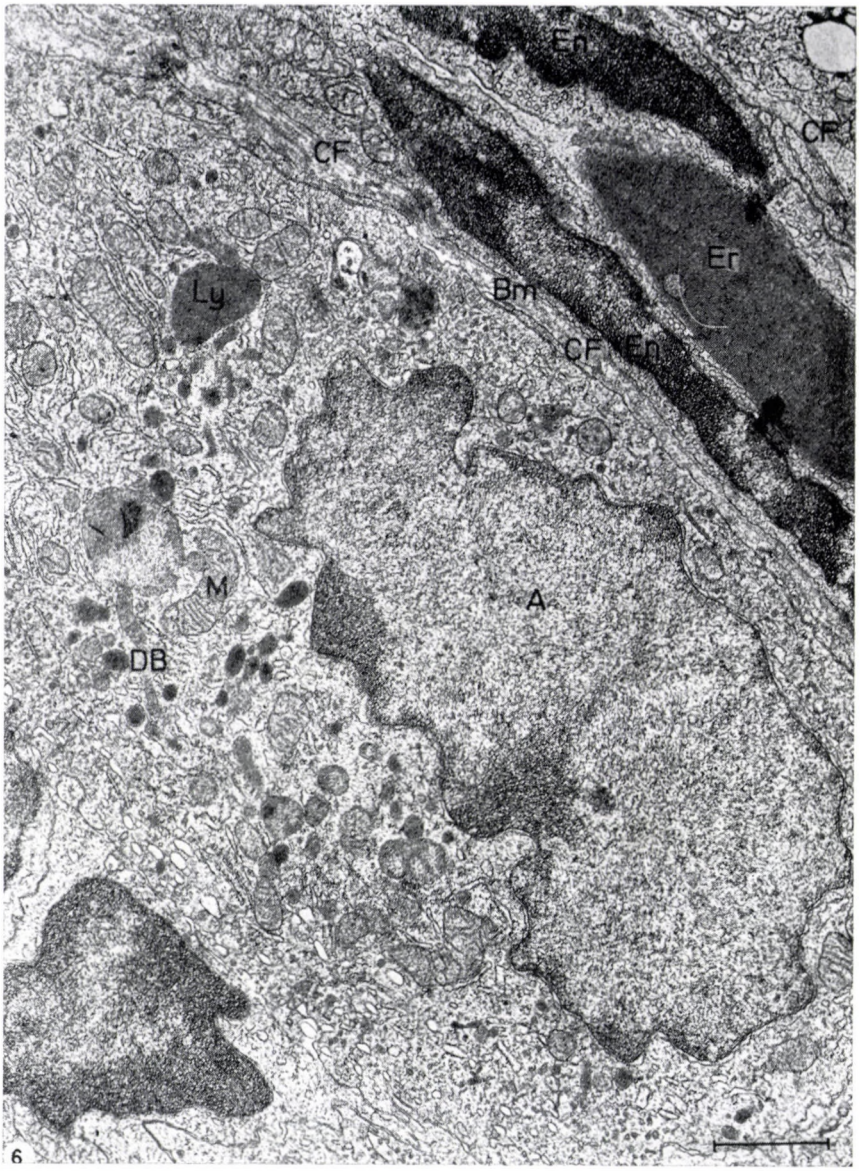

Fig. 6. Capillary in the cortex-medulla border of guinea pig thymus, longitudinal section with an erythrocyte. ×13,000

Bm—basal membrane; En—endothelial cell; Er—erythrocyte; CF—collagen filaments; A—adventitial cell; DB—dense body; Ly—lysosoma; M—mitochondrium

Other cell processes inserted like nails into the body of the adventitial cells show the very strong connection of the adventitial cells.

In Fig. 7 cross-sections of these processes are seen. The adventitial cells correspond to the so-called epithelial macrophages in which no tonofibrils are seen.

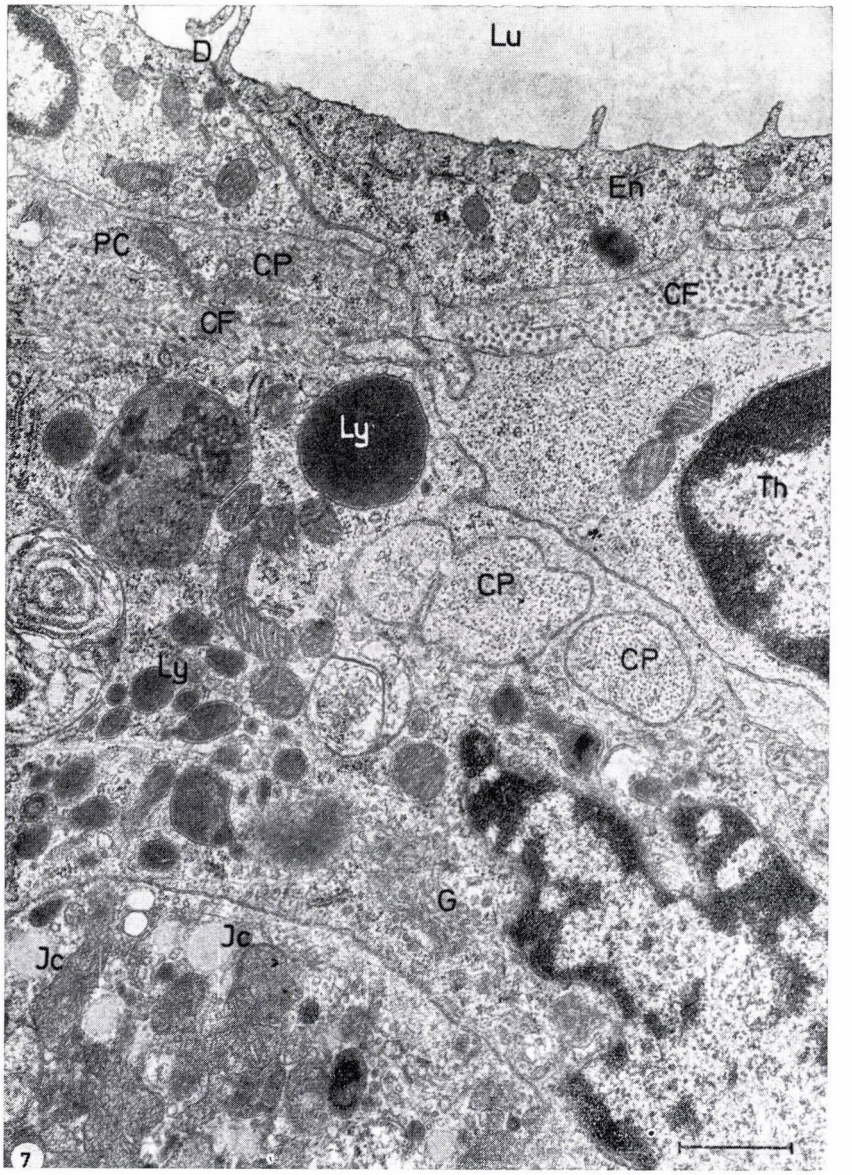

Fig 7. Vein-wall of guinea pig thymus cortex. × 15.400
D — desmosome; En — endothelial cell; CF — collagen filaments; CP — cell process; Pc — pericyte; Ly = lysosomes; G — Golgi apparatus; Jc — secretion; Th — thymocyte

Pinocytotic vacuoles are present in most endothelial cells in a remarkably high number (Fig. 8). The basal membrane is explicit and below it a thinner or wider fibrillar layer is seen. Beside this layer there are no intercellular fibres, the epithelial macrophage of the adventitia lies immediately beside the mem-

brane. In the macrophage a rough-surfaced endoplasmic reticulum and large-lysosomes are visible.

Fig. 9 represents a portion of the wall of a vein containing an erythrocyte. The endothelium rich in pinocytotic vesicles is flattened. The fibrillated basal membrane is obliquely, in some places tangentially sectioned; beside it a layer pricked with the processes of adventitial epithelial cells is seen. The adventitia consists of strikingly structured epithelial cells with mitochondria, dense bodies and endoplasmic reticulum.

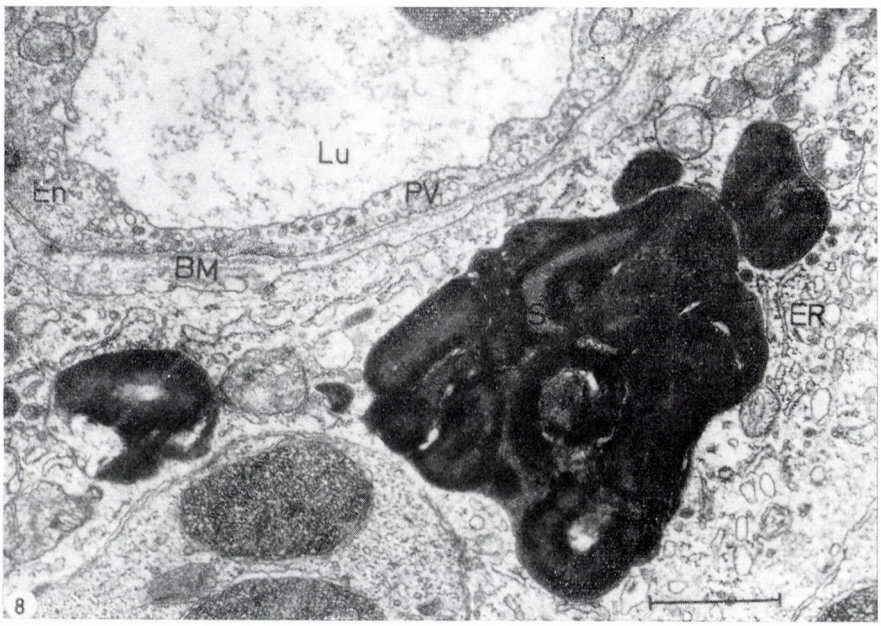

Fig. 8. Guinea pig, venous capillary. ×19,200
BM — basal membrane; En — endothelial cell; Lu — lumen; PV = pinocytotic vesicles; S — phagocytotic substance; ER — endoplasmic reticulum

In Fig. 10 a thymocyte is seen in the lumen of a vein. The endothelium is evenly stretched and surrounded by a basal membrane widened at places. Outside the membrane a layer containing the transections of bundles of collagen fibrils are seen. This is at some places indistinct, at others it runs parallel with the vein. Between the bundles adventitial cells, which with their processes lie close to each other, are inserted into each other; even intercytoplasmic fibres are visible.

In Fig. 11 there are several thymocytes in the lumen of a vein inside the endothelium, rich in pinocytotic vacuoles; further a well-defined basel membrane, followed by a layer containing indistinct and broad collagen fibrils. The adventitial cells lie immediately beside this layer.

Fig. 9. Guinea pig, venous capillary. ×20,200
Bm — basal membrane; Ly — lysosomes; Lu — lumen; Th — thymocyte; Er — erythrocyte; M — mitochondrium; En — endothelial cell; D — desmosome; EC — epithelial cell

In Fig. 12 the thymocyte and the cytoplasmic processes of the thymocyte in the lumen and processes of the endothel extending towards each other and the microvilli equally reflect a cytoplasmic restlessness. To this point the many

Fig. 10. Guinea pig, thymus cortical capillary. × 8,700
Bm—basal membrane; A—adventitial cell; CF—collagen filaments; PC—pericyte; En—endothelial cell; Th—thymocyte; CJ—cell junction

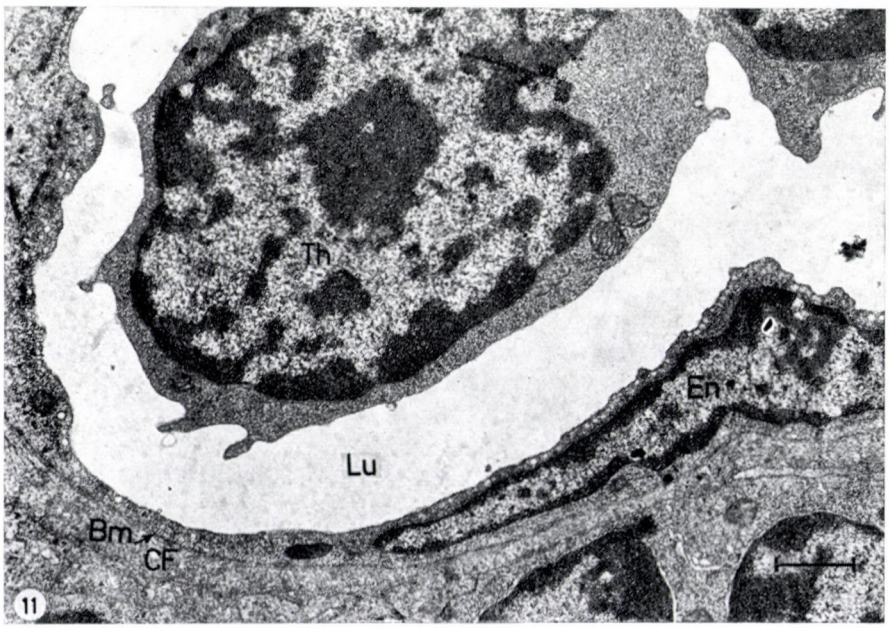

Fig. 11. Guinea pig, cortex-medulla border, small vein. × 9,200
Bm—basal membrane; Lu—lumen; CF—collagen filaments; En—endothelial cell; Th—thymocyte

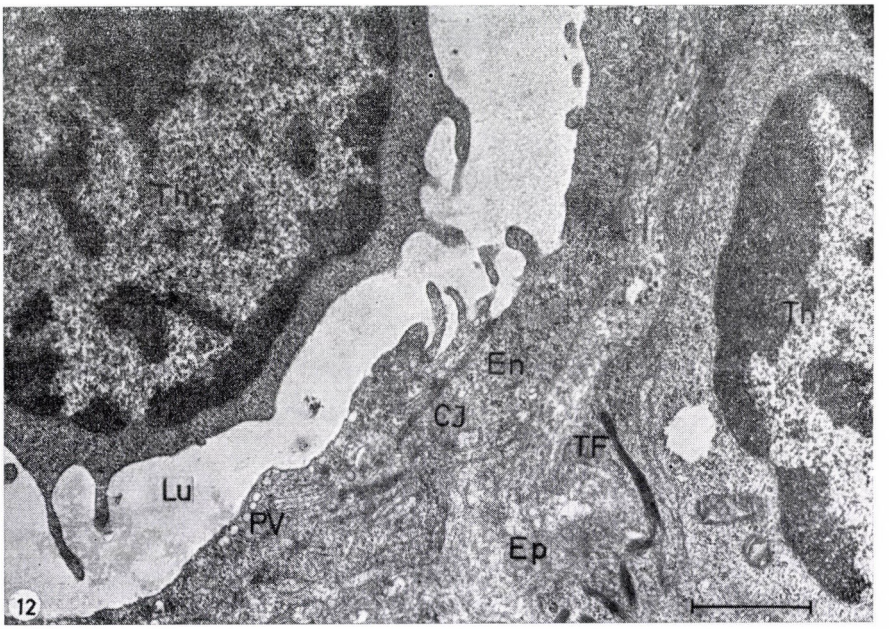

Fig. 12. Guinea pig, small vein in cortex. ×15,800
Lu—lumen; PV—pinocytotic vesicles; CJ—cell junction; TF—tonofilaments; Ep—epithelium; En—endothelial cell; Th—thymocyte

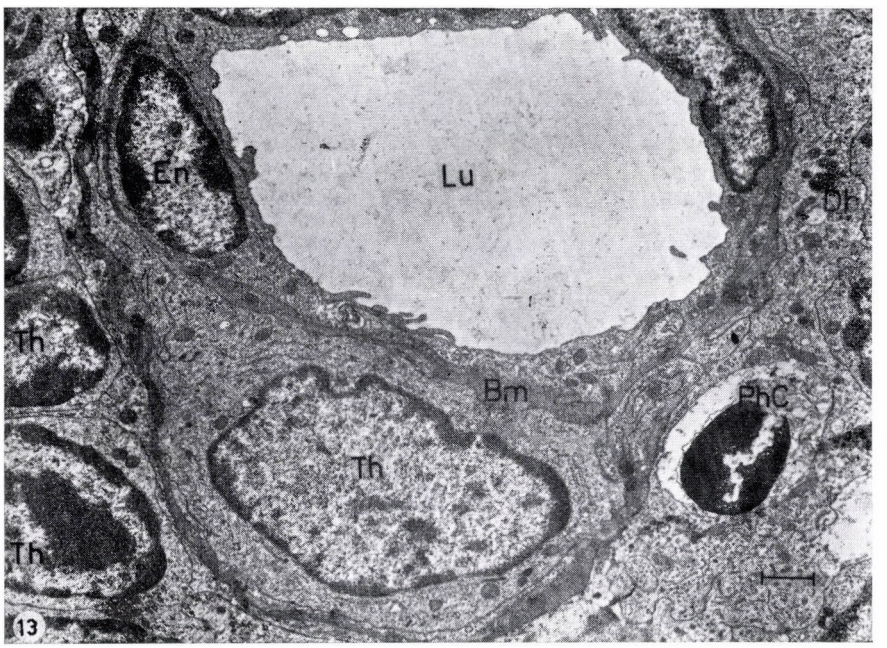

Fig. 13. Guinea pig, venous capillary in thymus cortex. ×4,600
Bm—basal membrane; Lu—lumen; Th—thymocyte; PhC—phagocyted cell; En—endothelial cell; Db—dense body

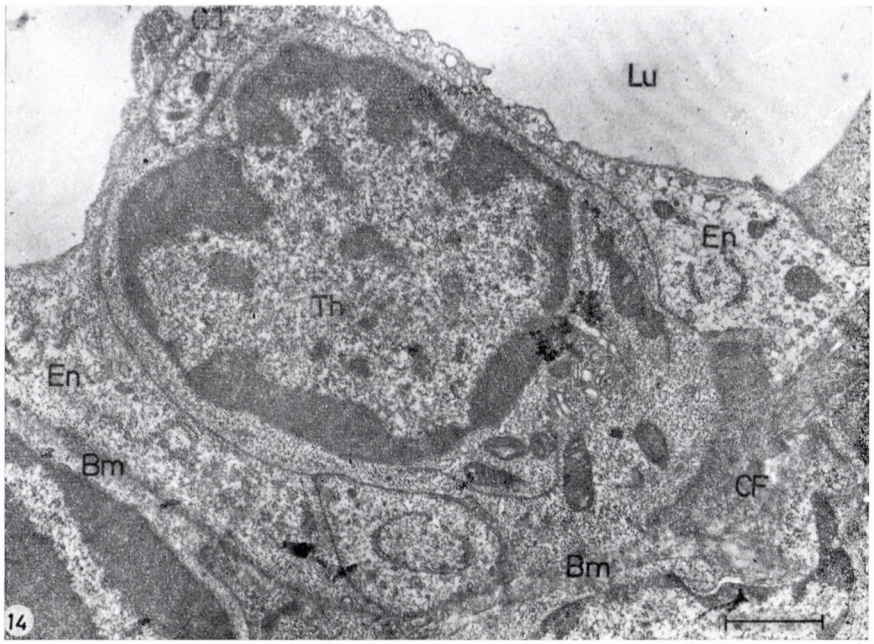

Fig. 14. Guinea pig, capillary in thymus cortex. ×11,400
Bm—basal membrane; Lu—lumen; Th—thymocyte; En—endothelial cell; CF—collagen filaments; CJ—cell junction

pinocytotic vesicles in the endothelium. The basal membrane is not everywhere visible because at some places it merges into a layer with indistinct fibres to which the thinned processes of epithelial cells containing intracellular fibre lie closely. To this layer thymocytes adhere.

In Fig. 13 the thymocyte is already seen in the vascular wall. The vein is lined with three endothelial cells. The basal membrane and the broad fibrous layer below it cannot be separated everywhere, in the latter the processes of endothelial and adventitial cells are embedded. Embedded in the vascular wall lies a thymocyte surrounded by the adventitial fibre layer separated in two parts. To the vascular wall where the penetrated thymocyte lies, thymocytes are adjacent while on the other sides adventitious cells are seen with a vacuole containing thymocyte fragments.

In Fig. 14 a thymocyte lies between the basal membrane and the endothelium directly corresponding to the place where the two endothelial cells contact each other. The basal membrane cannot be found everywhere; where it is absent, the adventitial collagen layer is of an undistinct structure and a thymocyte is adjacent to the endothelium.

The thymocyte, however, cannot only penetrate endothelial cells [11] but also adventitial cells shown in Fig. 15. In the interior of the adventitial cell a thymocyte of completely intact structure including Golgi area and cytocentrum is seen indenting the nucleus.

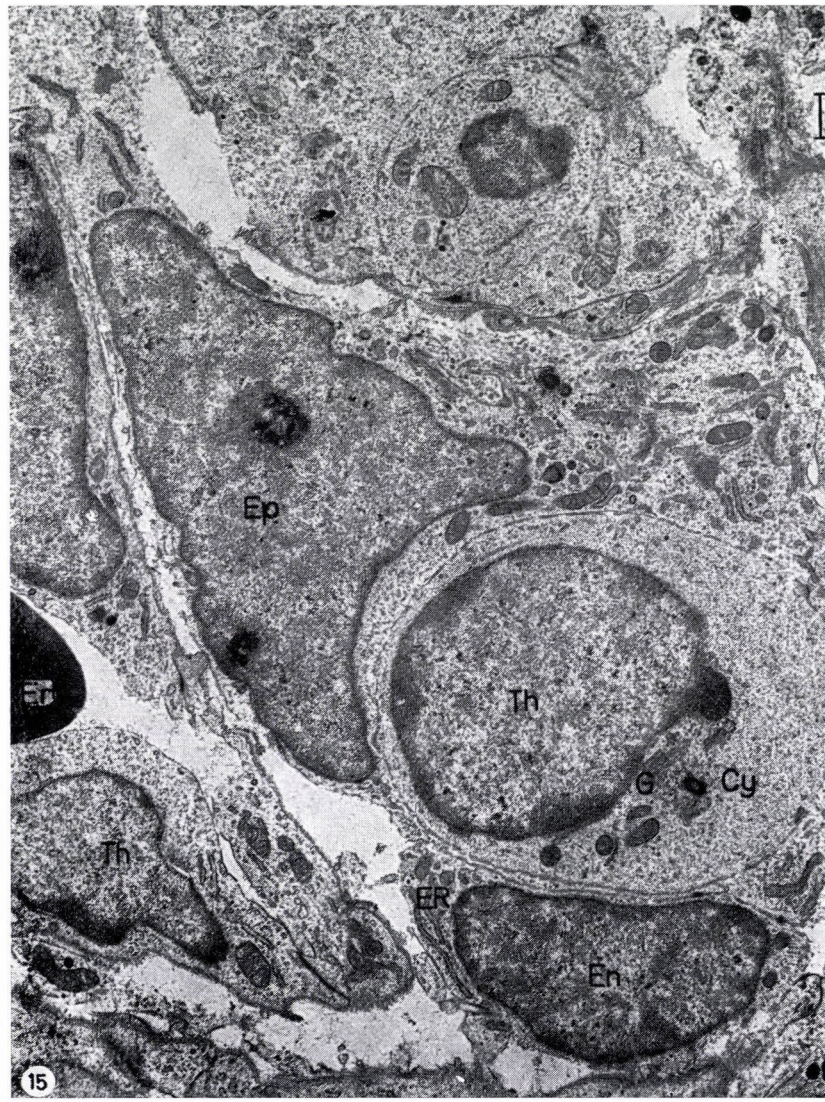

Fig. 15. Oblique section of a capillary in the thymus cortex of an 18-day-old rat embryo. ×5,000

Th — thymocyte; ER — endoplamic reticulum; En — endothelial cell; G — Golgi apparatus; Cy — cytocentrum; Ep — epithelial cell; Er — erythrocyte

Discussion

Our findings allow the conclusion that the thickness of the endothelium of capillaries and small veins of the thymus-cortex is very manyfold and differently structured. This is particularly remarkable on the sites where a thymocyte is in the lumen of a vein (Figs 1, 12) and both the endothelium and the thymocyte have microvilli-like processes. The pinocytotic vacuoles of the

endothelium reflect an active absorptive function of the cells. The endothelial cells are connected by easily discernible desmosomes but also deficiencies are found on them. The basal membrane lying outside the endothelium is also variable. It can be less explicit mainly at the sites where the structure of the collagen fibres is indistinct. At places the thin basal membrane is thickened by the processes of the endothelium and of the adventitial cells. In many cases the membrane is connected with perivascular collagen fibres and this is particularly pronounced where the collagen fibre layer includes the processes of the endothelia and adventitial cells. These processes, according to WEISS [14], may be connected to each other, ensuring a transport path. The perivascular epithelial cells, expanding sole-like, embrace the veins or surround them with their laminated processes and adhere directly to the endothelial cells. Here and there the endothelium and the epithelial cells are separated by collagen fibres. Not infrequently thymocytes are seen adjacent to the vascular wall.

The electron micrographs have verified that the thymocytes when returning into the blood may loosen the collagen fibre and the basal membrane and may migrate between endothelial cells into the lumen. In cinematographical photos of the cultures of thymus epithelial cells [13] it could be observed that between the epithelial cells and the thymocytes certain adhesive forces appear and the thymocyte can even penetrate the epithelial cell, may stay there for a while and then leave it. This phenomenon may repeatedly occur. All these are in good agreement with the great variability of the perivascular epithelial cell barrier. The cultured cells of the thymus in spite of their large cytoplasm moved intensively. They even left the cell wall, facilitating for the thymocytes to reach the vein-wall. Where there are also collagen fibres the thymocytes or the adventitial epithelial cells are able to dissolve these with their enzymes (Fig. 14), and reaching the vein-wall enter the lumen either between or through endothelial cells. These pictures confirm the idea already referred to [12] that when a thymocyte penetrates through the vein-wall it opens the pathway as a pioneer to the following ones and so by the same way several thymocytes after each other may get into the circulation. The major part of the collagen fibres form bundles running along the veins while another part from outwards irradiates into the basal membrane.

The present results support our opinion that the thymus—blood barrier is not a stationary structure but may dynamically change according to the possibility offered by the perivascular tissue in conformity with the momentary structural conditions in the given situation. The epithelial cells flattening in several layers may form a considerable barrier around the veins but may also retire from there and lay bare the vein-wall, thus promoting its permeability. To this point the perivascular tissue gaps found at some places. Our electron micrographs and microcinematographical examinations concerning the barrier

attract the attention to this dynamism. Thus it does not seem probable that antigens cannot penetrate the parenchyma of the thymus. Accordingly the reports on the presence of plasma cells in the thymus should be appreciated and the explanation of the peculiar behaviour of the thymus by the assumption of a blood—thymus barrier cannot be accepted without further consideration.

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ZUM PROBLEM DER BLUT—THYMUS BARRIERE

Im Thymus von Meerschweinchen und Ratten wurde die Frage der Blut—Thymus Barriere elektronenmikroskopisch untersucht. Hierbei wurde festgestellt, daß es keine stationäre Barriere gibt, die das Eindringen von Antigenen unmöglich machen würde. Die barrierebildenden Gewebeelemente wie Basalmembran, Adventitia, Kollagen Faserschicht sind zwar in einzelnen Fällen um einzelne Gefäße vorzufinden, sind aber meistens als Barriere ungenügend und in ihrer Zusammensetzung dynamisch veränderlich. Die Untersuchungen erlauben die Annahme, daß die Thymozyten zwischen oder auch durch die Zellen der Gefäßwand durchdringen vermögen.

ПРОБЛЕМА БАРЬЕРА МЕЖДУ КРОВЬЮ И ЗОБНОЙ ЖЕЛЕЗОЙ

Авторы проводили на зобной железе морских свинок и крыс электронномикроскопическое исследование проблемы о существовании барьера между кровью и зобной железой. Было установлено, что стационарного барьера, препятствующего проникновению антигенов, не существует. Тканевые элементы, образующие барьер, в том числе базальная мембрана, адвентиция, и слой коллагенных волокон иногда встречаются вокруг отдельных кровеносных сосудов, но они в большинстве случаев недостаточны для образования барьера, и, притом, их состав подчергается динамическим изменениям. Результаты исследований позволяют предположить, что тимоциты способны проникнуть через клетки стенки сосуда.

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MICRO-ORGANISMS IN THE WATER CIRCUITS EXPOSED TO RADIATION OF THE NUCLEAR REACTOR BUDAPEST—CSILLEBÉRC

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Synopsis

Living micro-organisms have been demonstrated in the waters of the 2.5 MW research atomic reactor of Csillebérc: in the secondary circuit, in the primary circuit and in the water of the container for the storage of the burnt out fuel elements. Four demonstrated micro-organisms are described and their conditions of life characterized. It is remarkable that we succeeded in demonstrating a blue alga in the water of the primary circuit.

Introduction

With the establishment of atomic reactors and nuclear power stations an artificial environment of a new style appeared the living world of which is worth while to be studied both from the theoretical and practical aspects. In the formation of this environment the high intensity nuclear radiations first of all gamma and neutron radiations have a decisive part. It has been the subject of a number of examinations to what extent the various organisms tolerate radiations. E.g. SHIELDS, DÜRELL and SPARROW studied the radiation tolerance of algae and fungi isolated from the soils of the experimental station of Nevada. The various radiation treatments are also well known in the practice of plant growing (new and better varieties, increased resistance to diseases). Radiation effects of atomic explosions have also been examined for a considerable time.

However, few authors have dealt with the living world of the water circuits of the atomic reactors. The papers of FOWLER, CHRISTENSON, JURNEY and SCHAFER describe the propagation of *Pseudomonas* bacteria in the water of the Omega West Reactor.

In the following, for the first time data are published from Hungary on micro-organisms acclimatized in the irradiated water circuits of a nuclear reactor. Let us first briefly describe the physical, radiation-physical and chemical conditions of the water circuits of the nuclear reactor Csillebérc, and then record the demonstrated and identified organisms.

Environmental conditions

Primary circuit

Cooling of the atomic reactor is solved by two series-connected water circuits. The water of the primary circuit flows through the uranium-containing reactor zone and transports the 2.5 MW heat developing during operation into the intermediary heat exchanger (in the following: heat exchanger). In the heat exchanger the heat arrives to the water of the secondary circuit. The water of the secondary circuit is cooled in a cooling tower by the free air.

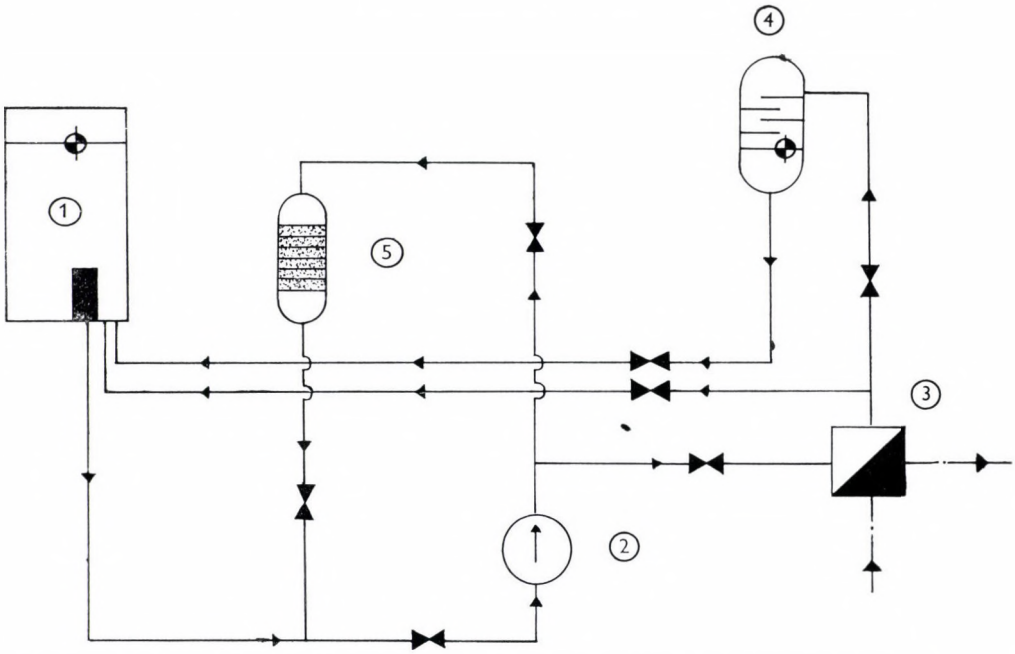

Fig. 1. The primary circuit. (1) = reactor container; (2) = pumps; (3) = heat exchanger; (4) = degasing unit; (5) = ion exchanging filter; ⋈ = valve; ⌖ = water-niveau

The main installations of the primary circuit are seen on Fig. 1. The reactor is placed in the (1) reactor container. The environment of the reactor in which radiation is most intensive may be estimated at 0.5 cu.m. Above the surface of the water, under the cover of the container there is a suction stub of the air exhaustor creating here a depression of about 5 mm water column.

The water of the primary circuit is kept in circulation by three pumps with a total performance of 1000 cu.m/h. A considerable part of the water gets from the pump into the heat exchanger (3). At the primary side of the heat exchanger the pressure is lower than on the secondary side and thus the water of the secondary circuit in the case of a defect may rush into the primary circuit but by no means can primary water find its way out. Only a small

portion of the streaming primary water gets into the degasing unit, where degasing is carried out by counter-coursing ordinary air. The ion-exchanger (5) (destionex, type AAK-5, semiautomatic, with a 500 l/hour performance) operates periodically.

The temperature of the primary water is in working about 28° C, during a standstill about 24° C, according to prescription it must never cool down below 20° C. The pH value of the water is kept around 5.0 most favourable from the viewpoint of aluminium corrosion. At the moment of sampling this value was 5.2. The total amount of the primary water is about 25 cu.m. It is a twice distilled water the salt content of which is maintained at the desirable level with the aid of ion exchanging filters. Before filtering, the conductivity of the water cannot exceed the 10 μS value. After filtration 1—2 μS must be reckoned with. In the moment of sampling 7.9 μS was measured. These values correspond to a salt content of 2—8 p.p.m.

Living organisms can get into the primary circuit through the degasing unit, the surface of the reactor container or through an accidental defect of the heat exchanger.

During operation in the primary circuit exceedingly high radiation levels arise. As a consequence of the neutron radiation of the zone the primary water, the nitrogen of the air solved in it and the salts dissolved in a minimum quantity become activated and thus the primary water itself intensively radiates too. In the most active environment of the reactor the intensity of the *neutron radiation* is in the order of 10^{13} n/cm^2 sec, the intensity of the *gamma radiation* is about 2×10^8 R/h. Beyond this it can be reckoned also with *alpha* and *beta* radiation. A secondary phenomenon is the so-called *Cherenkov-radiation*, which is induced by the strong *beta* radiation of the reactor. This can be regarded as a light of bluish colour. On account of the activity of the water the radiation level in the primary circuit nowhere declines below 10 R/h. As a consequence of the construction of the circuit an exceedingly small proportion of the primary water can avoid passing through the active zone every 1.5 minute.

Out of operation the water of the primary circle is at standstill, its specific activity is $2-3\times10^{-7}$ C/l. 1½ days after the stopping on the surface of the 50 cu.cm sample the radiation intensity was about 0.5 mR/h. The intensity of the radiation of the zone in such cases is about 10^6 R/h.

Secondary circuit

The water of the secondary circuit takes up the heat coming from the primary circuit in the heat exchanger and at the same time it gets here in the most radioactive environment. Considering that the radiation here consists almost exclusively of *gamma* rays, activation of secondary water is not to be reckoned with. In the secondary circuit about 250 cu.m desalinized water is

found, of which about 4 cu.m is in the heat exchanger. The intensity of radiation is here in the order of 10 R/h.

From the heat exchanger the secondary water arrives in the cooling tower with artificial circulation, where it is cooled by the external air being in 36.000 cu.m/h counter-course. Depending on the weather, a considerable drop and evaporation loss may arise which is continuously replaced from tap water with water desalinized with ion exchanging filters. Here an average amount of 400 cu.m per week is involved. Under the cooling tower a water basin of 150 cu.m volume is found. Here drops the water cooled in the tower. The wind drifted a considerable amount of leaves of trees into the basin. Through the free water surface here very manifold organisms may get into the secondary circuit.

From the basin the pumping plant of the secondary circle drives during operation an amount of water of 350 cu.m/hour into the heat exchanger.

The temperature of the secondary circuit is 17—18° C in the winter during operation. At the moment of sampling the hardness of the water was 2.2 degrees of hardness, its iron content was 0.2 mg/l.

Burnt out fuel receiver

The about 4 m deep container with 10 cu.m water serves to store the burnt out but still very intensively radiating uranium fuel element deep under the water on the bottom of the vessel. It is in no wet connection either with the primary or with the secondary circuits. About every two months some new burnt out fuel element bundles get here, which may carry living organisms originating from the primary circuit. Above the water level of the container is a steady circulation of air which also may drive living organisms. The container was filled up in 1959 with twice-distilled water of the same quality as that of the primary circuit. Owing to the natural evaporation, a water loss arises, which is replaced by desalinized water of 1 μS conductivity. The wall of the container is of aluminium. The pH of the water is 4.5—5.0—5.5, its temperature is permanently 18° C. In the receiver, for mechanical reasons, there are some rubber surfaces on which as well as on the bottom of the vessel a greyish-brownish deposit is found.

The intensity of radiation on the surface of the water is 10—20 mR/h. Its value depends upon when for the last time a burnt out fuel element came into the receiver. The activity of the burnt out fuel element bundle diminished at first rapidly, later more slowly. Similarly changed thei ntensity of radiation at the bottom of the receiver (Fig. 2) around 10^4 R/h. The radiation can be considered pure *gamma* radiation so that the water of the receiver is not active.

Natural light rarely reaches the water of the receiver or that of the primary circle (at the control made at about two months intervals).

Samplings

Water samples were taken from the primary circuit after proper discharge with the aid of the tubing. Two kinds of samples were taken from the fuel receiver: one from the vicinity of the water surface and the other from the bottom water, after having stirred up the deposits, to obtain information on the consistency of the deposits. From the secondary circuit samples taken from two sites deserve attention: one was taken from the sampling pipeline not far from the heat exchanger after proper discharge, while the other originates from the bottom water stirred up before sampling of the water basin below the cooling tower.

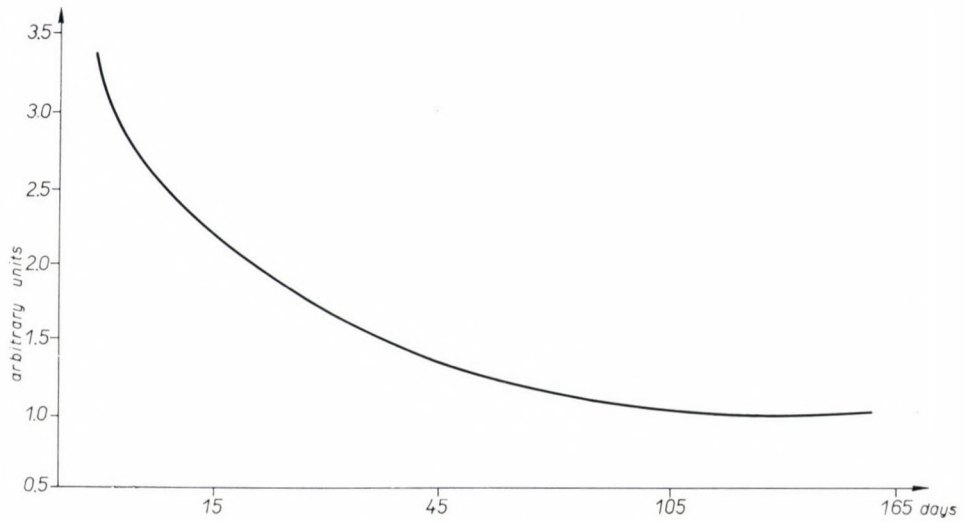

Fig. 2. Elimination curve of burnt out fuel element

The time of sampling was February 18th between 8.30 and 10.30 a.m. Previously the reactor was operated from February 7th 10 a.m. to February 12th 6 a.m. and from February 14th 10 a.m. to February 16th 4 p.m. with a power of 2.5 MW. The standstill of one day and a half was necessary in order to practically eliminate the activity of the primary water sample. The reactor operates 300 hours in monthly average.

In the course of elaboration we did not aim at publishing exact quantitative data because partly bottom waters cannot give a true picture, partly the collections cannot be compared. Determination of the algae was conducted with the aid of the taxonomic works listed in the References.

In the collections the following organisms were living.

Enumeration

Schizomycophyta

(1) *Schizomycophyta sp.* Tangled trichoma texture, the width of the threads is about 0.4 μ. No exact determination could be carried out up to the present.

Cyanophyta

Chroococcales

(2) *Synechocystis minuscula* WORONICHIN. The cells are globular, at division elongated, fingerbiscuit shaped. They appear by ones or by twos, their diameter is 1.6—2.6 μ. The membrane is thin, the cell content homogeneous, light blue.

Hormogonales

(3) *Romeria gracilis* KOCZW. The trichomas contain 1—4 cells, they are straight or bent. The equally straight or bent cells are 1.3—4 μ in length, 1—1.4 μ in width, oval, cylindrical, sometimes elongated oviform in shape, with rounded-off corners. The cell content is homogeneous, light blue. The cells or trichomas are solitary, not embedded in mucus.

Chlorophyta

Chlorophyceae, Chlorococcales

(4) *Ankistrodesmus falcatus* (CORDA) RALFS. var. *spirilliformis* G. S. WEST = *Raphidium contortum* (THURET) LEGN. The cells are of very manifold shape, both ends gradually tapering finish in pin-points. The cells are 14—28 μ in length and 1—2.6 μ in width.

Discussion

The least organisms were found in the primary circuit, where the water on the external surface of the glass contaning the sample showed a radiation of 0.5 mR/hour. In the active water a single species of blue alga lived, *Romeria gracilis*, the number of individuals of which was the least as compared to the organisms of all water samples.

In the water of the fuel element receiver and in the stirred up bottom water the blue alga *Romeria gracilis* also occurred, with comparatively many thalli living in the water while there were fewer in the bottom samples. In

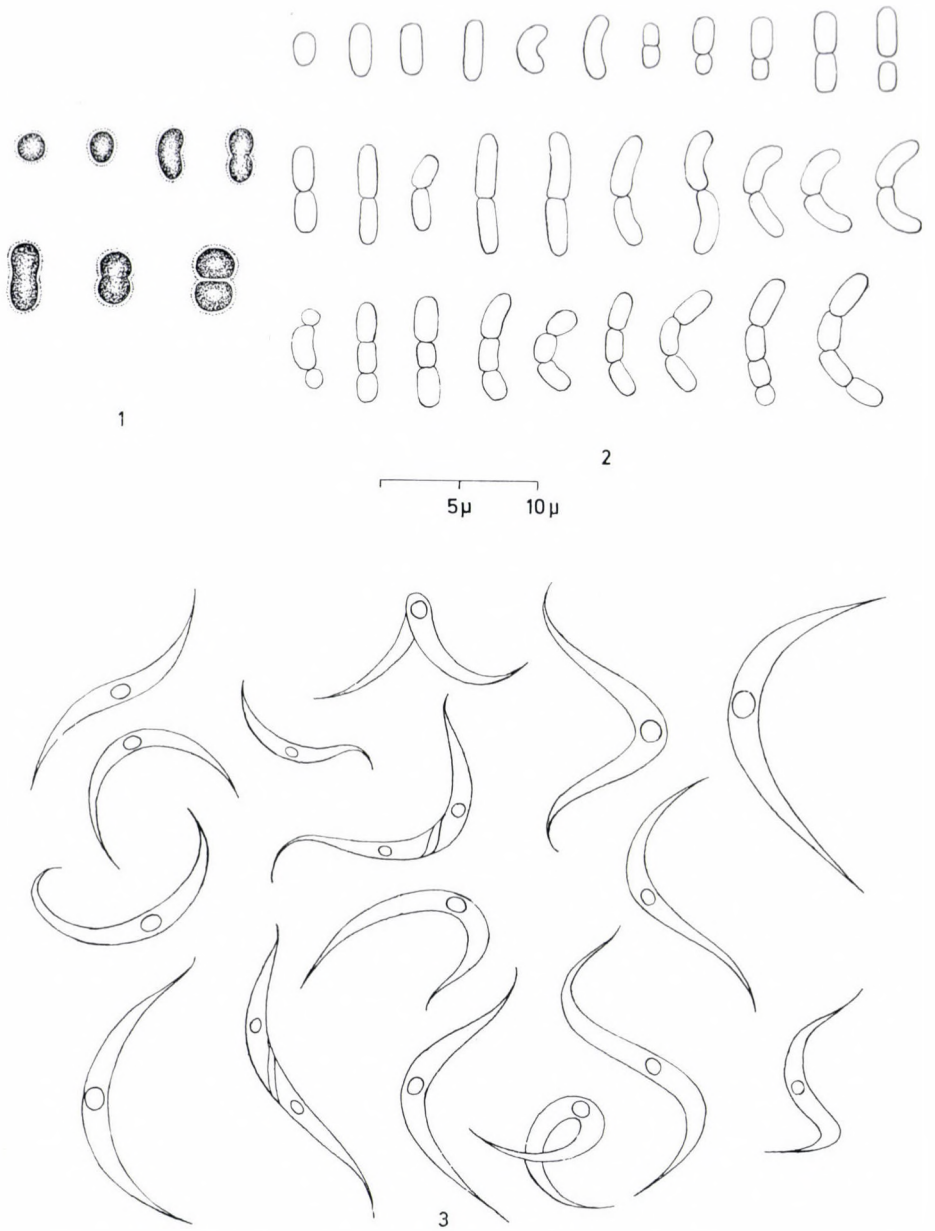

Fig. 3. Synechocystis minuscula WORONICHIN (1); *Romeria gracilis* KOCZW. (2); *Ankistrodesmus falcatus* (CORDA) RALFS. var. *spirilliformis* G. S. WEST (3)

both water samples there was one more kind of *trichoma bacteria* (Schizomycophyta). The sediment at the bottom of the fuel receiver is thus partly of biological origin. The twice-distilled water of the primary circuit and of the fuel

element receiver obtains natural light very rarely, usually once every two months (during control). Is it possible that for the assimilation of the blue alga *Romeria gracilis* living and propagating at both places in lasting darkness the radiations of the atomic reactor — e.g., the Cherenkov-radiation — have supplied the necessary energy?

In the secondary water circuit from the organisms referred to only the *trichoma bacterium* was observed with a low number of individuals. Besides, somewhat more *Synechocystis minuscula* blue algae and the green alga of very manifold shape *Ankistrodesmus falcatus* var. *spirilliformis (Raphidium contortum)* appeared.

In the water basin of the cooling tower all four kinds of organisms were living; the thread bacterium was present in a particularly high number. The water samples taken near the surface contained living cells. In the samples of stirred up water also destroyed cells are found. We could not reliably establish the ratio of the living and destroyed cells here.

Of the four kinds of micro-organisms *Romeria gracilis* is the most resistant, occurring both in the active and inactive water. The *trichoma bacterium* on the other hand lived in every water except the active ones.

The blue algae and the only green alga found belong within the strains Cyanophyta and Chlorophyta to the organisms standing on the most primitive organizatory levels. The blue algae are very resistant and widespread. According to FOTT, too, they are found in places where the living conditions for other plants are missing. The blue algae originating from the thermal springs can tolerate very low temperatures (—190° C) for several weeks [1]. They are the first to settle on volcanic soils, on new habitats. They are first of all of high significance in the soils of tropical regions. Most of them, however, are fresh water organisms and much fewer species live in the sea.

Table 1

Living organisms in the water basin of the cooling tower

Species	Water samples				
	Primary circuit	Fuel element receiver	Stirred up bottom water of fuel receiver	Secondary water circuit	Stirred up bottom water of cooling tower
Schizomycophyta sp.		+	+	+	+
Synechocystis minuscula				+	+
Romeria gracilis	+	+	+		+
Ankistrodesmus falcatus var. *spirilliformis (Raphidium contortum)*				+	+
	1	2	2	3	4

Of the Cyanophyta occurring *Synechocystis minuscula* was described by WORONICHIN from a bitter salt spring in North Caucasia. It belongs to the less frequent blue algae.

The other Cyanophyton, *Romeria gracilis*, was observed by KOCZWARA in a small lake near Lwow. It is also one of the blue algae the occurrence of which has been reported rarely.

Ankistrodesmus falcatus var. *spirilliformis* [newer name *Raphidium contortum* (THURET) LEGN.] belongs to most widespread Chlorococcales almost everywhere [1], including Hungary, Alaska, India, and Viet-Nam. It apparently tolerates under natural circumstances any radiation conditions and fluctuations of temperature. To its great ecological valence points, among others, its occurrence in the nuclear reactor. Chlorococcales — similarly to Cyanophyta — tolerate both normal and extreme conditions of life (ice, glacier, thermal springs, soil). They are characteristic of fresh waters.

The comprehensive work of FOTT does not refer to the radiation-tolerating capacity of the occurring organisms. Similarly, no relevant data are found in the new book on Cyanophyta of STARMACH. Its determination requires longer examinations also in our case.

Thus, there is life also in the primary water circuit of the WWRS type atomic reactor of the Central Physical Research Institute, demonstrating the high resistance of algae to radiations. The micro-organisms are involved in the deposits of the fuel element receiver.

Acknowledgements

Thanks are due to the leaders and workers of the Nuclear Reactor Csillebérc, Budapest, who helped us in the sampling of the waters and in measuring the life conditions.

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MIKROORGANISMEN IN DEN DER BESTRAHLUNG AUSGESETZEN WASSERKREISEN DES ATOMREAKTORS BUDAPEST-CSILLEBÉRC

Lebende Mikroorganismen wurden in dem bestrahlten Wasser des Versuchsatomreaktors Csillebérc von 2,5 MW Kapazität nachgewiesen, und zwar im sekundären Kreis, im primären Kreis, sowie im Wasser des Behalters für die Lagerung der ausgebrannten Heizelemente. Vier nachgewiesene Mikroorganismen werden beschrieben und ihre Lebensbedingungen gekennzeichnet. Es ist auffallend, daß auch im Wasser des primären Kreises eine Blaualge nachgewiesen werden konnte.

МИКРООРГАНИЗМЫ В ВОДЕ, ОБЛУЧЕННОЙ АТОМНЫМ РЕАКТОРОМ ЧИЛЛЕБЕРЦ В ОКРЕСТНОСТИ БУДАПЕШТА

В облученной воде экспериментального атомного реактора чиллеберц, мощностью в 2,5 МВ, живые микроорганизмы были выявлены во вторичном круге, в первичном круге и в воде приемника для хранения сожженного топлива. Дается описание четырех видов микроорганизмов и их жизненных условий. Заслучивает интерес, что удалось выявить синезеленую водоросль в воде первичного круга.

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THE FINE STRUCTURE OF ABDOMINAL PARAGANGLIA IN THE NEWBORN MOUSE

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Synopsis

The abdominal paraganglia (P.G.) and the adrenal medulla of newborn mice were examined by electron microscope. The fine structure of the large P.G. body and of the small P.G. cell groups is described in detail. In the P.G. body an irregular, in the small P.G. cell groups a regular structure suggestive of the ganglia of the peripheral nervous system was found. The P.G. cell groups seem to develop from the P.G. bodies (by cell migration). The fine structure of the cytoplasm and secretion granules in the P.G. cells was found to be identical with that of the adrenal medullary cells. Additionally some of the P.G. cells contained also autophagic vacuoles, in which destruction of secretion granules were found. Some of the P.G. cells of the newborn mouse showed regressive phenomena and signs of necrosis.

Introduction

Observations into the fine structure of different extraadrenal chromaffin cells including those of the corpus caroticum [31, 40], glomus pulmonale [29], intestinal tract [24], bronchi [4] and heart [7, 45] have been described. According to these investigations the subcellular organization of the extraadrenal chromaffin cells is basically similar to the adrenal medullary cells [3, 9, 17, 39, 41, 47, 48] and as expected they may also produce several vasoactive substances, (adrenaline, noradrenaline, serotonin, chinin activators [8]). Some differences however, in the size of secretion granules and in their histological structure have also been found, even in the chromaffin tissue producing the same chemical substances (e.g. glomus and adrenal medulla).

The abdominal paraganglia (P. G.) are remarkable chromaffin organs. Their embryonic development, light-microscopic structure and postnatal fate have been described in a number of studies [8, 10, 11, 12, 13, 14, 15, 42]. Some electron microscopic data were published by Diner [17] on the abdominal P. G. tissue in connection with the embryonic and postnatal development of the adrenal medulla.

This paper deals with the fine structure of the abdominal P. G. of the mouse, especially with their histologic and cytoplasmic structure. In man and in several animals the abdominal P. G. tissue disintegrates between birth and puberty [30, 36, 43, 49] by cell migration [12, 13, 15, 21] or by cell degeneration

[15, 28, 46], therefore we examined also the problem, how these changes do occur in the newborn mice.

Material and method

Investigations were made on one-, five- and ten-day-old albino mice. In each case the P.G. tissue of 8—10 decapitated animals was fixed in 1% osmic acid or 5% glutaraldehyde solutions buffered according to MILLONIG [33]. From some of the animals we also excised the adrenals and at one or two places cut off the cortical substance at the beginning of fixation in a drop of osmic acid or glutaraldehyde. Fixation of both tissues lasted for an hour at + 4° C. Then the material fixed in glutaraldehyde was washed for several hours with the buffer of the fixative and subsequently post-fixed for an hour with OsO_4. Dehydration was carried out with alcohol. In 70% alcohol the tissues were cut with a rasor blade into several pieces perpendicularly to the aorta, then stained with uranyl acetate. Oriented embedding — with the aid of a stereomicroscope — was done in Araldite, for the reason that the plane of the section should be perpendicular to the aorta. Sections 1 or 2 μ in thickness were examined by phase contrast microscope and toluidine blue staining in order to select the blocks suitable for electron microscopy. The ultrathin sections were stained with lead citrate and in some cases with the combined application of uranyl acetate and lead citrate. Photomicrographs were made by a JEM-6C electron microscope.

Results

Light-microscopic data

According to the description of COUPLAND [15] a large *P.G. body* was found at the origin of the renal arteries, in the connective tissue between the aorta and the peritoneum. This main P.G. body was fixed here and there to the wall of the vessels, its surface toward the connective tissue was covered by a single layer of elongated cells. The tissue of the main P.G. body was compact, showed an irregular structure, except for some places where cells arranged into short rows and small circular groups were also observed (Fig. 1a). The P.G. tissue contained many blood-vessels and nerve bundles of various thickness.

In the five- as well as ten-day-old animals beside the main P.G. body small P.G. *cell-groups* were found (Fig. 1b). In these cell groups the various cells forming the P.G. tissue seemed easier to be characterized than in the P. G. bodies, therefore, we begin the description with the small P.G. cell groups.

Electron-microscopic data

Histological structure

Among the *P.G. cell groups* blood capillaries, pericytes, fibroblasts, fibres and Schwann cells of the plexus sympathicus may be found.

The interstitial space between the P.G. cells and the capillaries was occupied by collagen fibres and by the cells referred to above (Fig. 2). The fibroblasts often followed with their long processes the surface of the P.G.

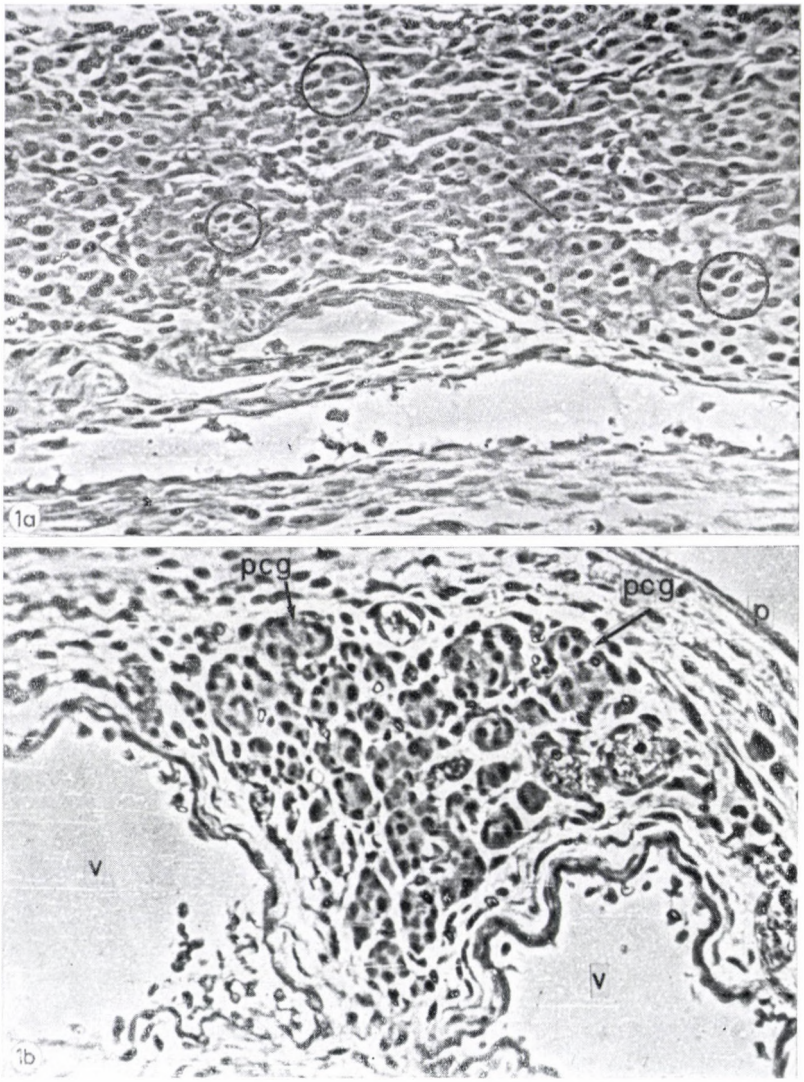


Fig. 1a—b. a— Phase contrast photo of a main P. G. body. The histological structure is rather irregular. A short cell row (arrow). and cell groups (circles). ×350

b— Phase contrast photo of P. G. cell groups (pcg). Also the peritoneum (p) and two veins (v) are visible. ×410

cells (Fig. 2). The fibroblasts themselves, however, are in contact with the P.G. cells only exceptionally, through the very ending of one or another of their processes (Fig. 3).

Some of the Schwann cells are not in contact with the P.G. cells (Fig. 3) while others tightly covered the surface of the P.G. cell group (Fig. 4). In one- to ten-day-old mice the majority of the axons of the abdominal sympathetic

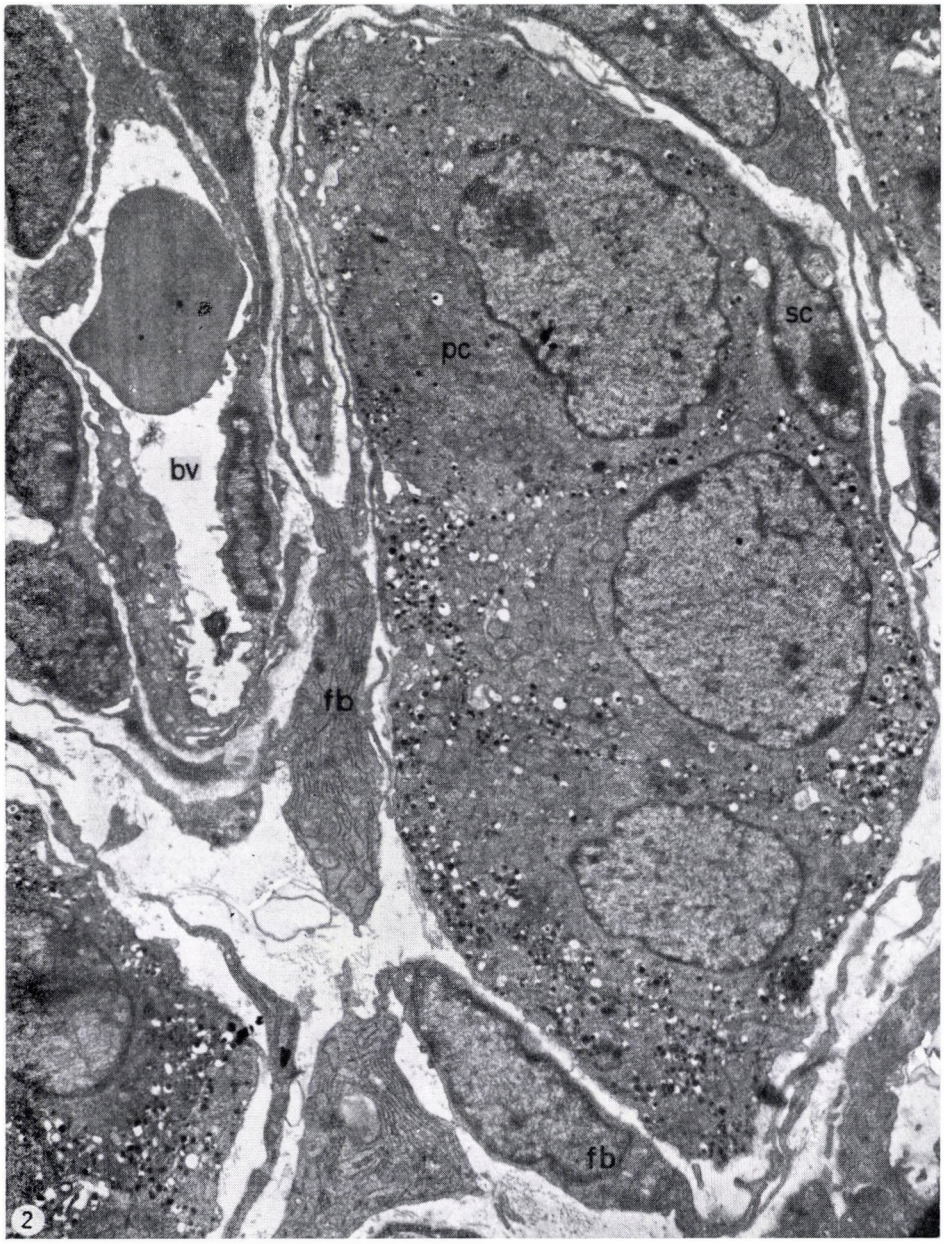


Fig. 2. Overall electron-microscopic view of a P.G. cell group. Three P. G. cells (pc) and a satellite cell (sc) are seen. The processes of the fibroblasts (fb) run parallel with the surface of the P. G. cells. A small blood vessel (bv). Glutaraldehyde fixation, post-fixation in OsO_4. ×4,550

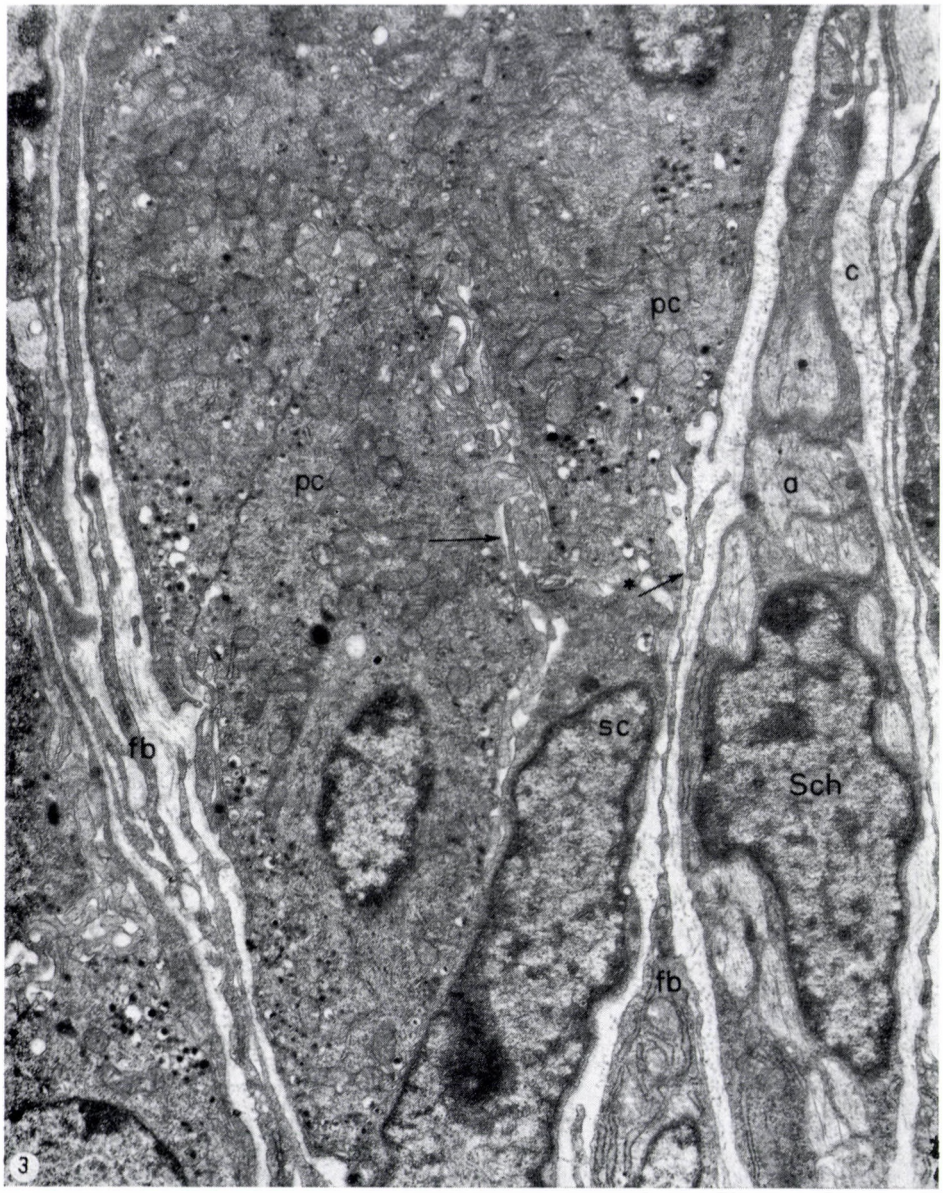


Fig. 3. P. G. cell group. Among the P. G. cells (pc) showing a separation tendency penetrates the process of a satellite cell (sc) (arrow). Between the flattened fibroblasts (fb) collagen fibres (c) and a Schwann-cell (Sch) with axons (a) are seen. One of the fibroblasts and the process of the P. G. cell are in contact with each other (arrow). Glutaraldehyde fixation, post-fixation in OsO_4. ×9,900

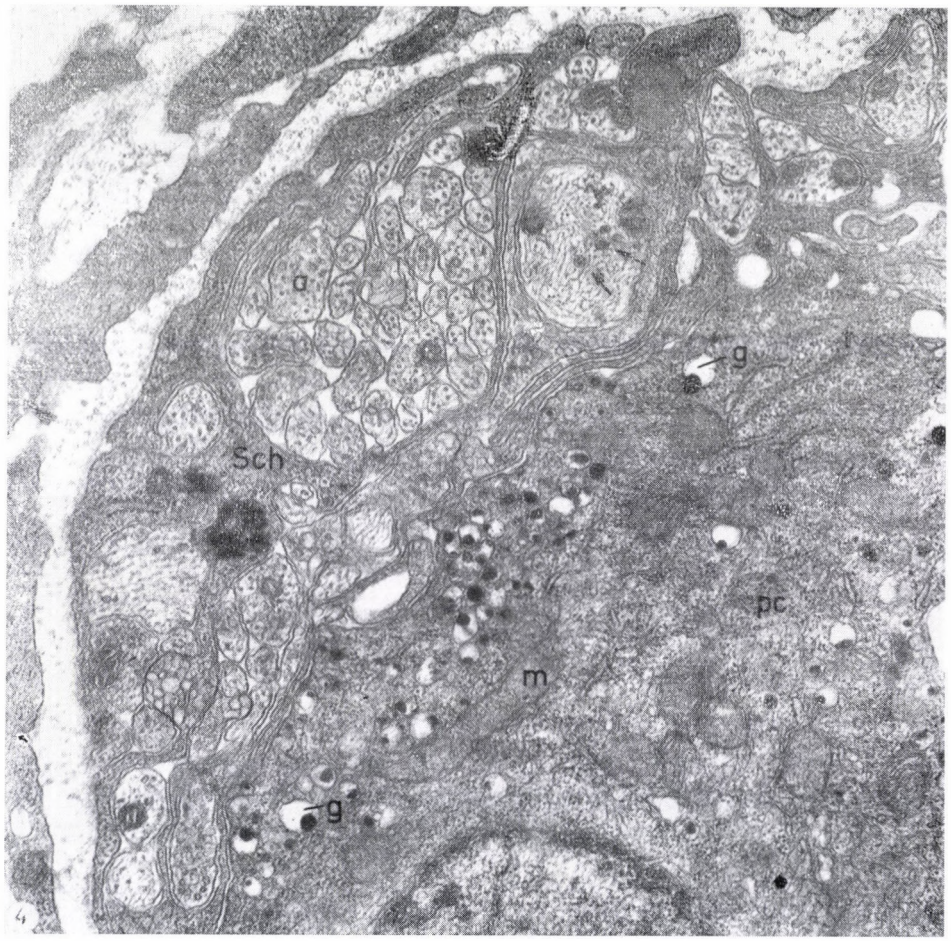


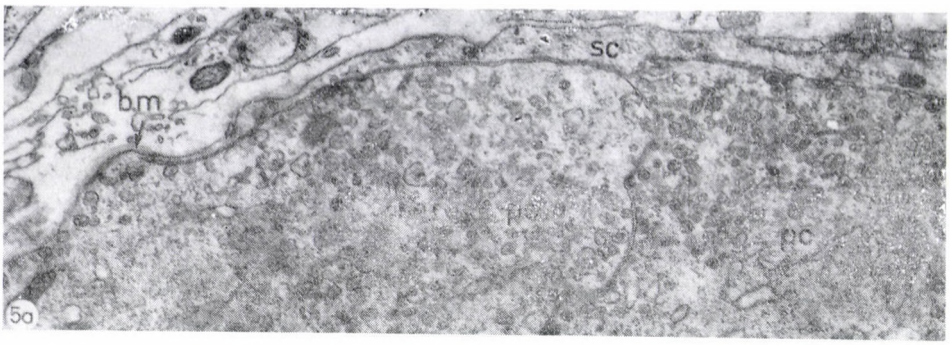
Fig. 4. Relation of a P. G. cell (pc) to a Schwann cell (Sch) which does not separate the axons (a) from each other. In the P. G. cell mitochondria (m), rough-surfaced tubules (t) of the endoplasmic reticulum, many free ribosomes and swollen secretion granules (g) are seen. In one of the axons dense core vesicles (arrow). Glutaraldehyde fixation, post-fixation in OsO_4. ×19,000

plexus were arranged by the Schwann cells in loose bundles, whereas the axons were only at places separated from one another by the cytoplasm of the Schwann cell (Fig. 4). Some of the imperfectly covered axons adhered here and

Fig. 5a—c. a— Close connection between the P. G. cells (pc) and the satellite cell (sc). Basal membrane (bm). OsO_4 fixation. ×14,500

b— The processes of the P. G. cell (pc) constitute a texture with the processes of the satellite cell (sc) (right). Portion of fibroblast (fb) above. The nucleolus (ns) is adjacent to the nuclear membrane. Glutaraldehyde fixation, post-fixation in OsO_4. ×14,600

c— Cilium of a P. G. cell. The basal body (bc) is in the Golgi region (G). In the cytoplasm multivesicular bodies (mvb) and normal as well as swollen (arrow) secretion granules (g) are seen. Glutaraldehyde fixation, post-fixation in OsO_4. ×16,500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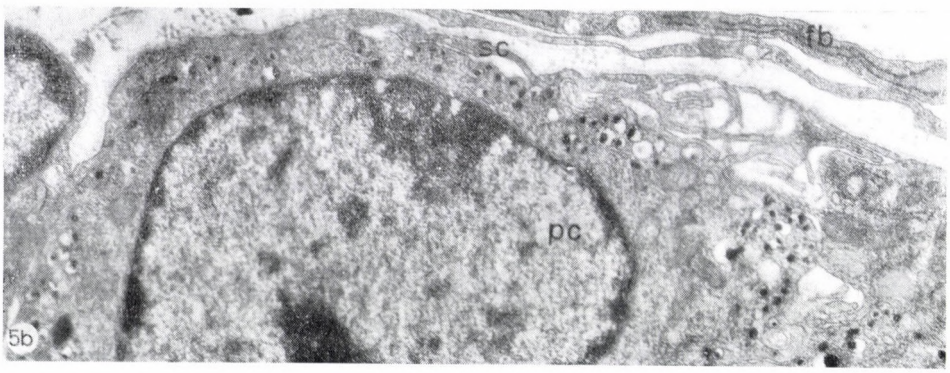
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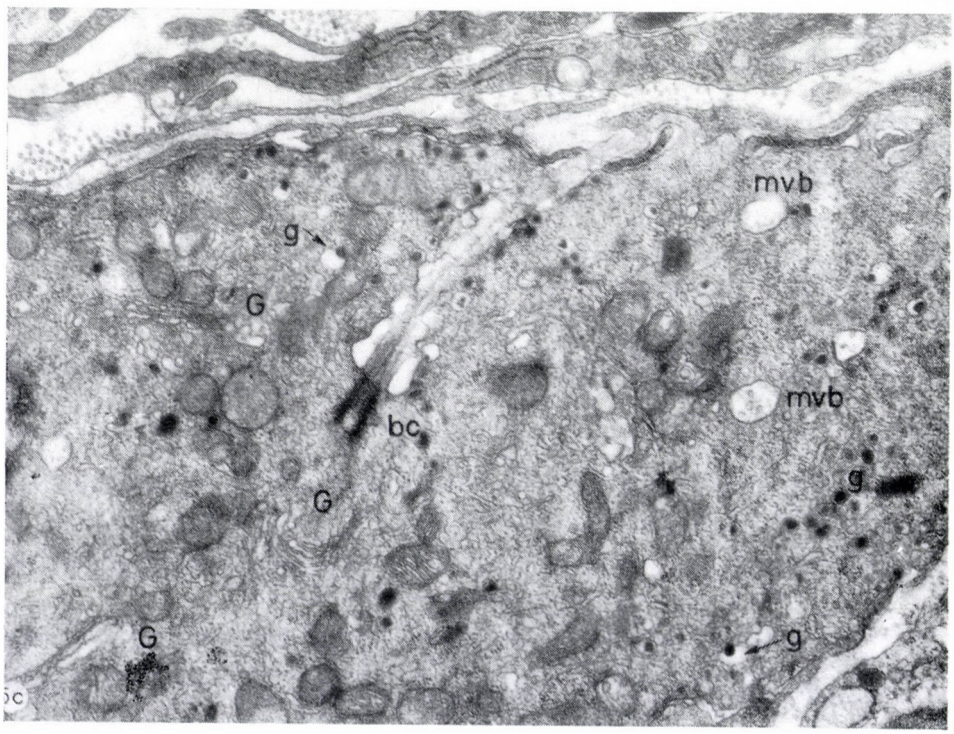
mvb
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5c

there to the surface of the P.G. cell, however, unlike in the rat [17] no true synaptic attachments were found between them.

On the surface of the P.G. cell groups a particular cell, identical with the satellite cells of the vegetative ganglia [2, 37, 38] has been also observed. These satellite cells lay sometimes in a hollow among the P. G. cells (Figs 2, 3). The flattened cytoplasm of the satellite cells and their process covered nearly everywhere the surface of the P.G. cell group in one or two layers (Fig. 3). At places where the external surface of the P.G. cell is rounded, the opposite membranes of the satellite cell and the P. G. cell run parallel, the intercellular gap between them is 200 Å wide (Fig. 5a). From the surface of the P.G. cells here and there processes arise and form an intricate labyrinth with the processes of the satellite cells. At such places wider intercellular gaps were found (Fig. 5b). The surface of the satellite cells toward the connective tissue is covered by a basal membrane which might extend to the surface of the P.G. cell as well (Fig. 5a). The satellite cells only penetrated into the P. G. cell group when the P. G. cells were separated more or less from each other (Fig. 3). In the perinuclear cytoplasm of the satellite cells a well-developed Golgi-apparatus, small mitochondria, few rough-surfaced tubules of the endoplasmic reticulum and many free ribosomes were found. The cytoplasmic processes, apart from the ribosomes, contained few cell-organelles.

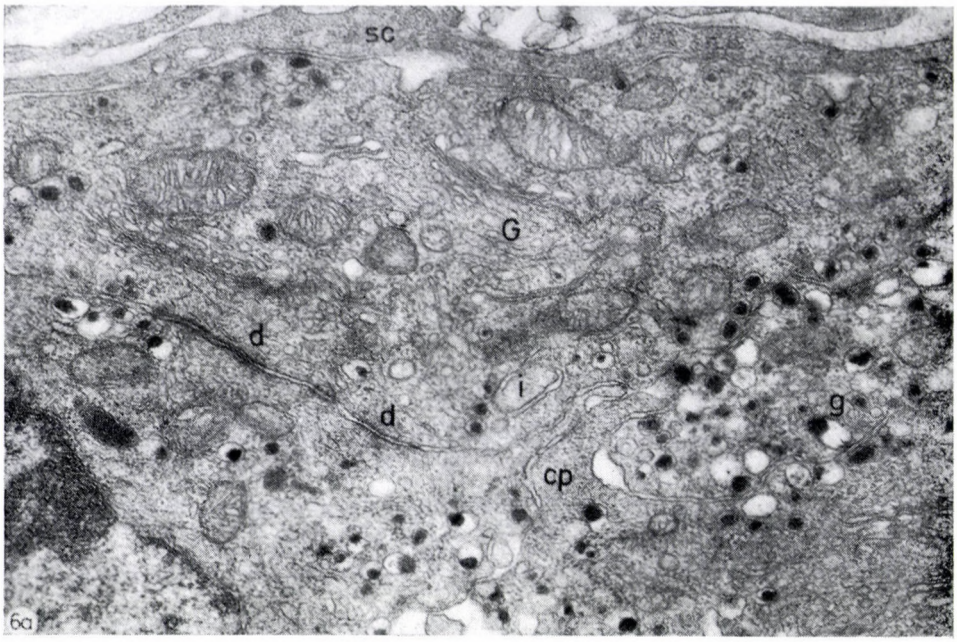
The shape of the P. G. cells when associated into small groups was generally polygonal (Figs 2, 3), their membranes running parallel to each other were found to be connected here and there by desmosomes (Fig. 6a). Interdigitation of the P. G. cells could be found mainly in the centre of the cell groups (Figs 3 and 6a).

A few P. G. cells are not arranged into groups. Isolated P. G. cells may be observed in the retroperitoneal connective tissue, along the fibres of the plexus sympathicus and in the ganglia. These cells having several processes are irregular in shape, their surface is covered by a common basal membrane.

The histological structure of the *P. G. body* is rather complex. The shape of the P. G. cells is also irregular, some of them, mostly the superficial ones might be even angular. These cells show deep interdigitations. At places the P. G. cells are flattened, and lie upon each other, their nucleus is elongated, bent, appearing semilunar in longitudinal sections (Fig. 6b). Compact tissue consisting exclusively of P. G. cells may be found in large areas without the participation of any other cell types.

Fig. 6a—b. a— The membrane of the P. G. cells is connected with desmosomes (d) and interdigitations (i). Well-developed Golgi-apparatus (G) in one of the P. G. cells. Part of the secretion granules (g) swollen. Satellite cell at the upper part of the figure. Glutaraldehyde fixation, post-fixation in OsO_4. ×24,000

b— Portion from a main P. G. body. Several interdigitations (i), a bent P. G. nucleus (n), Golgi-apparatus (G), secretion granules (g), multivesicular body (mvb) and mitochondria (m) are seen. OsO_4 fixation. ×13,200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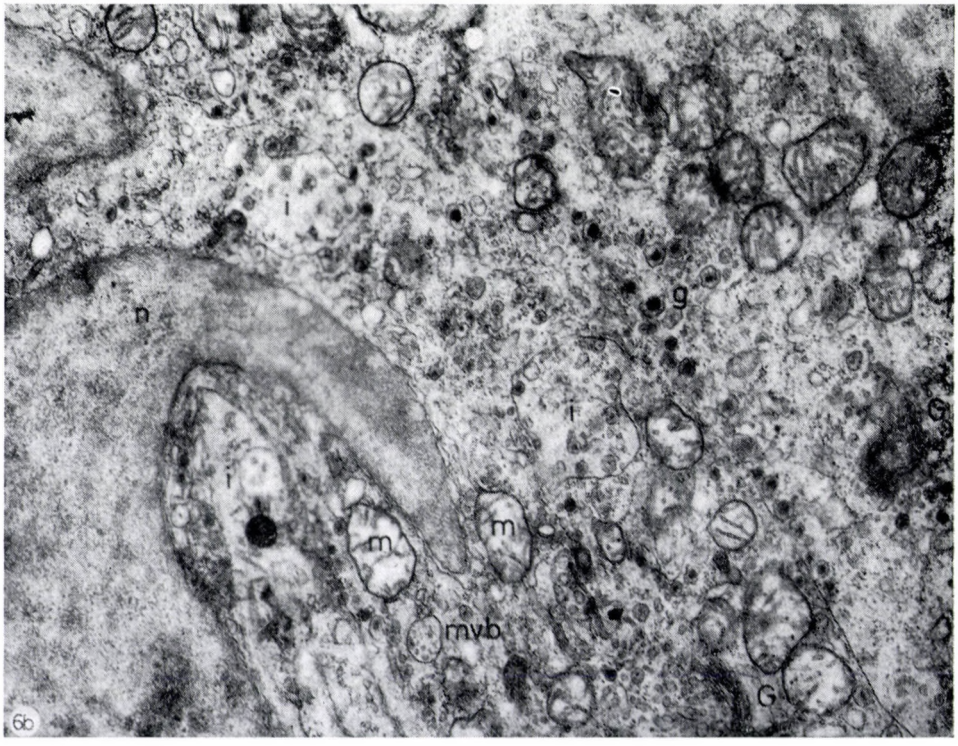
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The satellite cells are irregularly scattered, in the deeper region of the main P. G. body mostly their processes are visible (Fig. 7a). The satellite cells do not perfectly isolate the P. G. cells either from each other or from the surrounding fibres and cells of connective tissue. In the P. G. bodies numerous nerve bundles and at some places groups of Schwann cells in early stage of development [16] were observed. The capillaries and some of the P. G. cells are in close contact, at places their basal membranes being fused in a single layer. In the P. G. body the fibroblasts and pericytes are often in close contact with the P. G. cells. In some pericytes lysosomes could be observed. The capsule-like structures on the surface of the P. G. bodies proved to be a discontinuous layer of fibroblasts.

The fine structure of the P. G. cells

The cytoplasmic structure of the P. G. cells in one-, five- and ten-day-old animals is essentially identical. The nucleoli are large in all cases, and are often in close contact with the nuclear membrane (Figs 2 and 5b).

The cytoplasm is rich in free ribosomes (Fig. 4). The endoplasmic reticulum is rough-surfaced, the branching tubuli form a loose network in the whole cytoplasm (Fig. 7b). Small parallel-arranged ergastoplasmic structure was also found in some P.G. cells (Fig. 4). In the lumen of some tubuli osmiophilic granules were observed (Fig. 7b and Fig. 8a).

The well-developed Golgi-apparatus consists of several cisternae and vesicles variable in diameter. In the cavity of the Golgi elements a homogeneous substance more dense than the hyaloplasm was found. In the Golgi region the small secretion granules and multivesicular bodies (Figs 5c and 6a) are frequent and not rarely a centriolum or ciliary basal body can be found; the cell membrane is deeply invaginated to the latter (Fig. 5c and Fig. 7a). No more than one cilium was found in a cell and it is always convoluted in a cell membrane invagination.

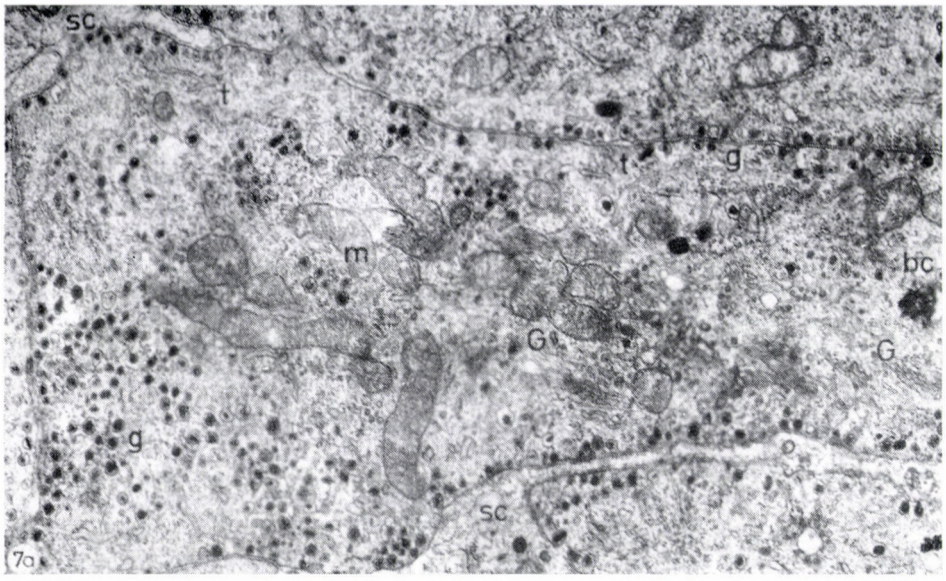
P.G. cells contain numerous elongated or round mitochondria, their inner membrane forms cristae.

In the glutaraldehyde-fixed tissue the P.G. cells contained dispersed microtubules.

The secretion granules of the P.G. cells are identical in fine structure with those in the adrenal medullary cells; these granules when fixed with OsO_4

Fig. 7a—b. a— Polygonal P. G. cell from a P. G. body. At places processes of satellite cells (sc) are seen. The secretion granules (g) are most numerous along the cell membrane. Ciliary basal body (bc), Golgi-apparatus (G), endoplasmic reticulum tubules (t), mitochondria (m). OsO_4 fixation. ×13,900

b— The secretion granules (g) of the P. G. cell are aligned along the tangentially sectioned cell membrane. The tubules (t) of the endoplasmic reticulum are readily visible, in some of them a granule-like dense substance (arrow) can be observed. OsO_4 fixation. ×25,400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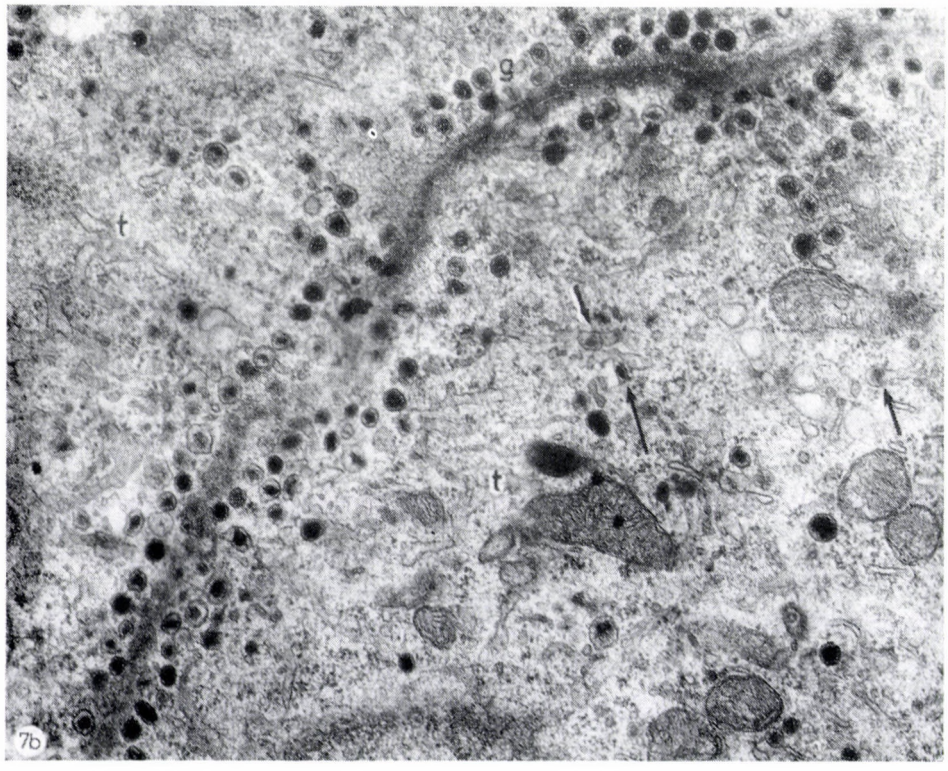
g
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7b

do not differ from the granules of the adrenal medulla of the adult mouse either [41, 47]: their electron-dense substance is surrounded by a smooth-surfaced membrane. The osmiophilic substance and the limiting membrane are separated by a halo of less dense material. In the P.G. cells the secretion granules are most numerous at the periphery of the cytoplasm, they are frequently attached to the cell membrane (Figs 7a, b and 8a). No secretion granule was found in the extracellular space.

The secretion granules of the P.G. (and adrenal medullary) cells fixed in osmic acid are somewhat different from those fixed in glutaraldehyde (Figs 6a, b). After OsO_4 fixation the granules are approximately of the same size with a maximum diameter of 200 mμ. The osmiophilic substance is of central location, it is usually round, less frequently elongated or of spindle shape (Figs 7b and 8a). After glutaraldehyde fixation some granules appeared swollen both in the P.G. cells and in the adrenal medullary cells, the diameter of the largest ones being about 600 mμ. It is interesting to note that large, swollen and small, 200 mμ granules may occur in the same cell. In the majority of the swollen granules the osmiophilic substance is attached to the limiting membrane (Figs 5, 6a and 8a). Rather often the osmiophilic substance of the swollen granules is rarified, the margins are indistinct, the density diminished (Fig. 8c). The fine structure of the granules of 200 mμ diameter is identical, either fixed in glutaraldehyde or in osmic acid only.

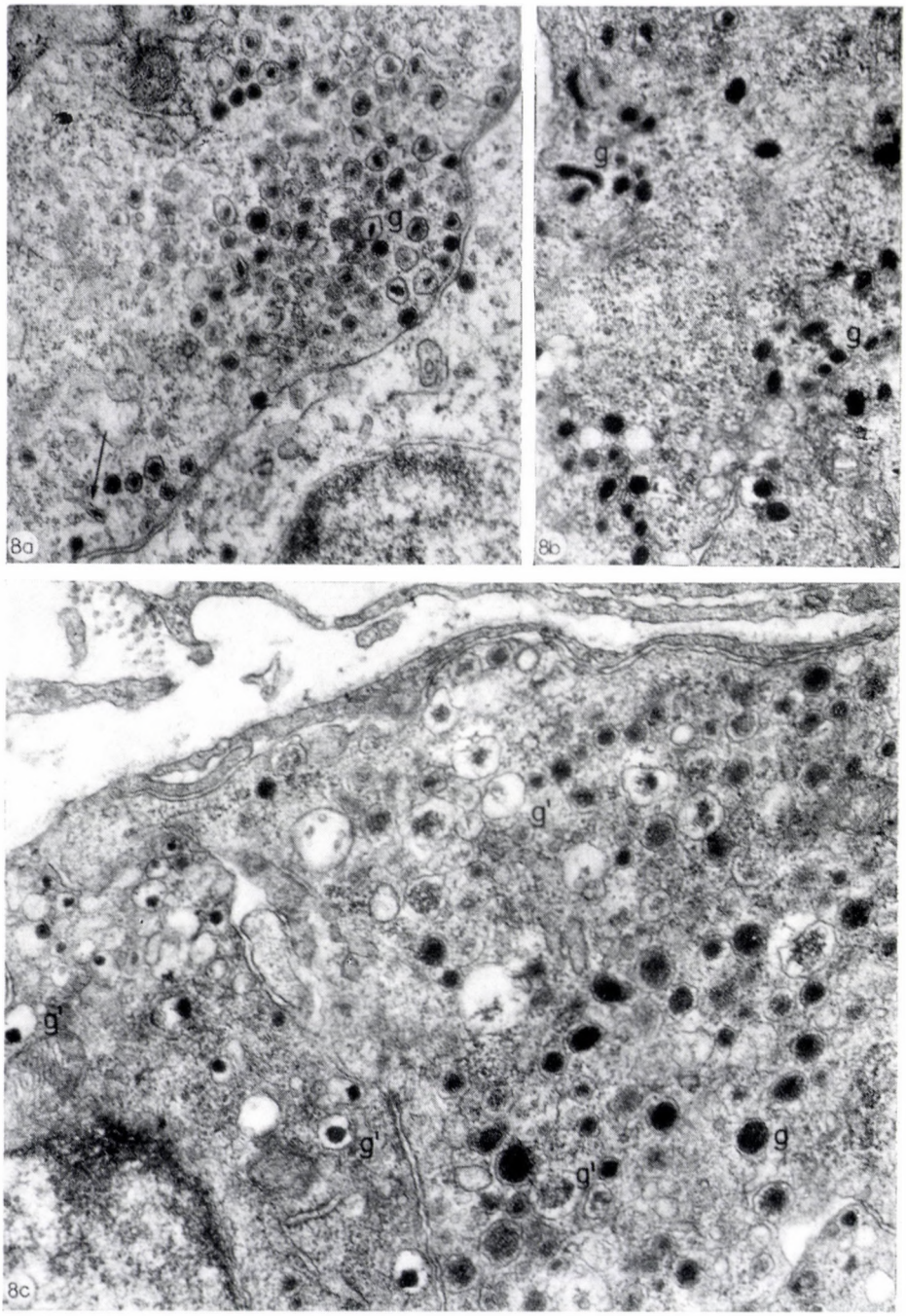
Regressive changes

Some of the secretion granules of the P.G. cells were observed to be taken up by cytolysomes [34] or autophagic vacuoles [18]. In some P.G. cells of the one-day-old animals the secretion granules aggregated into small groups, resulting the disappearance of their individual limiting membrane and the fusion of their osmiophilic substance (Fig. 9a). More frequently the aggregated granules were isolated from the rest of the cytoplasm by a membrane and so typical cytolysomes developed. In such structures the granules are usually seriously damaged (Fig. 9b). While in the P.G. cells of one-day-old animals only a few cytolysomes of 1—2 μ diameter were found (Fig. 9c), in the P.G. cells of five- and ten-day-old animals cytolysomes occupying large areas of the cytoplasm also occur. These may incorporate not only secretion granules but also mitochondria and other cytoplasmic components (Fig. 9d). In the cells with

Fig. 8a—c. a— Secretion granules (g) aggregated at the edge of a cell; the osmiophilic content is variable in shape. In one of the tubules of the endoplasmic reticulum a granule-like substance (arrow). OsO_4 fixation. ×32,800

b— Granules (g) of various shape in the cytoplasm of a P. G. cell. Glutaraldehyde fixation, post-fixation in OsO_4. ×28,400

c— Normal (g) and swollen (g') secretion granules; in part of the latter the osmiophilic substance adheres to the limiting membrane (below and left side), in another part it looks rarified (in the centre). Glutaraldehyde fixation, post-fixation in OsO_4. ×27,600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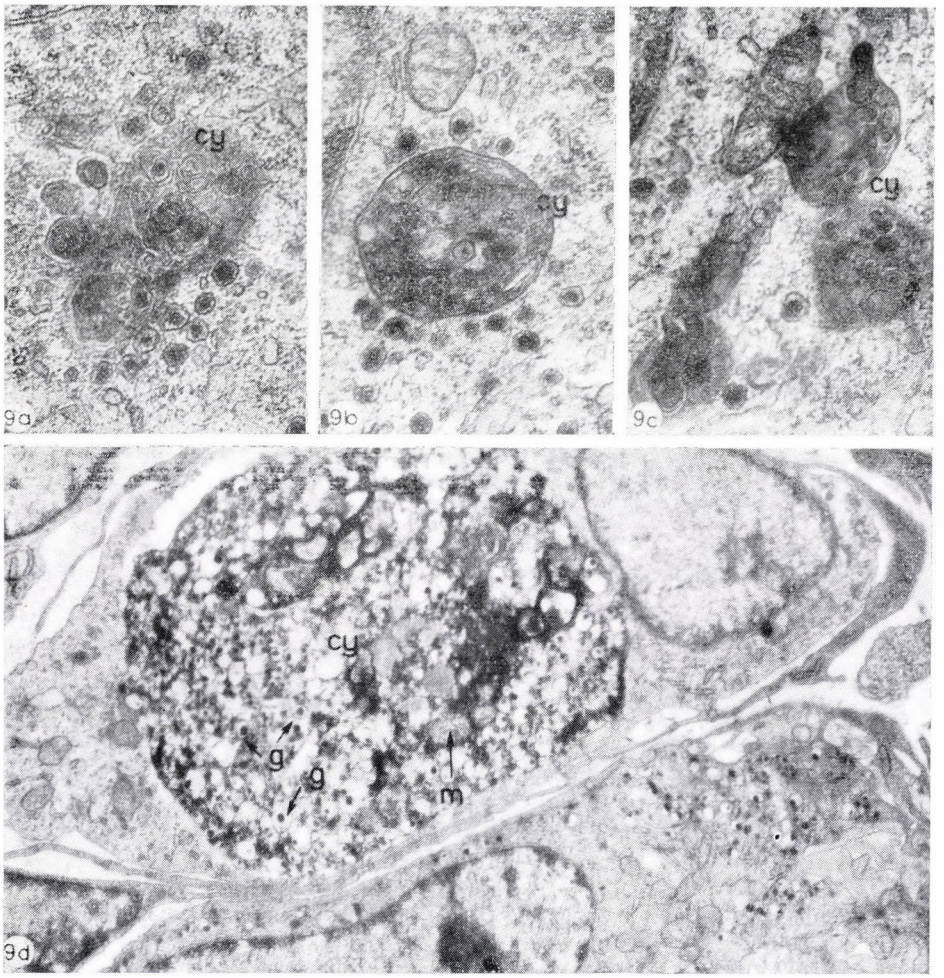
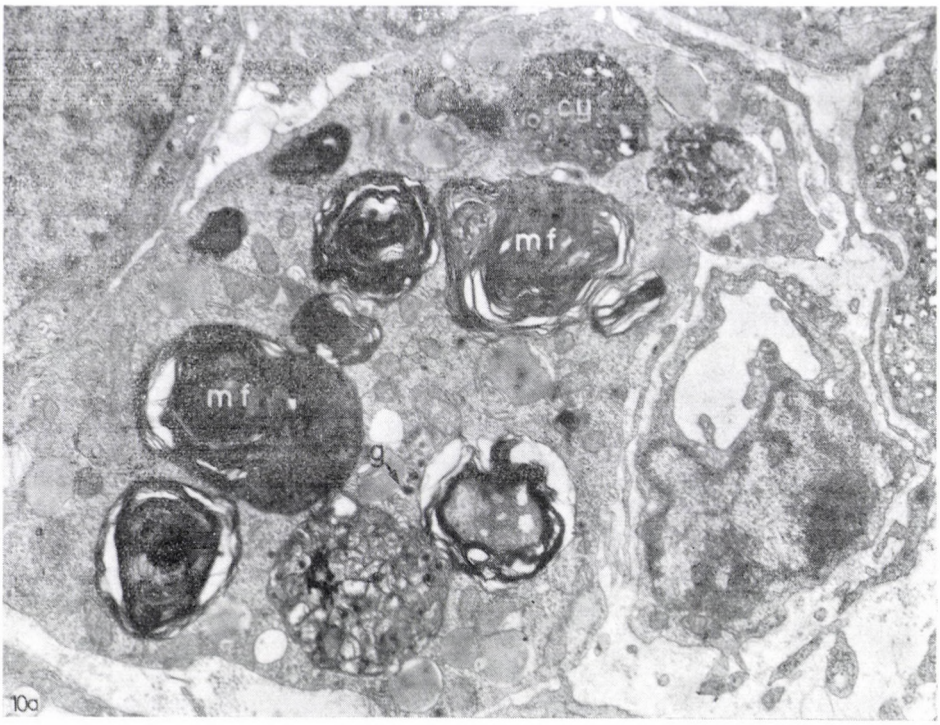


Fig. 9a—c Cytolysomes (cy) of various form containing not only partially destroyed secre tion granules (g) but also mitochondria (m). Glutaraldehyde fixation, post-fixation in OsO_4. ×9,470

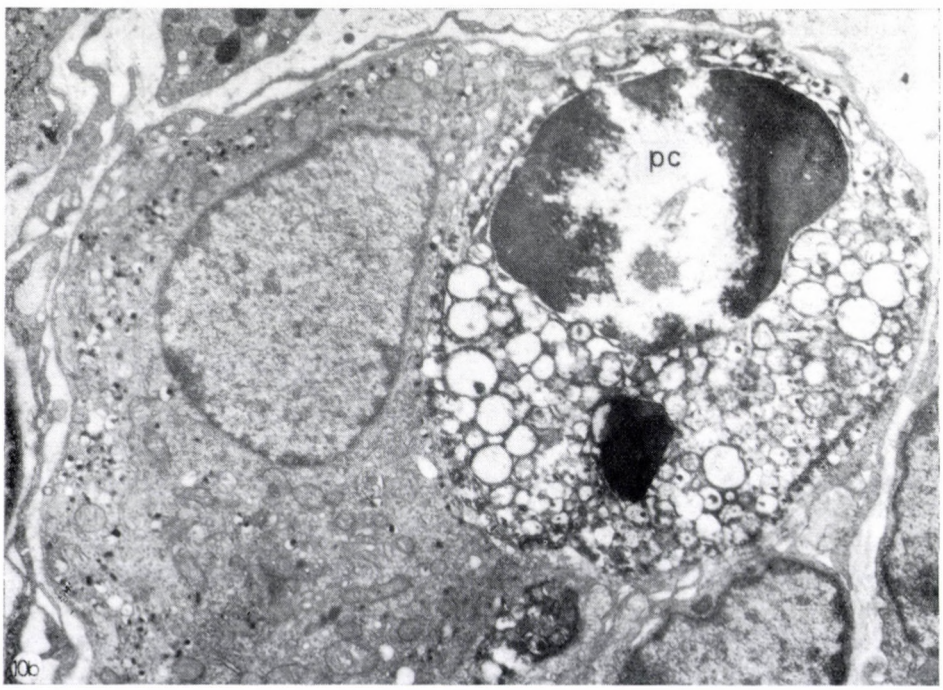
autophagic vacuoles also many myeline figures were observed. In such cells, besides the numerous, large autophagic vacuoles there are generally few dispersed secretion granules; the mitochondria, endoplasmic reticulum and other cytoplasmic components do not develop necessarily degenerative changes (Fig. 10a).

Fig. 10a—b. a— A number of cytolysomes (cy) and large, so-called myeline figures (mf) in a P. G. cell the cytoplasm of which contains very few free secretion granules (g). Glutaraldehyde fixation, post-fixation in OsO_4. ×11,250

b— Signs of nuclear and cytoplasmic necrosis in one of the P. G. cells (pc). Glutaraldehyde fixation, post-fixation in OsO_4. ×6,500



10a



10b

Notwithstanding, in the five- and ten-day-old animals some P. G. cells show obvious signs of nuclear and cytoplasmic necrosis (Fig. 10b).

Discussion

Few data are available on the function of the abdominal P. G. cells. It is well known for a long time that these cells contain pressor amines [5, 27] but the possible functional correlation between the paraganglia and the adrenals or other organs is still unknown.

According to the present observations the secretion granule of the abdominal P. G. cells is produced by the endoplasmic reticulum and the Golgi-apparatus as in the adrenal medullary cells [3, 9, 39]. In the mouse the granules in the P. G. cells and those in the adrenal medullary cells [41, 47] are identical. The same was observed by DINER [17] in the rat. As the cells of the two organs are of the same embryological origin [13, 17, 42] and as the secretion granules are the morphological equivalents of the adrenal medullary hormones [6, 22, 25, 26] it may be supposed that an identical granulation may indicate the production of the same hormone.

The swollen granules found both in the P.G. cells and adrenal medullary cells after glutaraldehyde fixation are noteworthy because they are similar to the granules found in the depleting adrenal medullary cells of reserpine-treated animals [9]. On the basis of our unpublished experience with other tissues, the 5 per cent glutaraldehyde fixation and the subsequent longer washing with 0.15 *M* phosphate buffer may result in an unequal swelling of the mitochondria and endoplasmic reticulum. Therefore, we are of the opinion that in our case the swelling of the secretion granules was an artefact.

The first investigations into the paraganglia had already shown that in most mammals the compact tissue of the main abdominal P.G. disappears between birth and puberty [30, 36, 43, 49]. GOORMAGHTIGH [21] and ZUCKERKANDL [49] tried to explain this phenomenon, suggesting a P.G. cell migration. According to COUPLAND [15] local factors as the traction of the nerves and the aorta of the growing animal may be involved in the disintegration of the P.G. tissue. In our opinion the transformation of the P.G. bodies into cell groups, and solitary cells along the plexus sympathicus suggests rather a cell migration.

The transformation of the compact P.G. body tissue into cell groups may be a result of tissue differentiation. Also some of the other extraadrenal chromaffin tissues which survive during the whole life (e.g., intracardiac chromaffin tissue [45], and glomus pulmonale [29]) show a structure similar to the abdominal P.G. cell groups. It is interesting that in the glomus caroticum all the chromaffin cells are separated by the "sustentacular cells" [40]. The transformation of the P.G. body into P.G. cell groups is analogous with the changes

observed in the course of the differentiation of the peripheral ganglia [23, 37, 38].

The autophagic and regressive phenomena which can be demonstrated already in the one-day-old animals are up to a certain point contradictory to the differentiation process of the histological structure. In some of the cells cytolysomes develop of which the smaller ones contain only secretion granules while in the larger ones other cytoplasmic components may be incorporated. Cytolysomes were described by many authors in various tissues [1, 18, 32, 35, 44]. The destruction of secretion granules in cytolysomes within the P.G. cells may be regarded as a peculiar degranulation process. Since the appearance of the cytolysomes is not related necessarily to the degeneration of the cell, it is possible that a part of the P.G. cells is not destroyed in the course of the above degranulation and eventually may survive in a degranulated form. It would be interesting to know whether in man the chromaffinomas might develop from chromaffin cells by reactivation in older age.

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DIE FEINSTRUKTUR DER ABDOMINALEN PARAGANGLIEN IN DER NEUGEBORENEN MAUS

Die abdominalen Paraganglien (PG) von neugeborenen Mäusen wurden mit dem Elektronenmikroskop untersucht und die Struktur der großen PG-Körper und der kleinen PG-Zellgruppen untersucht. In den ersteren ist eine unregelmäßige, in den letzteren eine an das periphere Nervensystem erinnernde regelmäßige Struktur zu sehen. Die PG-Zellgruppen entstehen durch die Trennung der PG-Körper (Zellwanderung). In den PG-Zellen und in den Nebennieren-Markzellen ist die Feinstruktur des Zellplasmas und der Sekretgranula identisch. In den PG-Zellen der neugeborenen Maus finden sich auch regressive Erscheinungen. In einem Teil der Zellen bilden sich autophage Vakuolen, in denen hauptsächlich Sekretgranula zugrunde gehen (nicht-sekretorische Degranulation). Selten können auch PG-Zellen beobachtet werden, die Zeichen des Absterbens zeigen.

МИКРОСТРУКТУРА БРЮШНЫХ ПАРАГАНГЛИЕВ У НОВОРОЖДЕННЫХ МЫШЕЙ

Брюшные параганглии (ПГ) новорожденных мышей исследовались под электронным микроскопом. Обсуждается структура крупных параганглиевых тел и малых параганглиевых клеточных групп. В первых видна нерегулярная, а в последних регулярная структура, подобная периферической нервной системе. Параганглиевые клеточные группы возникают в результате разделения параганглиевых тел (странствование клеток). В параганглиевых клетках и в миелоцитах надпочечников наблюдается одинаковая микроструктура цитоплазмы и секреторных зернышек. В параганглиевых клетках новорожденных мышей обнаруживаются также регерсивные явления. В части клеток образуются автофаговые вакуоли, в которых погибают главным образом секреторные зернышки (несекреторная дегрануляция). Изредка наблюдаются также параганглиевые клетки, показывающие признеки отмирания.

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ASSIMILATION OF NITRATES AND PHOSPHATES BY ASCORBIC ACID-FED MYCELIA OF CUNNINGHAMELLA ELEGANS

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Synopsis

Ascorbic acid did not accelerate phosphorus uptake but enhanced its incorporation in organic forms. Nitrate uptake and reduction were also increased but most of the produced ammonia accumulated as amides and acyclic nitrogen compounds; most of these were excreted into the external media. Nucleoprotein nitrogen slightly increased while protein amino nitrogen decreased with increasing the concentration of the vitamin.

Introduction

In a previous investigation, NAGUIB [8] studied the effects of ascorbic acid on the respiration and carbohydrate metabolism of *Cunninghamella*. He noticed that the gain in dry weight of the treated mats did not account for the high sugar uptake and suggested the possibility of alterations in the pathways of carbohydrate metabolism of the fungus. To elucidate such divergence, it was thought to investigate the effects of vitamin C on the nitrogen and phosphorus metabolisms of the same fungus.

Material and method

Standard mycelial mats of *Cunninghamella elegans* were prepared according to the methods previously mentioned by NAGUIB and SALAMA [9]. These mats were then floated over 50 ml Richard's solution containing no, or 10, 20, or 40 p. p. m. ascorbic acid. The flasks were incubated in the dark at 25° C for 48 hour. During this period nitrogen and phosphorus analysis of the media and tissues was carried out at 24-hour intervals. At least 5 replicates were used in each treatment. The nutrient solution contained 20 g sucrose, 10 g KNO_3, 2.5 g magnesium sulphate, 5 g potassium dihydrogen phosphate and 0.1 g ferrous sulphate per litre solution.

Fiske reagent [9] was used to determine both organic and inorganic phosphorus after complete digestion of the former with sulphuric perchloric mixture. The various nitrogen fractions in the media and tissues were estimated by the methods described by NAGUIB [7].

Results

Effect of ascorbic acid on phosphorus metabolism: Table 1 presents the mean values of the different phosphorus fractions estimated in the differently treated samples. It shows that ascorbic acid slightly increased the total phos-

Table 1

Analysis of the fungal mats of Cunninghamella elegans treated with various concentrations of ascorbic acid for their different phosphorus fractions

(mg P per fungal growth)

Medium		Inorg. P	Soluble org. P	Insol. org. P	Total org. P	Total P	% org. P
Initials		2.86	1.27	1.82	3.09	5.95	51.92
R. sol.	24 hrs	4.08	2.07	2.09	4.16	8.24	50.42
	48 hrs	6.54	5.06	5.00	10.06	16.62	60.53
R. sol. + 10 p. p. m.	24 hrs	3.54	2.80	3.46	6.26	9.80	64.00
	48 hrs	5.71	5.28	7.49	12.77	18.48	69.10
R. sol. + 20 p. p. m.	24 hrs	3.21	2.61	4.14	6.75	9.96	67.77
	48 hrs	3.85	5.73	6.58	12.31	16.16	76.18
R. sol. + 40 p. p. m.	24 hrs	3.90	2.63	4.66	7.29	11.19	65.15
	48 hrs	4.35	5.71	6.78	12.49	16.84	74.17

R. sol. = Richard's solution
p. p. m. = concentration of ascorbic acid

phorus content only when supplied in 10 p. p. m. concentration. Further, there is a noticeable increase in the organic phosphorus content, particularly of the insoluble fraction.

Effect of ascorbic acid on nitrate uptake: Table 2 records the mean values of nitrate nitrogen absorbed during the experimental period. It demonstrates that ascorbic acid enhanced nitrate uptake particularly during the second 24 hours of the experiment; the increase was almost unaffected by increasing the concentration of the vitamin.

Effect of ascorbic acid on nitrogen excretion: Table 3 illustrates the mean values of the different nitrogen fractions (other than nitrates) traced in the

Table 2

Uptake of nitrate nitrogen by Cunninghamella elegans in presence of various concentrations of ascorbic acid

(mg N per fungal growth)

Medium	1st 24 hrs	2nd 24 hrs	Total in 48 hrs
Richard's sol.	15.64	13.99	29.63
Richard's sol. + 10 p. p. m. AA	16.53	23.75	40.28
Richard's sol. + 20 p. p. m. AA	17.71	22.98	40.69
Richard's sol. + 40 p. p. m. AA	15.67	25.22	40.89

AA = ascorbic acid

Table 3

Nitrogen fractions excreted by mycelial felts of Cunninghamella elegans treated with various concentrations of ascorbic acid
(mg N per mycelial growth)

Medium		Ammonia N	Amide N	Nitrite N	Acyclic N	Total N
Richard's sol.	24 hrs	0.32	3.25	0.038	4.182	7.790
	48 hrs	0.42	3.75	0.057	6.553	10.780
R. sol. +	24 hrs	0.36	3.40	0.078	5.342	9.180
10 p. p. m. AA	48 hrs	0.48	8.21	0.141	14.099	22.930
R. sol. +	24 hrs	0.18	3.99	0.148	5.042	9.360
20 p. p. m. AA	48 hrs	0.24	8.52	0.212	17.958	26.930
R. sol. +	24 hrs	0.24	4.12	0.194	7.806	12.360
40 p. p. m. AA	48 hrs	0.36	12.52	0.262	21.398	34.540

AA = ascorbic acid

culture media of the variously treated samples. It shows that the level of all nitrogen fractions detected (lest ammonia) increased with increasing the concentration of ascorbic acid.

Effect of ascorbic acid on the nitrogen content of the tissues: The mean values of the different nitrogen fractions estimated in the variously treated samples are given in Table 4. It shows that ascorbic acid, in 40 p. p. m. only, lowered the total nitrogen content of the mycelial mats as a result of decreasing the different protein nitrogen fractions with a slight accumulation of nitrates. Although the other concentrations of ascorbic acid had no effect on the total nitrogen content, yet the amino-, nucleoprotein-, and protein amide-nitrogen increased while the protein amino-nitrogen decreased. In the meantime, the ammonia-, amide-, and acyclic-nitrogen content of the treated samples was almost unchanged with variation of concentration of the vitamin.

Discussion

The increased rate of formation of both forms of organic phosphorus in the ascorbic acid-treated samples in spite of the unchanged total phosphorus content compared with the controls indicates the accelerating effects of the vitamin on the phosphorylation enzymes; a phenomenon previously suggested by ARNON and co-workers [2] and AVRON and co-workers [3] in higher plants and confirmed by NAGUIB [8] in fungi where ascorbic acid increased both rates of sugar uptake and polysaccharide formation by *Cunninghamella*; both phenomena need phosphorylation. In the meantime the results of this investigation showed an increased rate of nucleoprotein nitrogen concurrent with the increased insoluble organic phosphorus content of the treated mats.

Table 4

Analysis of mycelial felts of Cunninghamella elegans treated with
(mg N

Medium		Ammonia N	Amide N	Amino N	Nitrate N
Initials		0.28	0.17	0.36	0.18
Richard's sol.	24 hrs	0.28	0.21	0.27	0.18
	48 hrs	0.35	0.31	0.58	0.16
R. sol. +	24 hrs	0.24	1.06	0.42	0.09
10 p. p. m. AA	48 hrs	0.38	1.40	0.82	0.10
R. sol. +	24 hrs	0.30	0.81	0.68	0.07
20 p. p. m. AA	48 hrs	0.42	1.23	0.94	0.09
R. sol. +	24 hrs	0.24	1.28	0.58	0.19
40 p. p. m. AA	48 hrs	0.36	1.18	0.54	0.33

AA = ascorbic acid

Furthermore, the absence in the various media of organic phosphorus might lead to the suggestion that the cytoplasm membrane of the fungal mycelia is impermeable for these substances, both in absence and presence of ascorbic acid.

The results of this investigation also demonstrated an increased rate of accumulation in the media of various forms of nitrogen, particularly of amide and acyclic nitrogen, both of which require a carbon skeleton. These observations might confirm the former suggestion [8] that most of the absorbed sugars, by the ascorbic acid-treated *Cunninghamella* mats were transformed to other non-sugar compounds most of which were excreted into the nutrient media, hence the observed low rate of dry weight compared with the excessive sugar absorption. In the meantime, it seems that ascorbic acid enhanced both nitrate uptake and reduction to ammonia *via* the nitrites; ammonia was transformed to amides and acyclic nitrogen while very little was used in building up proteins; an indication that ascorbic acid accelerated nitrate reductase but inhibited both amino acid and protein synthesis more prominently in higher than in lower concentrations.

In this connection it may be mentioned that VIRTANEN and SAUBERT v. HAUSEN [14, 15] and VIRTANEN [12] found favourable growth effects of ascorbic acid when added to nitrate but not to ammonium nutrient solutions. This effect was not specific but would be obtained by other reducing substances like glutathione and cysteine. Furthermore, inhibition of growth and metabolic processes by ascorbic acid were recorded by many investigators [1 5 6, 16] while stimulation was also observed [4, 8, 10, 11, 17].

various concentrations of ascorbic acid for their nitrogen fractions
per mat)

Acyclic N	Total sol. N	Nucleo-protein N	Protein amide-N	Protein amino-N	Total protein N	Total N
3.76	4.75	0.86	0.17	12.68	13.71	18.46
4.56	5.50	0.94	0.17	19.50	20.61	26.11
7.02	8.42	1.06	0.21	26.32	27.59	36.01
4.60	6.61	1.49	0.45	17.35	19.29	25.90
7.87	10.57	1.76	0.61	23.98	26.35	36.92
6.81	8.67	1.34	0.86	15.84	18.04	26.71
7.73	10.41	1.69	1.17	21.81	24.67	35.08
5.20	7.49	1.04	0.17	12.84	14.05	21.54
6.91	9.32	1.39	0.19	14.69	16.27	25.59

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NITRAT- UND PHOSPHATASSIMILATION IN MIT ASKORBINSÄURE ERNÄHRTEM MYZELIUM VON *CUNNINGHAMELLA ELEGANS*

Die Askorbinsäure bewirkte keine gesteigerte Phosphoraufnahme, erhöhte aber die Phosphorinkorporation in die organischen Verbindungen. Auch die Nitrataufnahme und -reduktion waren gesteigert, doch der Großteil des Ammoniums erschien in Form von Amiden und azyklischen Stickstoffverbindungen, deren Mehrheit in das äußere Medium ausgeschieden wurde. Mit der Zunahme der Vitaminkonzentration zeigte der Stickstoffgehalt der Nukleoproteide einen leichten Anstieg und der Aminostickstoff der Proteine eine Abnahme.

АССИМИЛЯЦИЯ НИТРАТОВ И ФОСФАТОВ МИЦЕЛИЕЙ ГРИБОВ *CUNNINGHAMELLA ELEGANS*, ПРОРАСТАЮЩИХ НА СУБСТРАТЕ, СОДЕРЖАЩЕМ АСКОРБИНОВУЮ КИСЛОТУ

Аскорбиновая кислота не ускоряет поглощение фосфора, а повышает его инкорпорацию в плодовые тела. Поглощение и восстановление нитратов также возросло, однако, преобладающая часть образовавшегося аммония была аккумулирована в виде амидов и ациклического азотного соединения, большая часть которых была выделена в наружную среду. Содержание нуклеопротеинового азота несколько повысилось, в то время как по мере повышения концентрации витамина в содержании протеинового аминоазота отмечалось понижение.

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CHANGES INDUCED BY ADRENALECTOMY IN NERVE ENDINGS OF THE HYPOTHALAMIC MEDIAN EMINENCE (ZONA PALISADICA) IN THE ALBINO RAT

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Synopsis

Dense (osmiophilic) vesicles, occurring normally in a moderate number in the median eminence and pituitary stalk (zona palisadica) nerve endings of the rat, increase in number and size during the second week after bilateral adrenalectomy. Their dark osmiophilic cores are progressively resolved, a process that appears to start from the centre. Enlarged synaptic vesicles of more ordinary structure seem to open up (or the reverse) towards a relatively wide system of interterminal, and possibly partly intraterminal, spaces. The changes are at their maximum around the end of the second week and tend to abate during the third. — The possible relations of these changes to increased discharge of Corticotrophin Releasing Factor (CRF) are discussed.

Introduction

The existence of specific nerve terminals situated around the vascular plexus and capillary loops of the median eminence, i.e. around the vessels draining towards the anterior lobe of the pituitary gland has been postulated long ago [5]. These vessels have been supposed to be capable of releasing substances stimulating the various trophic functions of the anterior lobe. Although the existence of nerve endings in this region have been described by numerous authors since the first observation by CAJAL [2], the large abundance of specific nerve terminals in the surface zone (zona palisadica) both of the median eminence and the proximal segment of the pituitary stalk was first shown by SZENTÁGOTHAI and co-workers [16], using the Golgi procedure. The same authors have brought forward conclusive anatomical evidence for a hypothesis — proposed first by SPATZ [13] — of a parvicellular, probably neurosecretory, neuron system situated mainly in the lower tuberal nuclei (nucl. arcuatus and nucl. periventricularis ant. [ventral part]), and sending their delicate axons to the zona palisadica. This system appears to be completely independent of the magnocellular neurosecretory system of SCHARRER and SCHARRER [12] originating in mammals from the supraoptic and paraventricular nuclei giving rise to the supraoptico-hypophyseal tract, which terminates in the posterior lobe. For distinction from this latter system the parvicellular system was termed *tubero-infundibular* by SZENTÁGOTHAI and co-workers [16].

The first description of the zona palisadica nerve endings at the electron microscope level was given by T. KOBAYASHI and co-workers [9] in the rat and by SZENTÁGOTHAI and HALÁSZ [17] in the cat. A detailed study of the ultra-architecture of the median eminence of the rat was made by RÖHLICH and co-workers [11]; the present work can strongly rely on this paper for comparison to the normal structure. H. KOBAYASHI and co-workers [8] discuss the various types of vesicles and granules in these terminals, considering that FUXE [3] found a high catecholamine content in the surface zone of the median eminence. AKMAYEV and DONÁTH [1] recorded a considerably increased catecholamine fluorescence and widening in depth of the fluorescent zone of the median eminence after bilateral adrenalectomy.

Material and method

Twenty-four adult albino rats of both sexes were used altogether: 5 normal controls, three groups of 5 animals each (these groups were sacrificed 10, 14 and 17 days respectively after bilateral adrenalectomy), and finally twice 2 animals which were killed 14 days after bilateral thyroidectomy and ovariectomy, respectively. Animals were killed by decapitation, the calvarium was removed as quickly as possible, cold 3.4% glutaraldehyde solution buffered with isotonic Millonig phosphate buffer of pH 7.3 was dropped immediately between the carefully elevated brain and the cranial base. Then, after transecting the cranial nerves, the brain was removed from the skull, while glutaraldehyde solution was continuously dropped on the basal surface. The origin of the pituitary stalk, including a small part of the median eminence, was cut off with scissors and the fixation of this piece was continued for one hour in the same fluid. After being washed in Millonig phosphate buffer, the pieces were post-fixed in 1% buffered osmic-acid solution (4° C) for an hour, dehydrated in alcohol and, after propylene-oxide treatment, embedded in Araldite (Fluka). Orientation of the tissue block was ensured by semithin sections; thus ultrathin sections (LKB ultramicrotome) of the zona palisadica and adjacent parts of the pars tuberalis could be made starting from the origin of the pituitary stalk at the median eminence. Sections were contrasted with lead citrate as described by REYNOLDS and investigated in the Tesla Electron Microscope BS 242 B. The micrographs of Figs 6 and 8 were made in the Hitachi HU 10 Electron Microscope.

Results

The normal structure of the zona palisadica nerve endings, as it appears in our control animals, is seen in Figs 1 and 2. It is consistent in every respect with the description of RÖHLICH and co-workers [11]; the only differences, especially in appearance of the dense vesicle, are obviously caused by the difference in fixation, only osmic acid having been used in the latter study. Their number, size, localization and density of distribution seem to be identical. Profiles of glial end-feet and processes can be recognized easily from their numerous dark glycogen bodies. Large, probably lipid droplets frequently occur in the glial profiles (Fig. 5).

Ten days after bilateral adrenalectomy fewer true dense vesicles were seen, larger vesicles with light centres having taken their place (Figs 3, 4, 5, 6]. As also in the normal material, a few large empty vesicles can be observed

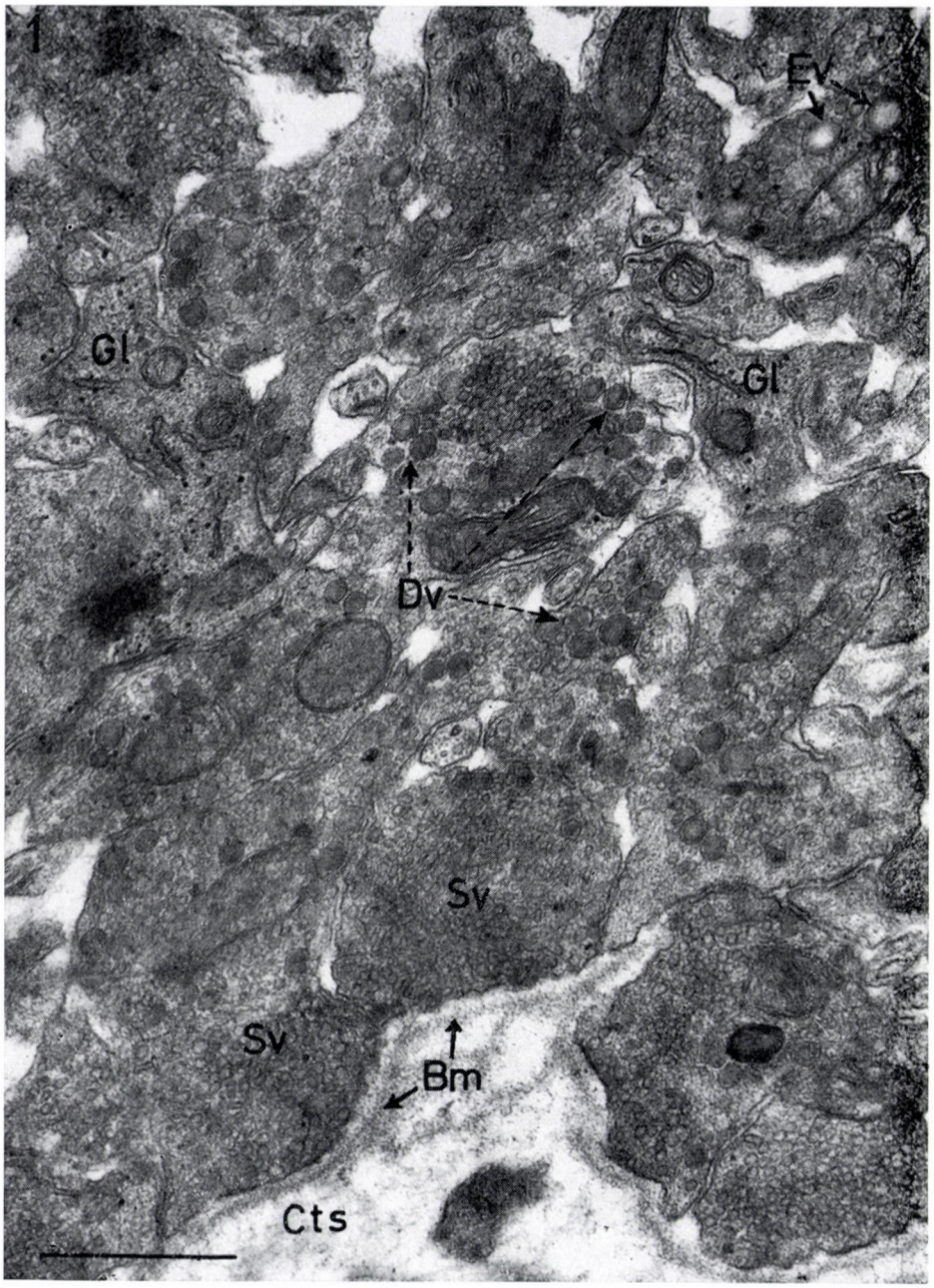


Fig. 1. Surface zone (zona palisadica) of the median eminence in normal control rat. Closely packed nerve terminals, with some glial feet (Gl) profiles intermingled are separated from connective tissue space (Cts) of the surface only by thin basement membrane (Bm). Nerve terminals contain synaptic vesicles (Sv) of usual character and—generally not very close to the outer surface—large vesicles with pale rims and dense centres (Dv). Only in few of the largest vesicles the interior becomes progressively lighter towards the centre (Ev). Scale 1 μ

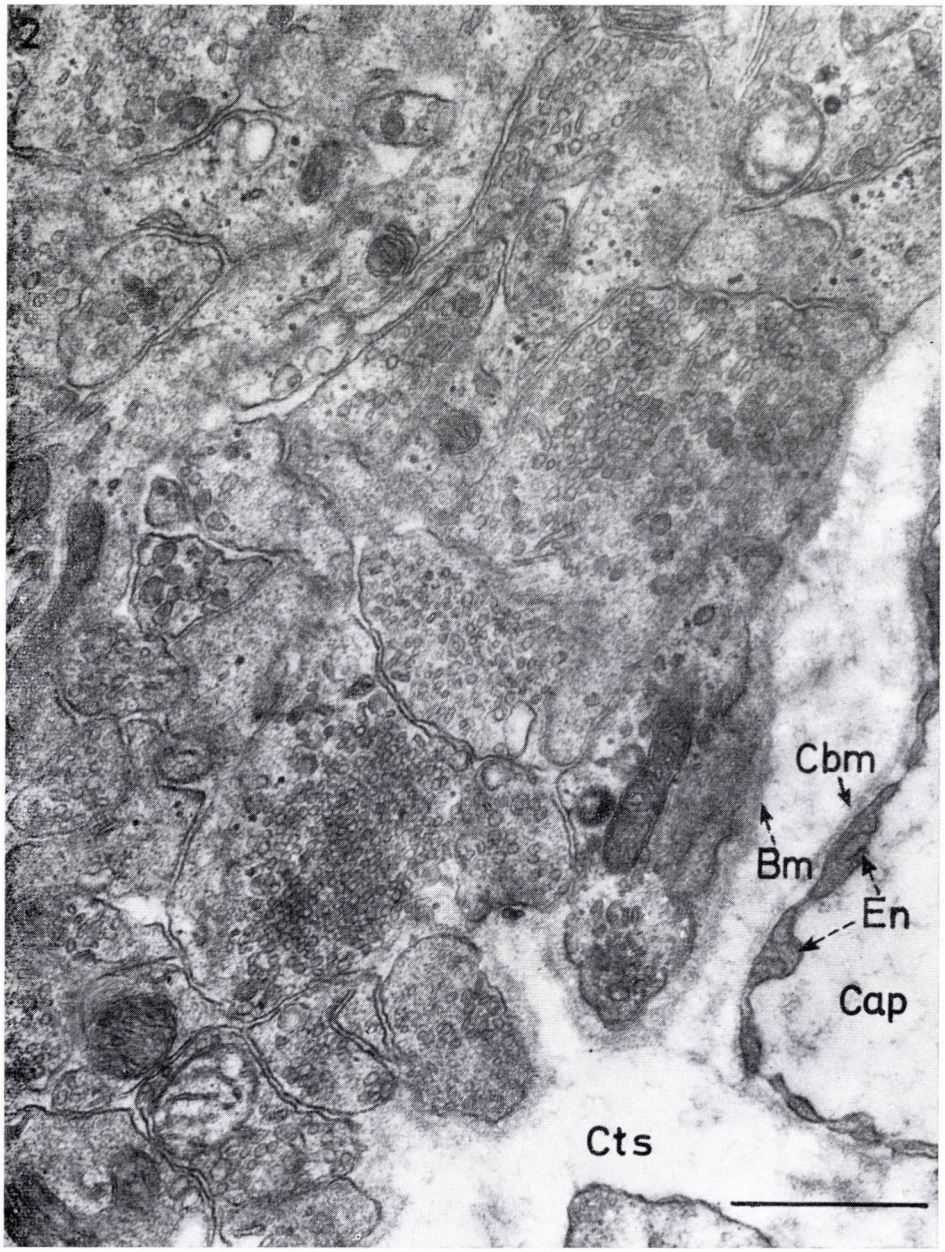


Fig. 2. Same as Fig. 1, showing the close relation of surface zone nerve endings to capillaries (Cap) draining towards the portal veins. The nerve terminals are separated from the interior of the capillary only by the outer basement membrane (Bm) of the median eminence, the thin connective tissue (Cts), the capillary basement membrane (Ctm), and the fenestrated endothelium (En). Much fewer larger vesicles appear in this visual field. Scale 1 μ

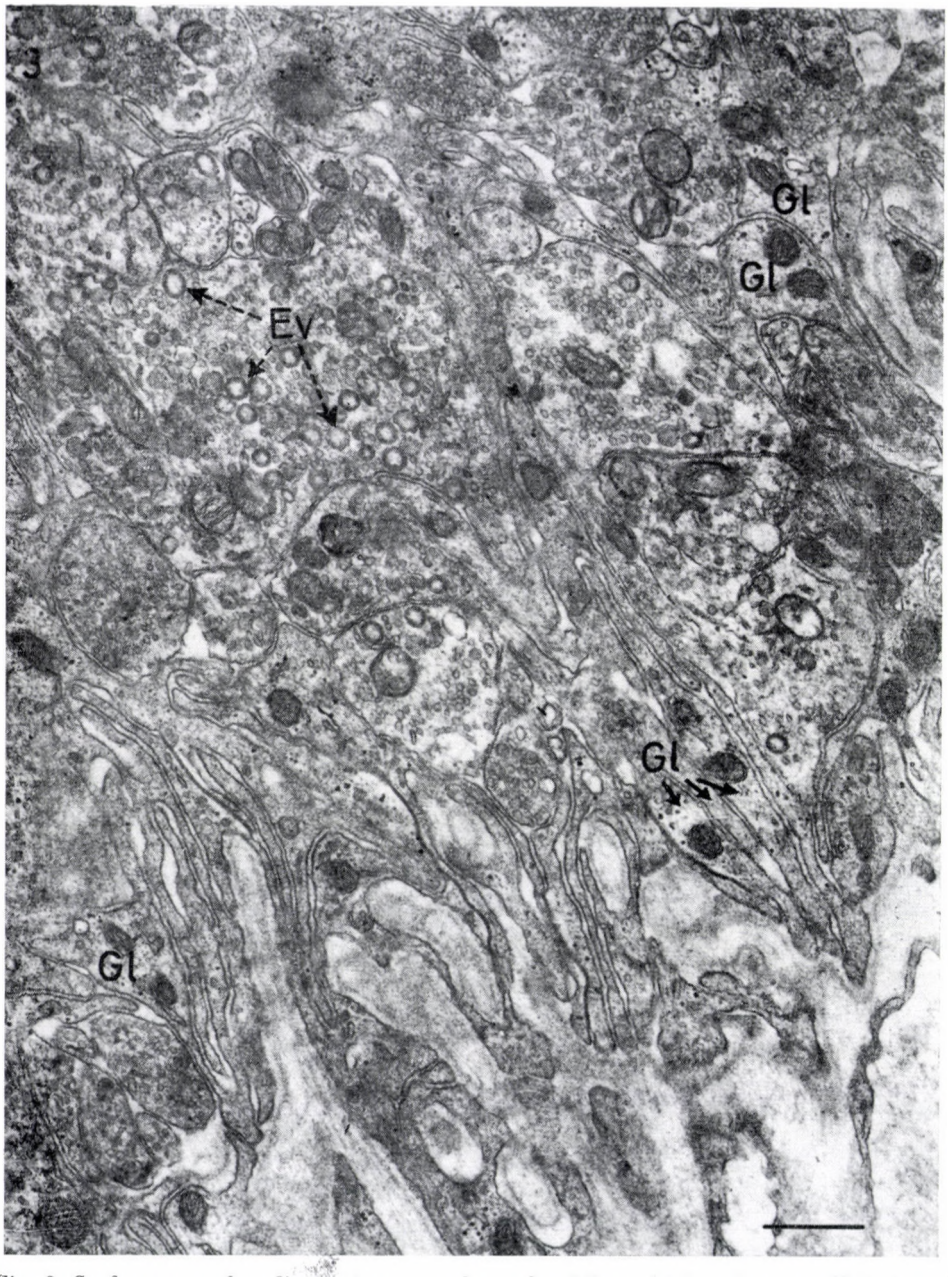
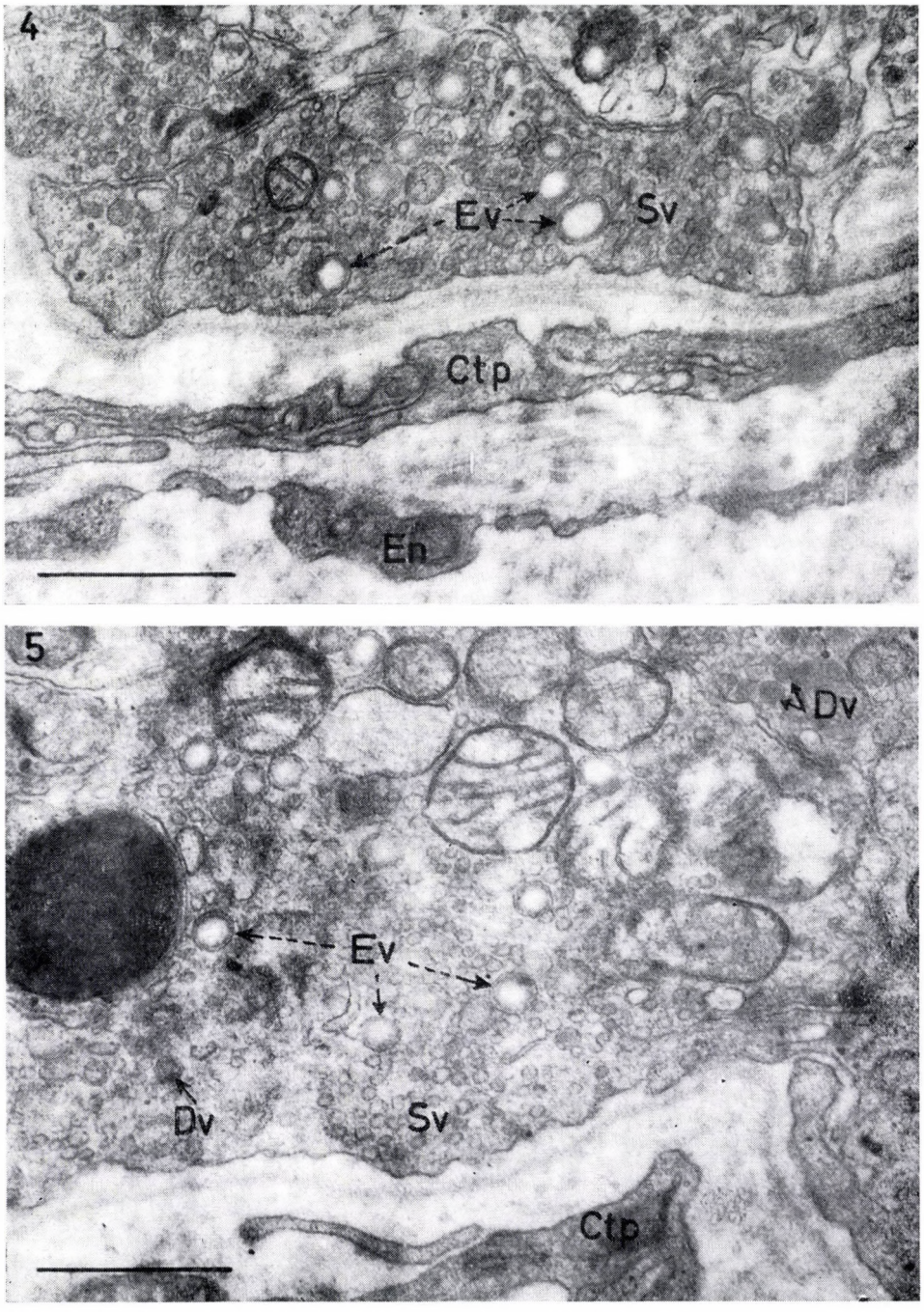


Fig. 3. Surface zone of median eminence 10 days after bilateral adrenalectomy. Note in this low-power general view the abundance of large vesicles with "emptied" centres (Ev). Surface area made irregular by "microvilli" of glial end feet is seen at lower left. Glial profiles (Gl) contain dense glycogen bodies. Scale 1 μ



Figs 4 and *5*. Surface zone of median eminence 10 days after bilateral adrenalectomy. Same structural details are seen in Fig. 3 with larger magnification. Besides synaptic vesicles (Sv) of ordinary size large "empty" vesicles (Ev) and some smaller dense vesicles (Dv) can be seen.

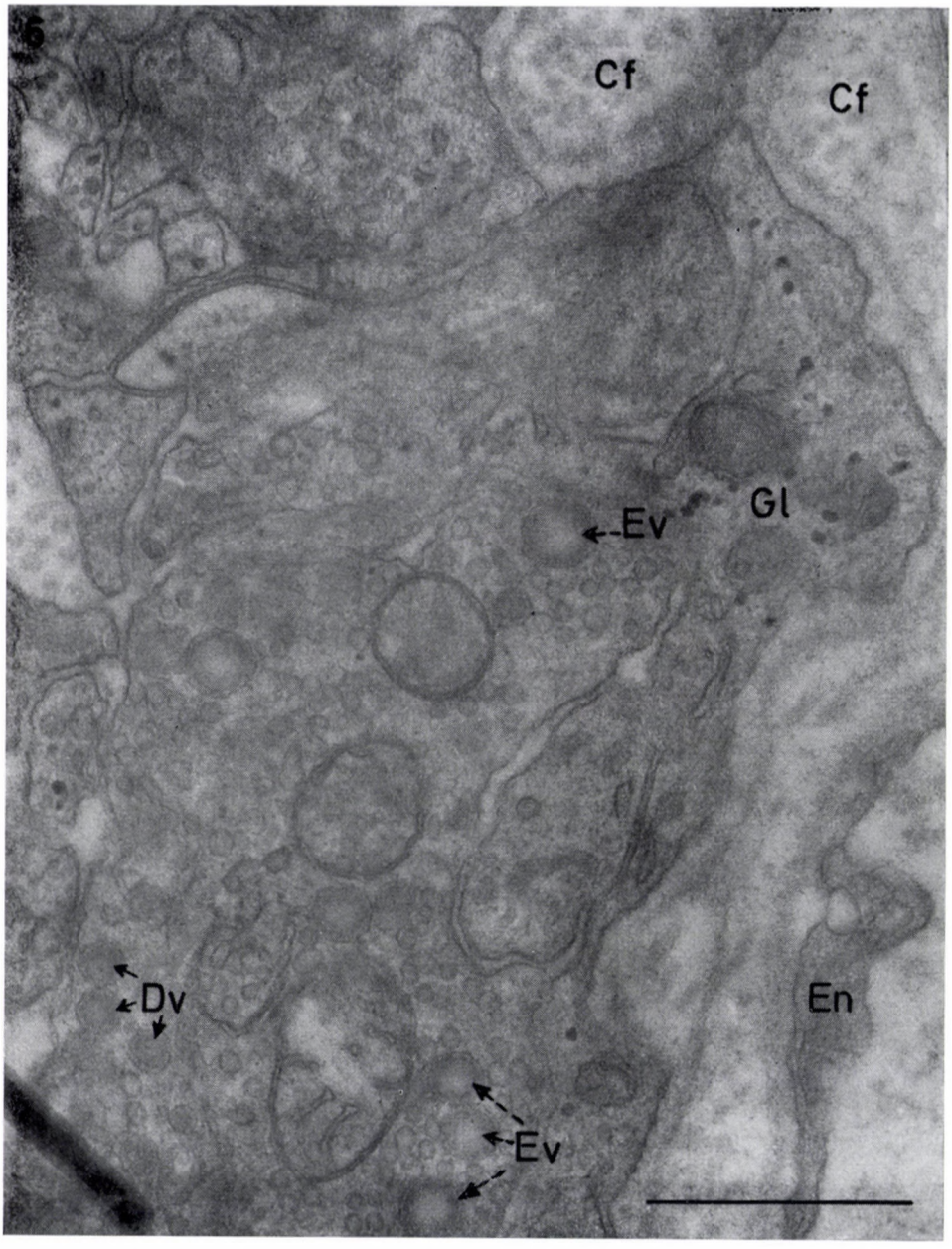


Fig. 6. High-power electron micrograph of nerve ending in surface zone of the median eminence. Same labels as in preceding figures. Note the collagen filaments (Cf) in the outer connective tissue space and their niches entering the zona palisadica. Scale 0.5 μ

In outer connective tissue space, processes of connective tissue cell (Ctp). En = fenestrated capillary endothelium. Large, probably lipid, droplet (at left in Fig. 5) may occur both normally and after adrenalectomy. Scale 1 μ

occasionally (Fig. 1), and true dense-core vesicles occur also in adrenalectomized material (Figs 6, 8), the difference being not qualitative only quantitative. This emerges more clearly from Fig. 7 giving the distribution by diameter for 300 large vesicles in the normal controls and 300 in the adrenalectomized animals. It is shown that the vesicles grew in diameter after adrenalectomy. (The ordinary synaptic vesicles were neglected in the comparison.) As the great majority of the vesicles up to 1,200 Å diameter have dense cores, whereas the larger ones appear to be empty, irrespective whether in normal or adrenal-

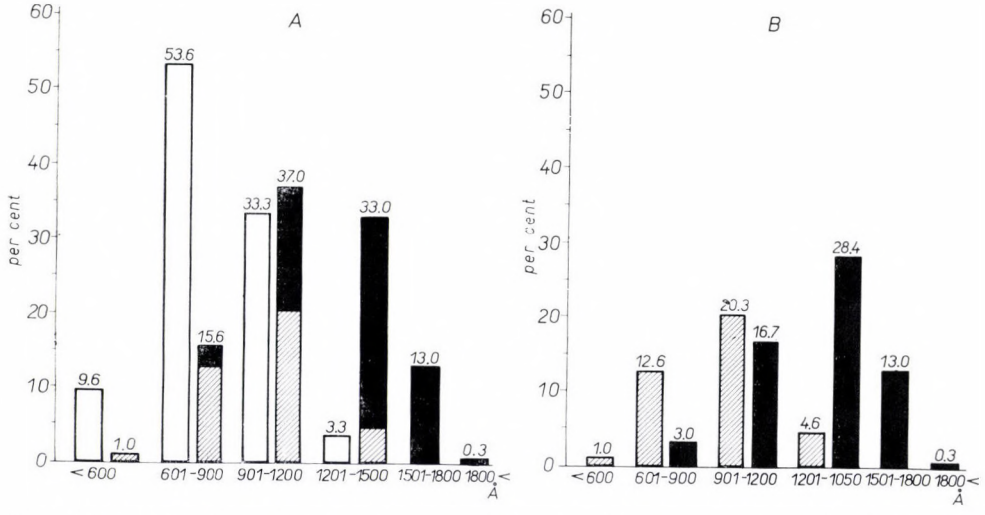


Fig. 7. Diameter histogram of various types of large vesicles in nerve terminals of the surface zone of median eminence. Comparison between dense vesicles (white bars) in normal controls and dense (hatched) + empty vesicles (black bars) in adrenalectomized animals. (Empty vesicles are so few in the normal terminals that they do not show up as separate fraction but are included in the dense group.) — B. Dense (hatched) and empty vesicles (black) after bilateral adrenalectomy distributed by size

ectomy material, the most obvious interpretation is to assume that the loss of the dense core is a consequence of the growth of the vesicles. The question whether their contents is removed, changed, or whether it is simply the distribution of the same amount of electron-dense material in much larger space that causes the vesicles appear "empty", remains open. Opening up of these large vesicles and discharge of their contents have not been so far observed.

The nerve terminals are separated from one another and from glial endfeet by relatively wide (300—400 Å) interspaces, which in some cases seem to extend into the nerve terminals themselves (Fig. 8) inasmuch as it can be established without plastic reconstruction of an uninterrupted series. Enlarged vesicles of the more conventional synaptic vesicle type open up towards these inter- or intraterminal spaces (Fig. 8). The interterminal spaces open up freely

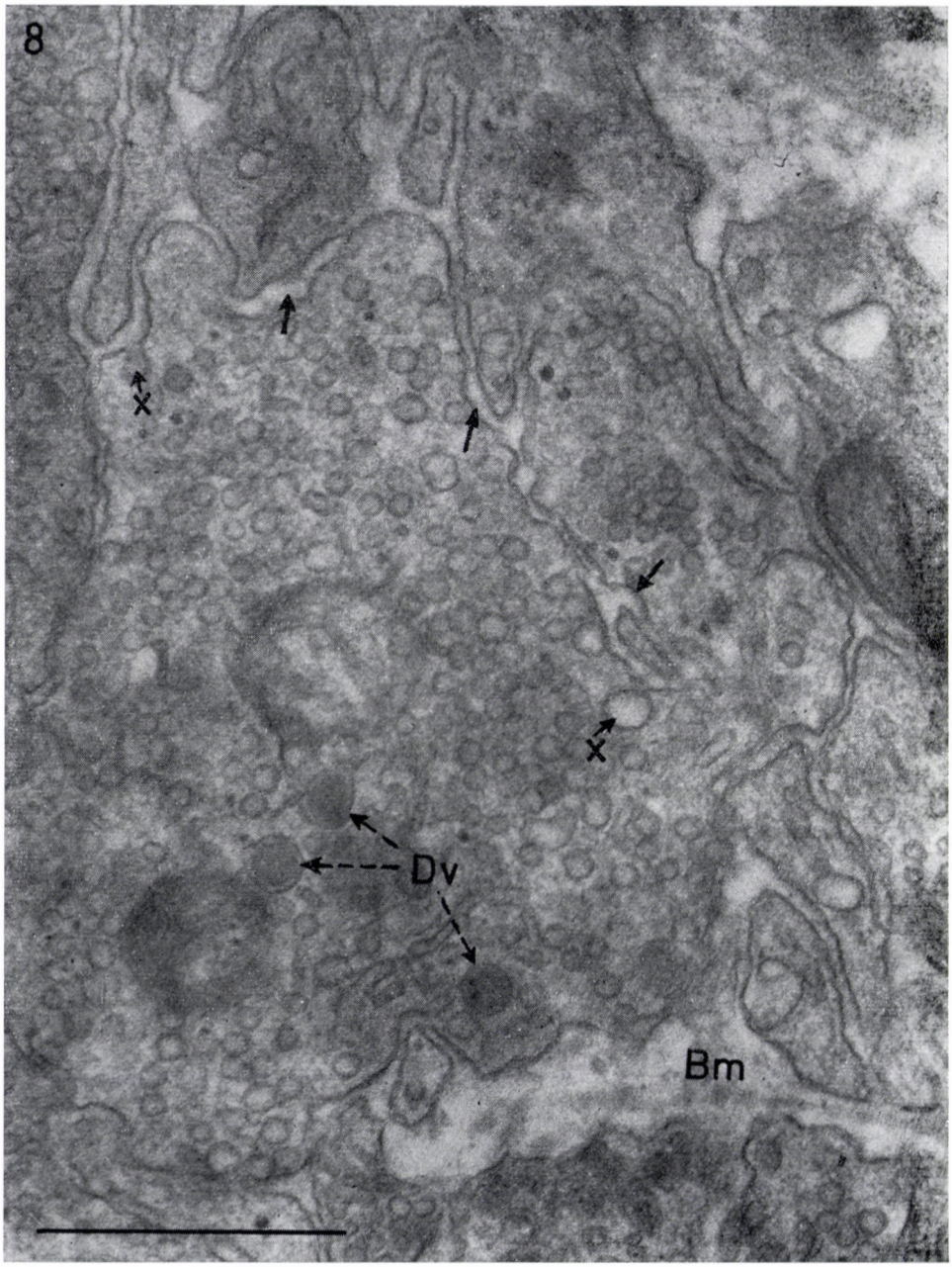


Fig. 8. Interterminal and partly perhaps intraterminal (arrows) spaces with enlarged synaptic vesicles of usual type opening up (x). Some dense vesicles (Dv) are also present in this region. — 10 days after bilateral adrenalectomy. Scale 0.5 μ

into the connective tissue space surrounding the primary capillary plexus of the portal vessels (Figs 6 and 8). The fenestrated endothelium of the capillaries is separated from the endings by a space of often less than 1 μ in diameter, although two unusually thin basement membranes are regularly present: one immediately neighbouring the nervous tissue, the other belonging to the capillary.

No significant changes of similar kind have been recorded in the thyroidectomized and the castrated controls. The changes after bilateral adrenalectomy are at their height between the 10th and 14th postoperative days and diminish considerably towards the 17th day.

Discussion

There can be little doubt left about the specific nature of the nerve terminals accumulated in the surface zone (zona palisadica) of the median eminence and the most proximal part of the pituitary stalk. The nerve endings can be traced back to thin fibres crossing the coarse-fibred supraoptico-hypophyseal tract and originating from small nerve cells situated mainly in the arcuate nucleus and some more rostral adjacent regions in the anterior periventricular nucleus of the hypothalamus. These nuclear regions, according to HALÁSZ and co-workers [6] and KNIGGE [7] contain the substances essential for the maintenance both of the normal structure and the function of anterior pituitary tissue. The magnocellular neurosecretory nuclei (nucl. supraopticus and paraventricularis) do not contain such substances. — The present electron microscopic study has revealed that the zona palisadica does not contain else than glial end-feet and a condensed sheet of nerve terminals without any postsynaptic structure. Accumulation of nerve endings seems to be less pronounced around those special capillary loops entering the median eminence before merging into the portal vessels than on the surface of the median eminence, i.e., at the zone adjacent to the pars tuberalis. As predicted from light-microscopic findings by SZENTÁGOTHAI and co-workers [16] and widened by electron microscopy the capillary loops penetrating the median eminence are less important for the neurovascular connection between the hypothalamus and the anterior pituitary than the primary capillary plexus (Mantelplexus) situated between the median eminence (proximal stalk) and the pars tuberalis. The zona palisadica ends abruptly at the lower margin of the pars tuberalis [14], i.e. at the site wherefrom the last capillaries can join the portal vessels. — All this is in complete accordance with all known facts about hypothalamo-adenohypophyseal neurovascular transmission and shows that the parvicellular (tuberoinfundibular) system is clearly separated from the classical magnocellular (supraoptico-, paraventriculo-hypophyseal) neurosecretory system of the posterior lobe.

Although only quantitative, the changes in the median eminence nerve endings after bilateral adrenalectomy are marked and unequivocal. It is obviously tempting to bring them into connection with the enhanced CRF release well known under these circumstances. So far very equivocal, if any, acute structural changes attributable to increased functional load have been observed in nerve terminals and synapses, even by electron microscopy. The functional load used in the present experimental series is less acute than it is usual in stimulation experiments but less chronic than in experiments of "neurotrophic" character, e.g., when a sensory (usually visual) system is completely deprived from external stimuli. In the latter cases marked changes can be recorded even at the level of light microscopy. — As a rather unsophisticated and superficial kind of explanation it would be conceivable that the large "dense" vesicles are in some immediate connexion with CRF storage, their growth in size with increased demand for, and their empty centres with the discharge of CRF. Such a direct and mechanistic explanation would, however, meet many difficulties.

(1) One of the main difficulties is that no comparable changes occur in any of the nerve endings after thyroidectomy or castration. Obviously, some of these nerve endings must discharge substances stimulating the thyrotrophic and gonadotrophic functions of the anterior pituitary tissue. We do not know whether there are specific neurons in the parvicellular system for CRF, TRF and LRF and even other similar substances. It is also unclear whether there are enough specific organelles in the nerve endings to be related with each of these substances. — Strangely, it is only adrenalectomy that causes obvious electron microscopic changes not only in the median eminence nerve terminals but also in cellular and synaptic structures of the ventromedial nucleus of the hypothalamus [15]. One would have, of course, to look very carefully for comparable changes in many different parts of the hypothalamus after thyroidectomy and castration and at various postoperative periods. For the time being, the suspicion that the changes following adrenalectomy are nonspecific — or at least not directly connected with CRF production and release — cannot be flatly rejected.

(2) Dense-core vesicles are usually brought into connexion with catecholamines. The view that these are contained by the dense vesicles of the median eminence terminals is favoured by (a) the known large content of the median hypothalamus, (b) the strong fluorescent effect after formaldehyde-gas condensation of the zona palisadica, (c) the pharmacological evidence that it is due largely to dopamine and noradrenaline [3], and (d) the high monoamine-oxydase activity of the same region [10]. The strong enhancement of the fluorescence effect in the surface zone of the median eminence after adrenalectomy as reported by Akmayev and Donáth [1], could be well in accord with the growth and structural changes experienced in the dense vesicles in this series. As according

to AKMAYEV and DONÁTH [1] administration of Cortisone causes the fluorescent strip at the surface of the median eminence to narrow down considerably without any diminution of the total fluorescence, we initiated similar experiments simultaneously with this series. However, no significant electron-microscopic changes were recorded in the nerve terminals. Such experiments with pharmacological effects at the electron-microscopic level have to be continued on broader scale before drawing any conclusion.

(3) Finally, the smaller vesicles resembling the usual type of synaptic vesicles should also be taken into consideration. As seen in Fig. 8, some larger vesicles of this kind appear to open up towards their inter- and perhaps even partly intraterminal tissue spaces. Although this phenomenon appears to be more frequent after bilateral adrenalectomy, it is by no means a proof of the discharge of the contents of the vesicles into the intercellular space. The micrographs might be interpreted otherwise, i.e., they might be signs of vesicles closing down from the surface and ingesting some material into the terminals from the outside. This idea might seem fantastic at this particular site, but completely similar pictures can be seen at a number of interneuronal synapses even with intrusion of microvilli (or microspines) of the postsynaptic element into the invagination of the presynaptic membrane. In this case the interpretation of surface invaginations closing down is more easy to accept than the reverse. — It cannot be decided whether the usual type of synaptic vesicles — in some median eminence endings the only vesicular structures — are in connection with some cholinergic mechanism, or whether they would be the morphological representative of one or the other of the releasing factors. A cholinergic (or an adrenergic) mechanism (or both) might supposedly be needed for the control of permeability which is obviously altered during the discharge of the releasing factors.

Direct identification of the releasing factors with any type of vesicles would be difficult also with respect to the observations of HALÁSZ and coworkers [6] suggesting that at least some of the releasing factors — or, at any rate, substances essential for structural maintenance of the anterior pituitary — are present in active form even at the site of the parvicellular neuron system. Although descent of neurosecretory elementary bodies through the fibres of the supraoptico-hypophyseal tract can be well observed both with light- and electron microscope, this is not clear for the parvicellular system. As catecholamines are present also in the cells of this system [4], the descent of dense vesicles along its axons towards the median eminence endings could by no means be interpreted as a histological proof for the transport of releasing factor substances by the axonal current. — Obviously, many further investigations are necessary before the results presented could be interpreted with fair security.

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VERÄNDERUNGEN AN DEN NERVENENDIGUNGEN DER EMINENTIA MEDIALIS DES HYPOTHALAMUS (ZONA PALISADICA) NACH NEBENNIERENENTFERNUNG

Bei Ratten sind an den Nervenendigungen der Eminentia medialis und des Hypophysenstiels unter normalen Verhältnissen dunkle, osmiophile Körnchen zu sehen. In der zweiten Woche nach Nebennierenentfernung vergrößern und vermehren sich diese Körnchen. In einem Teil der Körnchen ist der dunkle Kern »aufgelöst«. Parallel diesen Veränderungen öffnen (oder schließen) sich die vergrößerten synaptischen Bläschen in Richtung des ziemlich breiten interterminalen, evtl. zum Teil intraterminalen Spaltensystems. Die Veränderungen sind am Ende der zweiten Woche am ausgeprägtesten und bilden sich im Laufe der dritten Woche zurück. Der vermutliche Zusammenhang zwischen den beschriebenen Veränderungen und der erhöhten Ausschüttung des »Corticotrophin Releasing Factor«s (CRF) wird erörtert.

ИЗМЕНЕНИЯ В НЕРВНЫХ ОКОНЧАНИЯХ СРЕДНЕГО ВОЗВЫШЕНИЯ ГИПОТАЛАМУСА (ПАЛИСАДНАЯ ЗОНА) ПОСЛЕ УДАЛЕНИЯ НАДПОЧЕЧНИКОВ

У крыс в нервных окончаниях среднего возвышения гипоталамуса и ножки гипофиза в норме встречаются темные, осмиофильные зернышки. На второй неделе после двустороннего удаления надпочечников, наблюдается увеличение размеров и числа этих зернышек и в части зернышек «растворение» темного ядра. Параллельно с этим увеличенные синаптические пузырьки раскрываются (или наоборот) в направление довольно широкой межтерминальной, возможно и внутритерминальной щелевой системы. Эти изменения достигают своего максимума к концу второй недели; в течение третей недели они уменьшаются. Обсуждается предположительная связь между указанными изменениями и повышенным выделением так наз. Corticotrophine Releasing Factor (CRF).

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NUCLEIC ACID CONTENT OF THE NICTITATING MEMBRANE AFTER CERVICAL SYMPATHECTOMY

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Synopsis

The nucleic acid content of the denervated smooth muscles of nictitating membranes was investigated in 16 mature cats of both sexes. The nucleic acid content of the denervated membrana nictitans showed no alteration during the 30 days' investigation period in comparison with the contralateral innervated controls. Experimental results show that sympathetic innervation is of no significance in the regulation of the nucleic acid metabolism of the examined type of muscle.

Introduction

Relatively very few data are available pertaining to the neural regulation of the nucleic acid metabolism of the different types of muscle tissues. The majority of relevant studies are limited to the analysis of somatic nervous-system effects [1, 2, 5, 7, 8, 10]. Still scantier is our knowledge regarding the role of vegetative innervation played in keeping the nucleic acid content of smooth muscles at a physiological level. The problem in question has been rendered difficult by the fact that neurons of diffuse dispersion are found among the myons, so that the denervation of the tissue cannot be carried out in the usual manner. However, neuron-free smooth muscles are known to exist, too, as for instance the smooth muscles of the membrana nictitans of cat, which are sympathetically innervated. Taking advantage of this anatomical circumstance, we have made a study of the changes in the nucleic acid content of sympathectomized nictitating membrane in our present investigations for the purpose of obtaining data referring to the effect of sympathetic innervation on the nucleic acid metabolism of smooth muscles.

Material and method

The right nictitating membrane of domestic cats (*Felis domestica*) of both sexes (weighing 2.40—2.90 kg) was denervated by extirpating the superior cervical ganglion of the identical side. The operation was performed under intratracheal ether anaesthesia in aseptic circumstances. The left innervated nictitating membranes were used as controls. The animals of one group were overnarcotized with ether 5 days, those of the second group 10 days, those of the

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third group 30 days later. After the eyes had been cut round with a scalpel and lifted out of their sockets, the nictitating membrane was detached. The weight of the latter was quickly weighed and, after freezing with dry ice, it was used for determining nucleic acid content. The nucleic acids were determined on the basis of their base components according to TSANEV and MARKOV [11]. Na-DNA (Light) and Na-RNA (Fluka) were used as standards. The UV adsorption measurements were carried out with a Beckman DU-type spectrophotometer.

Results

On the basis of the data shown in Table 1, it may be established that the weight of the denervated membranes does not undergo any change in consequence of surgical intervention.

Nor can any significant change be observed in the nucleic acid content of denervated membranes. The 8.24% decrease in the total deoxyribonucleic acid (DNA) content, measured 30 days after denervation, as well as the 9.51% decrease in the amount calculated for 100 mg wet weight are of no significance ($p > 0.70$, Table 2). The ribonucleic acid (RNA) content of the sympathectomized membranes remains also unchanged (Table 3).

Discussion

The negative results of the investigations carried out on sympathectomized smooth muscles indicate that the sympathetic neural effects do not influence their nucleic acid metabolism. We reported similar observations on sympathectomized myocardia of mammalian hearts [4, 6]. Neither has lumbar

Table 1

Weights of innervated and denervated nictitating membranes (mg)

Animals No.		Postoperative days					
		5.		10.		30.	
		I.	D.	I.	D.	I.	D.
1, 6, 11		267	261	282	288	255	271
2, 7, 12		239	236	237	253	253	246
3, 8, 13		264	266	242	228	293	299
4, 9, 14		260	248	291	286	267	266
5, 10, 15		251	262	288	280	227	236
16		—	—	—	—	251	245
	Mean:	256	254	268	267	257	260

I. = innervated nictitating membrane
D. = denervated nictitating membrane

Table 2

DNA content and concentration of the innervated and denervated membranes

Postop. day	Animals No.		DNA content (μg)		Percentage I.—D. diff.	DNA concentration (μg/100 mg wet tissue)		Percentage I.—D. diff.
			I.	D.		I.	D.	
	1		58.7	52.7		21.9	20.1	
	2		49.4	47.7		20.6	20.2	
5	3		58.8	59.3		22.2	22.2	
	4		46.6	50.5		17.9	20.3	
	5		47.9	46.0		19.0	17.5	
		Mean ± S. D.	52.2±2.6	51.4±2.3	−1.54	20.3	20.0	−1.48
	6		64.2	60.5		22.7	21.0	
	7		45.8	55.0		19.3	21.7	
10	8		51.6	59.4		21.3	26.0	
	9		68.2	70.4		23.4	24.6	
	10		67.7	73.6		23.5	26.2	
		Mean ± S. D.	59.5±4.5	63.7±3.5	+7.05	22.0	23.9	+8.63
	11		49.3	53.8		19.3	19.8	
	12		55.5	49.6		21.9	20.1	
	13		62.9	58.0		21.4	19.3	
30	14		64.8	55.6		24.2	20.9	
	15		49.7	44.3		21.8	18.7	
	16		60.8	53.3		24.2	21.7	
		Mean ± S. D.	57.1±2.7	52.4±3.9	−8.24	22.1	20.0	−9.51

sympathectomy any effect on the nucleic acid content of mammalian skeletal muscles [3].

Though the above-mentioned conclusions regarding mammalian muscles cannot be generalized for all vertebrate muscle types on account of the lack of comparative studies, we are obliged to assume on the basis of the experimental data available so far that the sympathetic neural effects are of no significance in the regulation of nucleic acid metabolism of various types of muscle tissues.

Table 3

RNA content and concentration of the innervated and denervated membranes

Postop. day	Animals No.		RNA content (μg)		Percentage I.—D. diff.	RNA concentration		Percentage I.—D. diff.
			I.	D.		I.	D.	
	1		139.6	123.0		52.3	47.1	
	2		118.4	108.0		49.5	45.7	
5	3		140.1	140.2		53.0	52.7	
	4		142.2	135.5		54.6	54.6	
	5		127.4	129.3		50.7	49.3	
		Mean ± S. D.	133.5±4.4	127.2±5.6	−4.72	52.0	49.8	−4.24
	6		146.6	124.8		51.9	43.3	
	7		124.3	132.8		52.4	52.4	
10	8		119.3	122.6		49.2	53.7	
	9		151.5	133.1		52.0	46.5	
	10		144.9	140.4		50.3	50.1	
		Mean ± S. D.	137.3±6.4	130.7±3.2	−4.81	51.1	49.2	−3.72
	11		142.2	159.4		55.7	58.8	
	12		141.8	137.0		56.0	55.6	
	13		154.7	138.2		52.7	46.2	
30	14		159.9	149.9		59.8	56.3	
	15		121.9	128.1		53.7	54.2	
	16		129.8	119.4		51.7	48.7	
		Mean ± S. D.	141.7±5.8	138.6±5.9	−2.19	54.9	53.3	−2.92

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DER NUKLEINSÄUREGEHALT DER MEMBRANA NICTITANS NACH ZERVIKALER SYMPATHEKTOMIE

An 16 geschlechtsreifen Katzen beiderlei Geschlechts wurde der Nukleinsäuregehalt der denervierten glatten Muskulatur der Membrana nictitans untersucht. Während der 30-tägigen Untersuchungszeit zeigte der Nukleinsäuregehalt der denervierten Membrana nictitans, gegenüber den innervierten kontralateralen Kontrollen, keine Veränderung. Die Versuchsergebnisse weisen darauf hin, daß der sympathischen Innervation in der Steuerung des Nukleinsäurestoffwechsels keine Bedeutung zukommt.

СОДЕРЖАНИЕ НУКЛЕИНОВОЙ КИСЛОТЫ В МИГАТЕЛЬНОЙ ПЕРЕПОНКЕ ПОСЛЕ УДАЛЕНИЯ ШЕЙНОГО УЧАСТКА СИМПАТИЧЕСКОГО НЕРВА

Авторы исследовали на 16 половозрелых кошках обоих полов содержание нуклеиновых кислот в денервированных гладких мышцах мигательной перепонки. В течение 30-дневного периода исследования содержание нуклеиновой кислоты в денервированных мигательных перепонках, по сравнению с контралатеральными иннервированными контрольнами, не показало изменений. Результаты экспериментов указывают на то, что симпатическая иннервация не имеет значения в регуляции обмена нуклеиновой кислоты в исследовавшемся типе мышц.

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STUDIES ON THE ORGANON VASCULOSUM LAMINAE TERMINALIS

I. COMPARATIVE EXAMINATION IN TELEOST FISHES (A MORPHOLOGICAL STUDY)

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Synopsis

The organon vasculosum laminae terminalis (OVLT) of teleost fishes was studied by comparative morphological methods. OVLT is present as a well-developed circumventricular organ in all the 13 examined species and exhibits three characteristic zones: a vascularized outer layer containing elements of connective tissue, an inner layer covered by specially differentiated ependyma, and an intermediate layer of parenchyma. Blood is supplied to the OVLT from the outer surface. Parenchyma cells appear to be predominant: they are provided with well-developed processes, which connect them with the hypothalamo-retinal, and also with the preoptico-hypophyseal, tract. Being well-developed in teleost fishes, the OVLT should be regarded as a phylogenetically ancient organ. Its function may consist in overcoming the blood-liquor barrier by anastomoses or shunts.

Introduction

The wall and hypendyma of the third cerebral ventricle contain severa organs such as the subcommissural organ, the subfornical organ, the paraventricular organ and the recessus organ [2, 17, 28, 29, 34, 35]. Being similar to these organs the organon vasculosum laminae terminalis (OVLT) also belongs to this group [7, 8, 9, 17, 25]. The lamina terminalis is a thin membrane which is localized in the anterior wall of the third ventricle [1]. The term OVLT means according to HOFER [17] and MERGNER [25] the microscopically well distinguishable, strongly vascularized part of the lamina terminalis which, structurally similar to the other circumventricular organs, extends from the anterior commissure to the optic chiasma. It had been nominated crista supraoptica by WISLOCKY and KING [38].

PUTMAN [30] was the first to describe the region in question, and it was then discussed by BEHNSEN [4], who observed a striking avidity for trypan blue in this region of the mouse brain. Similar observations were made by other authors in rabbits, cats and monkeys. WISLOCKY [38, 39] found very similar staining properties in the subfornical organ, in the posterior pituitary, in the area postrema and in the OVLT. KUHLENBECK was the first to describe the OVLT in the human brain. MERGNER [26] and HOFER [17] considered it to be an organ on account of its similarity to the organs that had been called "paraependymal" or "juxtaependymal" areas.

DUVERNOY and KORITKÉ [9, 10, 11, 12], studying the structure of the OVLT, found that it was provided with a characteristic capillary network. The subdural vessels, running towards the interior, divide into delicate branches in the middle zone. In certain species a so-called subependymal network has been observed. This is strikingly similar to that of the median eminence; a similarity explained by their common embryological origin [7, 16]. The preoptic artery is the principal artery of the OVLT, while its venous blood is drained by the preoptic vein. KAWAKATSU [20] and HOLZMANN [18] observed numerous nerve fibres with fibrous neuroglial cells in the middle zone of the OVLT of mammals. They encountered furthermore characteristic parenchymatic cells. Similar observations were made by other authors [15, 19].

FLEISCHHAUER [14] provides information about the turtle (*Testudo graeca*), MURAKAMI and BAHN [27] about the gecko (*Gecko japonicus*). WEINDL [36] reports on the results of histochemical studies without expatiating upon the chemical properties of the OVLT. As an accidental finding, TAGUCHI and co-workers [32] observed intensive incorporation of radioactive cystein in the OVLT of the sparrow.

The function of the organ is obscure. Certain authors suggest a correlation with the hypothalamic-retinal fibres [7, 31], others suppose a communication between its fibres and the neurosecretory pathways [18].

A few data are regarding the fishes. BRAAK [5], describing the OVLT of *Chimaera monstrosa*, refers to the specially differentiated ependyma and the abundance of neural and neuroglial fibres in the middle zone. He observed Gomori-positive reaction in the ependymal layer without interpreting its significance. It was likewise a cartilaginous fish (*Scyliorchinus caniculus*) in which MELLINGER [24] studied the organ in question. Compared to the surrounding neural and hypendymal structures, it revealed a fairly high degree of differentiation. This author describes a preoptico-portal-hypophyseal system that would connect the OVLT with the portal vessels of the pituitary gland.

In our best knowledge no data have been published of teleost fishes; since the other circumventricular organs are characteristically differentiated in the members of this subclass, it seemed worth while to subject the structure of the OVLT of teleost fishes to a morphological study.

Material and method

The following species are used in the present study.

Cyprinidae:	*Cyprinus carpio* (True carp)
	Leuciscus rutilus (European roach)
Percidae:	*Perca fluviatilis* (European perch)
	Acerina cernua L. (Ruff)
	Alburnus alburnus L. (Bleak)
	Lucioperca lucioperca (Pike perch)

Centrarchidae: *Eupomotis aureus* (Sunfish)
Salmonidae: *Salmo irideus* (Rainbow trout)
Anabantidae: *Colisa lalia* (Ham.) (Dwarf guramie)
Poecilidae: *Platypocylus macalatus*
Xyphophorus helleri
Lebistes reticulatus (Guppy)
Mollienisia spenops × *Mollienisia formosa* (Black molly).

The fishes derived partly from our own stock, partly from the Zoologico-Botanical Garden of Budapest and the State Fishery of Szilvásvárad. After being embedded in paraffin, the material was serially sectioned in the frontal, horizontal and sagittal planes. Five to six individuals per species were used. The sections, 7 μ in thickness, were stained with haematoxylin-eosin, Gomori's chrome haematoxylin phloxine, aldehyde-fuchsin, Heidenhain's iron haematoxylin, and complex metal-ion haematoxylin. Staining with the last named dye was performed *ad modum* TÖRÖK [33] in the following manner: deparaffinize and hydrate through 96% alcohol; rinse in distilled water; treatment with 5% ammonium molybdate for 10 min.; running tap water for 2 min; rinse in distilled water; treatment with 5% iron alum for 10 min.; running tap water for 30 min; rinse in distilled water; staining with Heidenhain's haematoxylin for 15 min. (if necessary, under microscopic control); differentiation in 1% hydrochloric alcohol or in 5% iron alum; running tap water for 2 min; rinse in distilled water; dehydration; mounting.

Results

The OVLT was found as a distinctly separate circumventricular organ in all examined species.

Characteristics observed in general

The OVLT extends from the anterior commissure to the optic chiasma and closes the anterior wall of the third ventricle (Fig. 1). It has three zones morphologically well distinguishable. Blood vessels and the meninges with the

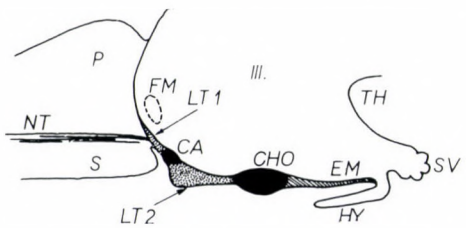


Fig. 1. Schematic illustration showing location of the lamina terminalis in teleost fishes. LT1 = dorsal aspect of lamina terminalis separating the nuclei (nerve cells) from the diencephalon. LT2 = organon vasculosum laminae terminalis, extending from the anterior commissure (CA) to the optic chiasma (CHO). P = putamen. S = striatum. NT = terminal nerve. EM = eminentia medialis. HY = hypophysis. SV = saccus vasculosus. TH = thalamus. III = 3rd ventricle. FM = Monroi's foramen

joined fibrous tissue constitute the main features of the outest zone. The inner zone consists of differentiated ependymal cells poor of chromatin, provided with long processes, while a great number of nerve fibres, neuroglial cells and parenchymatic cells characterize the middle zone of this organ. The fibres

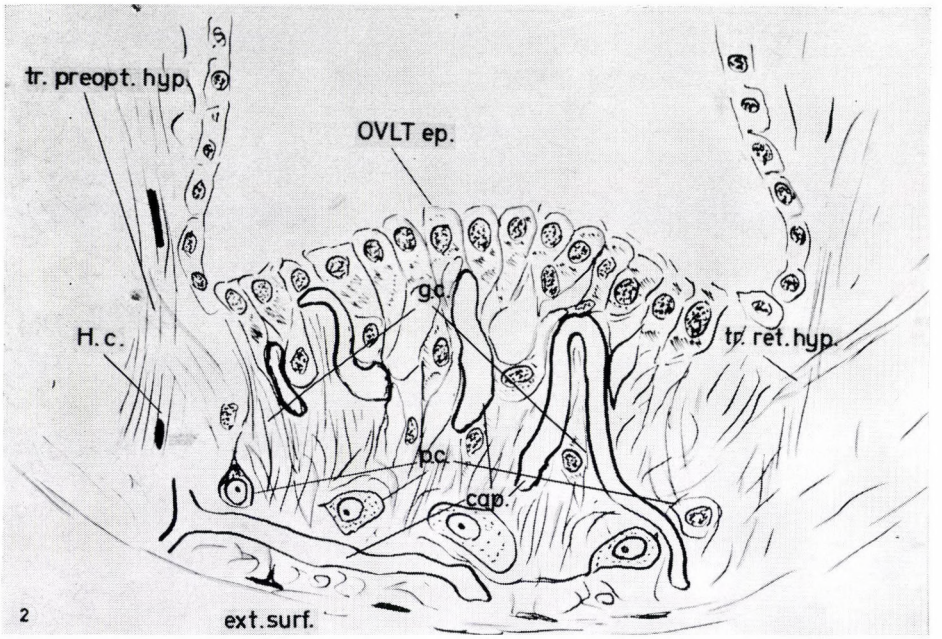


Fig. 2. Schematic illustration of the organon vasculosum laminae terminalis (OVLT) in teleost fishes. — tr. preopt. hyp. = preoptico-hypophyseal tract. — H. c. = Herring body. — OVLT ep. = special ependymal cells of the organ. — g. c. = glial cells. — p. c. = parenchymal cells. — cap. = capillaries. — tr. ret. hyp. = retinohypothalamic tract. — ext. surf. = external surface of brain

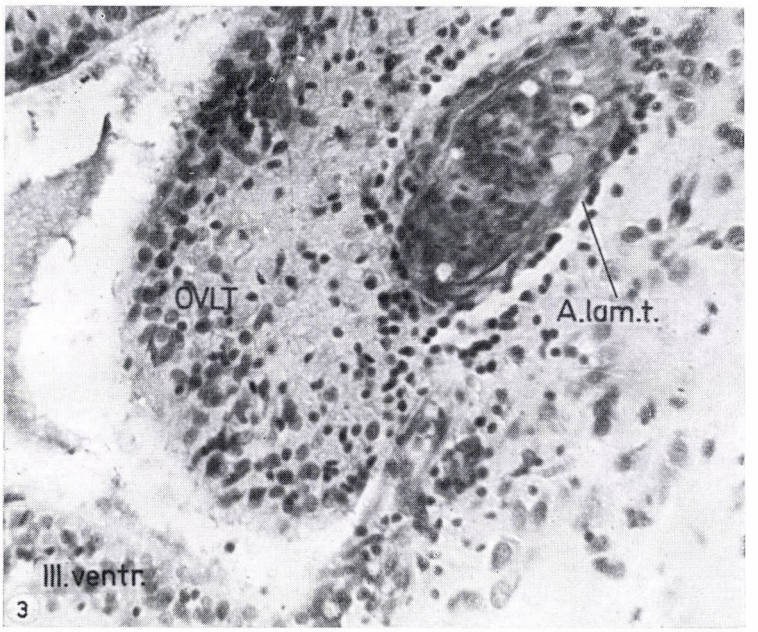


Fig. 3. OVLT in the carp (*Cyprinus carpio*). A. lam. t. = arteria laminae terminalis; III. ventr. = 3rd ventricle. Gomori's stain, ×80

are those of the hypothalamic-retinal tract; the parenchymal cells are smaller than the nerve cells of this region, they have nuclei of medium size and well-developed nucleoli. The parenchymal cells are strikingly similar in all the examined species.

The organ is adjoined by the fibres of the preoptico-hypophyseal tract in which numerous Herring's bodies can be seen.

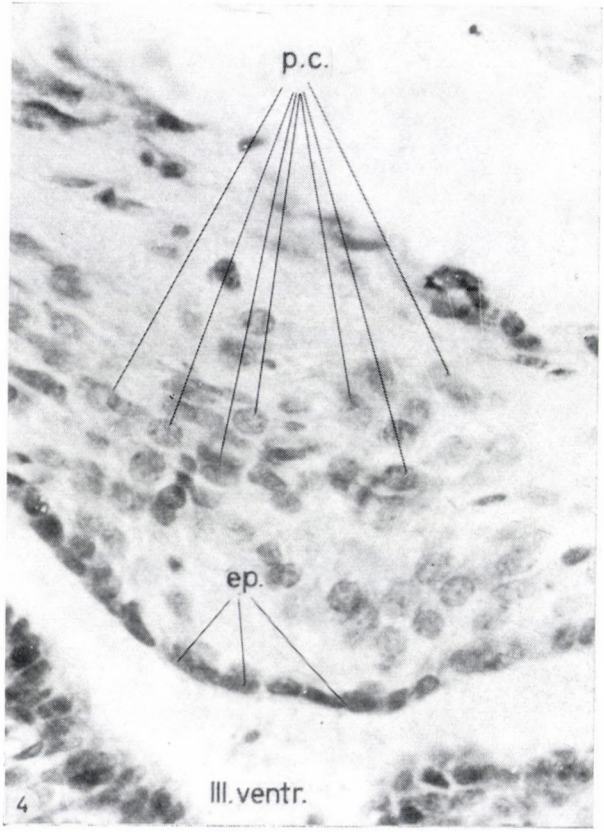


Fig. 4. OVLT in the perch (*Perca fluviatilis*). p. c. = parenchymal cells; ep. = ependyma; III. ventr. = 3rd ventricle. Haematoxylin-eosin, ×160

All vessels penetrate the organ from the brain surface. Part of them reach the ependymal level forming there a subependymal plexus which runs parallel to the ependyma. Other vessels, turning back above the ependyma, supply the middle zone. Capillaries of the outer layer invade this zone in the form of direct branches (Fig. 2).

Characteristics observed in some of the examined species

In *Cyprinus carpio* the OVLT projects into the third ventricle (Fig. 3). The parenchyma is longitudinally traversed by an artery that has been termed by us "arteria laminae terminalis". It originates from the preoptic artery in the area of the optic chiasma, penetrates the lamina terminalis at the height of the anterior commissure and breaks through the lamina closely to its inside

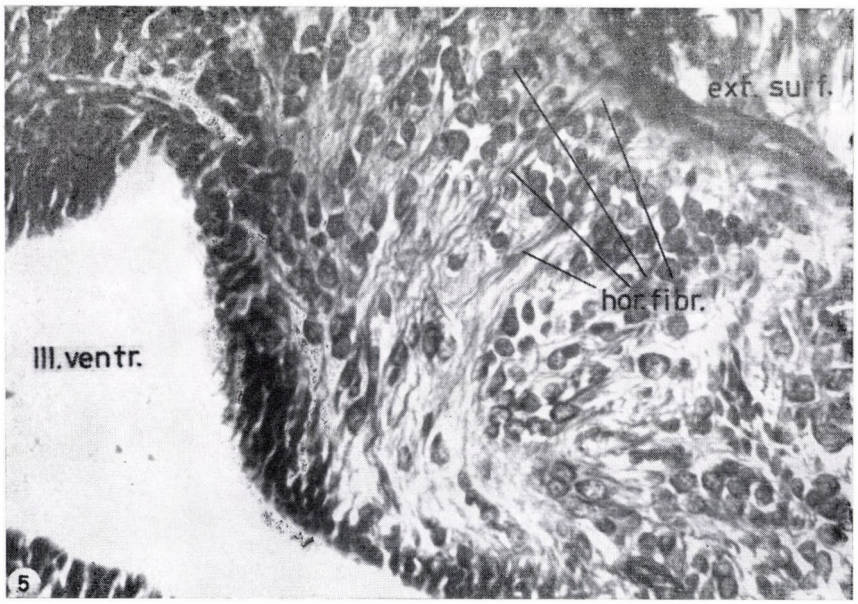


Fig. 5. OVLT in the rainbow trout (*Salmo irideus*). hor. fibr. = fibres running between outer and inner surfaces; ext. surf. = external surface; III. ventr. = 3rd ventricle. TÖRÖK's staining technique, ×100

wall. It emits no collaterals. The situs was the same in *Lebistes reticulatus* and in *Xyphophorus helleri*, too.

Perca fluviatilis. The predominance of parenchymatic cells characterizes the organ (Fig. 4). No neural cells were observed in the OVLT of the Percidae.

Salmo irideus. The OVLT contains horizontal fibres connecting the outer and the inner layers. Subependymal capillaries, or capillary network were observed in some cases (Figs 5, 6, 7). The fibres of the ependymal and parenchymal cells adjoin partly those of the hypothalamic-retinal, and partly those of the preoptico-hypophyseal tract. The number of parenchymatic cells is fairly large and their form is characteristic (Fig. 8).

In *Leuciscus rutilus* an intraependymal plexus in the OVLT is a characteristic feature. The ependymal cells are also well- developed (Fig. 9).

In *Colisa lalia* the connection of the OVLT with the preoptico-hypophyseal tract was well observable. It was found that in some cases also the parenchyma of the organ contained Gomori-positive Herring bodies (Fig. 10), which in all individual derived from the preoptico-hypophyseal tract. In general, the OVLT and the neurosecretory fibres were situated side by side. No Gomori-positive secretion was observed in the OVLT.

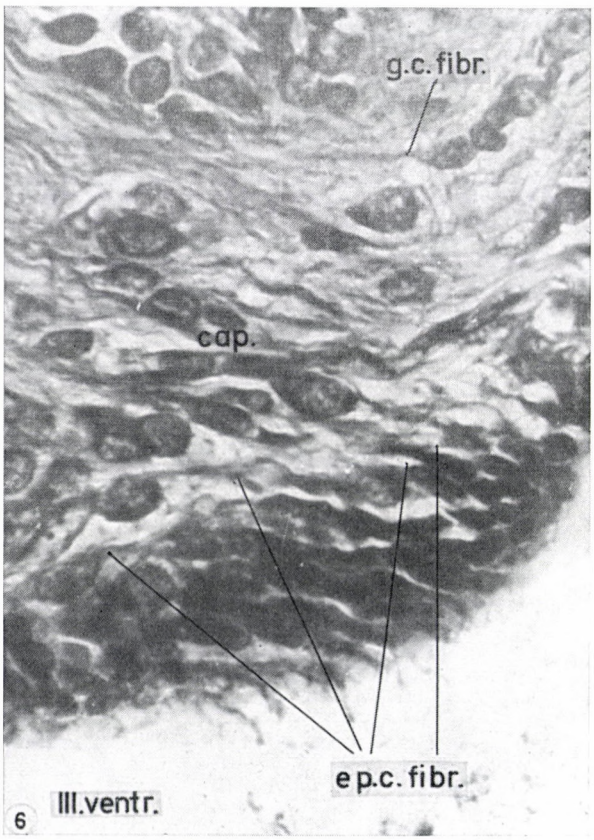
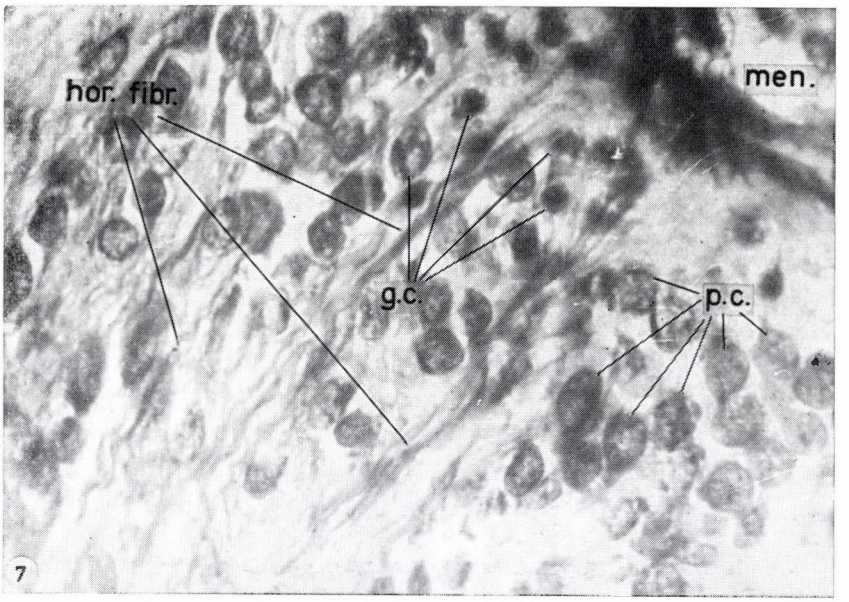


Fig. 6. Periependymal detail from the OVLT in the rainbow trout (*Salmo irideus*). g. c. fibr. = glial cells with process; ep.c. fibr. = ependymal cells with processes; cap. = capillary; III. ventr. = 3rd ventricle. TÖRÖK's staining technique, ×250

The cells of the OVLT of *Perca fluviatilis* and *Salmo irideus* were subjected to karyometric studies. Having measured 300 nuclei by means of a special instrument (made in own institute) at a magnification of × 2.500, we computed the logarithm of their volume from the diameter with the aid of FISCHER's and INKE's formula [13]. Results are illustrated in Figs 11a and 11b. There were two kinds of cells, of which the parenchymatic cells were predominant. A



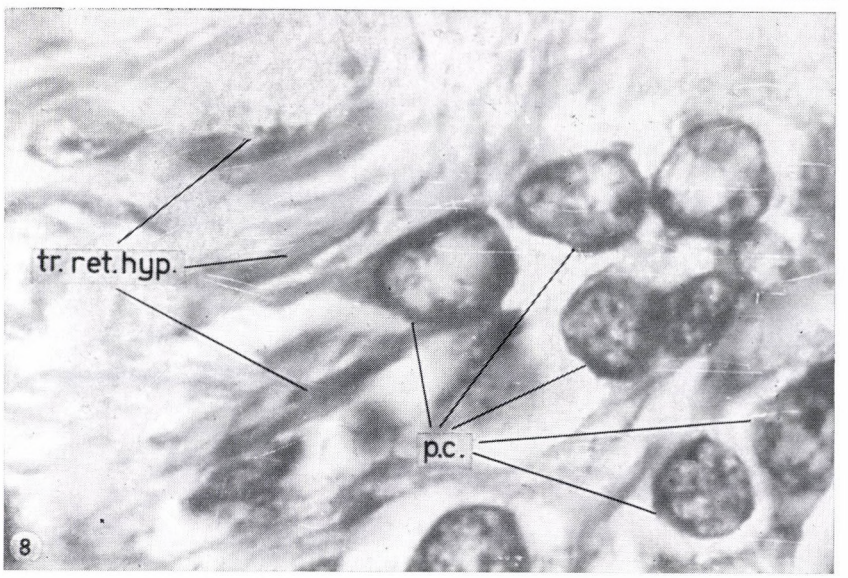


Fig. 7. External aspect of the OVLT in the rainbow trout (*Salmo irideus*). hor. fibr. = horizontal fibres; g. c. = glial cells; p. c. = parenchymal cells; men. = meninx. TÖRÖK's staining technique, ×250

Fig. 8. Parenchymatic cells of the OVLT, high-power magnification. tr. ret. hyp. = fibres of the retinohypothalamic tract. TÖRÖK's staining technique, ×1,250

smaller group represented neuroglial cells. There were, besides, a few nerve cells in the organ; these surpassed of the other cells in size, they were of no significance.

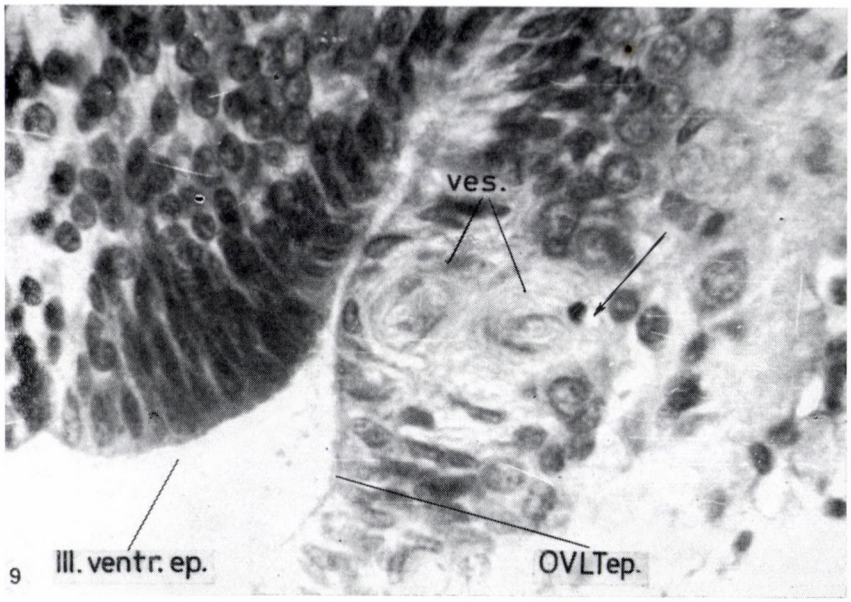


Fig. 9. Detail from the OVLT of the roach (*Leuciscus rutilus*). OVLT ep. = ependyma; III. ventr. ep. = normal mural ependyma of the 3rd ventricle. Arrow points to a dividing cell, Gomori's stain, ×250

Discussion

OVLT was present in all examined bony fishes; since it exists in cartilaginous fishes as well [5, 24], the organ presumably represents a phylogenetically ancient structure.

The crest-like projection found in the carp corresponds to the supraoptic crest observed in the higher orders by WISLOCKY and KING [38]. The arteria laminae terminalis encountered in this species emits no collaterals, and is of no significance. It presumably supplies the dorsal portion of the lamina terminalis (Fig. 1).

MELLINGER [24] describes a preoptico-hypophyseal system of vessels for the *Scyliorhinus caniculus*, which would establish communication between the OVLT and the portal vessels. We failed to trace it in the examined teleost fishes, so that it may be a special feature of Chondrostei.

Otherwise the vascular pattern of the OVLT of teleosts is strikingly similar to that of higher vertebrates as described by DUVERNOY and KORITKÉ [8, 12], the course of the vessels and the presence of ansae being highly characteristic of the OVLT both in vertebrates and teleosts. Rich capillary network separates the OVLT from its surroundings in all species.

BRAAK [5] described Gomori-positive granules in the ependyma of the OVLT of *Chimera monstrosa*. Unlike this in the course of the present study we

found no Gomori positivity in the OVLT except in the Herring bodies which, however, derived in all cases from the adjacent preoptico-hypophyseal tract.

The fibres of the cells of the OVLT may adjoin the fibres of the hypothalamic-retinal tract running in the same area [21, 22, 31]. In some cases it was the fibres of the neuroglial cells which adjoined the fibres of the tract in ques-

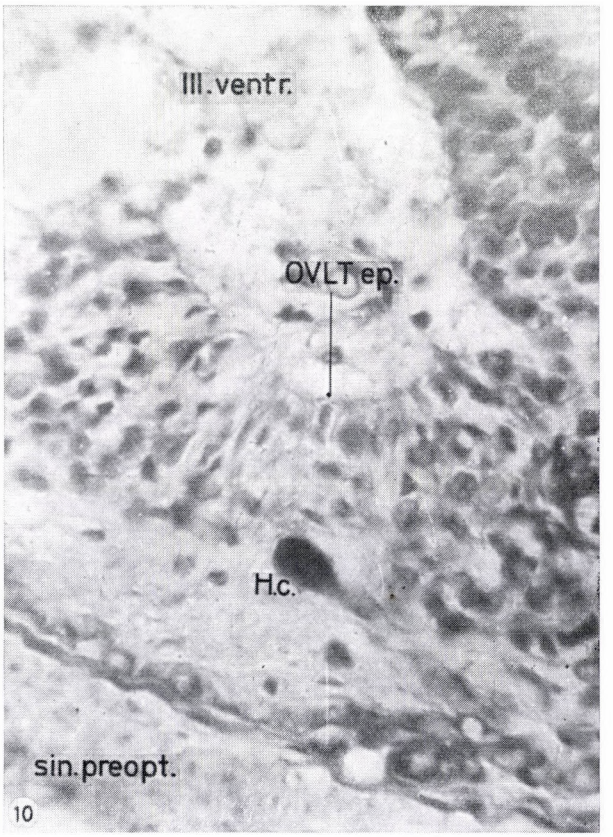
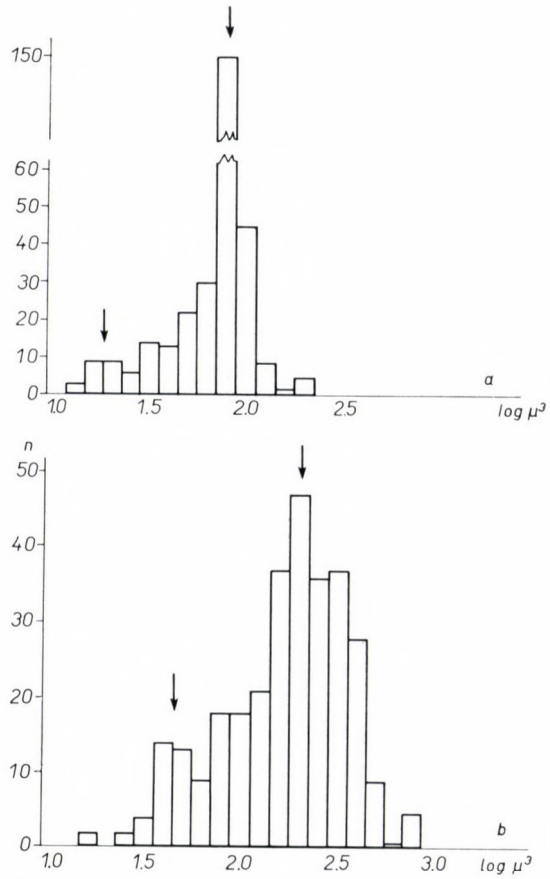


Fig. 10. OVLT of dwarf guaramie (*Colisa lalia*). OVLT ep. = ependyma; H.c. = Gomori-positive Herring body; sin. preopt. = sinus preopticus; III. ventr. = 3rd ventricle. Gomori's stain, ×160

tion. Further investigations are needed to elucidate the nature of these connections. It is anyway sure that the processes of the parenchymatic cells and the neuroglial cells mostly disappear among the fibres of the hypothalamic-retinal tract, although some cellular processes have been observed to reach, and disappear among, the fibres of the preoptico-hypophyseal tract.

The parenchymatic cells are dominant among the cells of the OVLT. The illustrations clearly show that the bulk of the OVLT consists of parenchymatic

cells. These resemble the cells described by DELLMAN [6] and KAWAKATSU [20], and presumably derive from undifferentiated cells of the ependyma, which are always present as described in one of our earlier studies [37]. The neuroglial cells correspond to those observed by FLEISCHHAUER, HOFER and MERGNER [15, 17, 26], the main difference being a quantitative one, the number of neuroglial cells is comparatively small in the OVLT of teleosts; nerve cells have



Figs 11 a—b. Diagram showing distribution of cells in the OVLT. *a* = Perch (*Perca fluviatilis*), *b* = Rainbow trout (*Salmo irideus*). The logarithm of cell volumes is plotted on the abscissa, the number of cells on the ordinate. Arrows point to projections characteristic of parenchymal cells and neuroglial cells. For detailed explanation see text

occasionally been observed, but these probably do not belong to the OVLT. They might have gained an accidental access to our sections from the adjacent regions of the brain in the course of preparations.

The function of the OVLT is unknown. It may represent an anastomosis or shunt serving for overcoming the blood—cerebrospinal fluid barrier, as

suggested by MELLINGER [24] and supported by the fact that the organ is comparatively thin, that it is richly provided with capillaries and has a loose structure. The connection by fibres between the outer and inner layers, as observed in the trout, is a further argument in favour of MELLINGER's theory. Of course, further investigations are needed in respect of this theory.

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UNTERSUCHUNG DES ORGANON VASCULOSUM LAMINAE TERMINALIS

I. Vergleichende Untersuchung des Organon vasculosum laminae terminalis bei Knochenfischen. Morphologische Analyse

Das Organon vasculosum laminae terminalis (OVLT) von Knochenfischen wurde mit Hilfe vergleichender morphologischer Methoden untersucht. Bei sämtlichen 13 untersuchten Arten wurde das OVLT in Form eines deutlich ausgeprägten zirkumventrikulären Organs gefunden. Im OVLT lassen sich drei typische Zonen unterscheiden: eine äußere, Blutgefäße und Bindegewebselemente enthaltende Zone, eine innere, mit einem speziell differenzierten Ependym bedeckte Schicht, und das zwischen diesen beiden Schichten gelagerte Parenchym. Das OVLT erhält seine Vaskularisation von der äußeren Oberfläche. Für seine Zellen ist das Vorherrschen der Parenchymzellen charakteristisch. Die Parenchymzellen verfügen über deutlich ausgeprägte Fortsätze, die in erster Linie die Verbindung mit dem Tr. retino-hypothalamicus, jedoch auch mit dem Tr. praeoptico-hypophysealis sichern. Auf Grund seines hohen Entwicklungsgrades bei den Knochenfischen kann das OVLT phylogenetisch als ein primordiales Organ betrachtet und bei den Knochenfischen den zirkumventrikulären Organen zugezählt werden. Es wird angenommen, daß das OVLT funktionell eine in den Weg der Blutliquorschranke gestellte Anastomose oder ein Shunt ist.

ИССЛЕДОВАНИЕ ORGANON VASCULOSUM LAMINAE TERMINALIS (OVLT)

I. Сравнительное исследование Organon vasculosum laminae terminalis у костистых рыб. Морфологический анализ

Organon vasculosum laminae terminalis исследовался у костистых рыб при помощи морфологическиих методов. У всех 13 исследовавшихся видов **OVLT** был обнаружен в виде выраженного околовентрикулярного органа. У костистых рыб в **OVLT** можно определить три типичные зоны: внешнюю зону, содержащую кровеносные сосуды, и элементы соединительной ткани, внутреннюю часть, покрытую специфически дифференцированной эпендимой, и, между этими двумя слоями, состав паренсимы. **OVLT** получает васкуляризацию из наружной поверхности органа. Для его клеток типично господство паренхимных клеток, обладающих ясно выраженными отростками. Последние обеспечивают прежде всего всязь с **tr. retinohypothalamicus**, но и с **tr. preoptico-hypophysealis**. На основании его высокой степени развития у костистых рыб **OVLT** филогенетически следует рассматривать как примордиальный орган и причислять к околовентрикулярным органам костистых рыб. Предполагается, что **OVLT** играет роль анастомоза или шунта по пути гематоэнцефалографического барьера.

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EFFECT OF TSH ON THE RATE OF DNA AND PROTEIN SYNTHESIS IN EMBRYONIC CHICKEN THYROID CELLS, INVESTIGATED WITH ^{3}H-THYMIDINE AND ^{3}H-METHIONINE AUTORADIOGRAPHY

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Synopsis

The rates of DNA and protein synthesis in the thyroid glands of chicken embryos were investigated with ^{3}H-thymidine and ^{3}H-methionine autoradiography. Normal and decapitated embryos of different ages were used. The rate of DNA synthesis was calculated from the percentage of autoradiographically labelled cell nuclei (labelled index), protein production from the silver grains over the cytoplasm of the epithelial cells and over the follicular colloid of the thyroid gland. It was found that in normal embryos there is a critical drop in the labelled index of thyroid cells at the time when endogenous TSH secretion sets in (about the 11th day of incubation). In decapitated embryos, this drop does not occur but it can be evoked by a single injection of exogenous TSH. In normal embryos of the early stages of development (before the onset of TSH secretion), and in decapitated embryos irrespective of age, ^{3}H-methionine was incorporated only into the cytoplasm of the cells and was not present in the lumina of follicles, whereas in the normal embryos in which endogenous TSH secretion has already set in, ^{3}H-methionine could be found in great amounts also in the colloid. The conclusion is drawn that TSH is the stimulus under which the embryonic thyroid gland "switches over" from the rapid self-reproduction to specific hormone secretion.

Introduction

Previous experiments had suggested that TSH is essential for the structural differentiation of the thyroid epithelial cells as well as for the onset of hormone secretion in the thyroid gland. In the following the observations made in this regard are summarized.

Electron microscopic studies on chicken embryos [2] revealed that (1) the ergastoplasm of the thyroid epithelial cells is undifferentiated in normal embryos until the 11th day of incubation and in decapitated embryos irrespective of age; (2) in the intact embryo the rapid differentiation of the ergastoplasm ensues on the 11th day of incubation, i.e., on the day when TSH secretion presumably starts. The differentiation is characterized by the formation of a very extensive lamellar ergastoplasm rich in ribosomes; (3) in decapitated embryos three hours after injection of exogenous TSH the undifferentiated ergastoplasm of the epithelial cells shows a marked development. Similar

observations were reported by STOLL and co-workers [5, 6, 10, 11] in avian thyroid glands.

Further it has been shown [4] that (1) in the thyroid of the chicken embryo accumulation of ^{131}I begins about the 11th day of incubation, i.e., at the 37th developmental stage of HAMBURGER—HAMILTON [3]; (2) this ^{131}I accumulation does not occur in embryos decapitated at the mesencephalic level on the second day of incubation; (3) if adenohypophysis is transplanted from donors of the same age under the skin of the breast of decapitated embryos on the 7th day of incubation [7], the ^{131}I accumulation starts exactly on the 11th day of incubation; (4) if the donors are two days older than the decapitated acceptors, the ^{131}I accumulation starts already on the 9th day of incubation, i.e., on the day when the hypophyseal graft is 11 days old. It seems very likely that the onset of ^{131}I accumulation in the thyroids is a consequence of TSH release from the pituitary, starting on the 11th day of incubation; (5) in decapitated embryos the accumulation of ^{131}I in the thyroids attains the normal level after a single injection of TSH.

The mentioned findings seem to indicate that TSH might act first on the ergastoplasm of the thyroid epithelial cells and then, by activating these structures, starts hormone secretion in the gland.

Since ergastoplasm, protein synthesis and hormone synthesis are very closely related, the present experiments were designed to study the rates of DNA and protein synthesis in the thyroid epithelial cells of chicken embryos in the presence and absence of TSH. An abstract of this paper has already been published elsewhere [12].

Material and method

The experiments were performed on 138 chicken embryos. The embryos were divided into two main groups.

Group A served for the determination of DNA synthesis; 78 embryos of different ages received 40 μC ^{3}H-labelled thymidine injected into the allantoic sac.

In *group B* protein synthesis was investigated; 60 embryos of different ages received 40 μC tritiated methionine injected into the allantoic sac. Each group contained normal embryos (76) altogether and decapitated embryos (62). Decapitation was performed at the midbrain level on the 2nd day of incubation by the method described by SZÉKELY [13].

Five decapitated embryos formed a separate subgroup. These were injected with TSH (Ambinon, Organon 2 USPmU) on the 12th day of incubation and 12 hours later they received 40 μC ^{3}H-thymidine. After another 12 hours the embryos were killed.

With the exception of the TSH-treated subgroup, the embryos were sacrificed 3, 12 or 24 hours after the isotope administration. Thyroid glands were quickly removed, fixed in a mixture of 4 parts of 96% alcohol and 1 part of 40% formaldehyde. After two hours of fixation the thyroids were embedded into polywax and 5 μ thick sections were covered with Kodak AR10 stripping film. Autoradiographs were developed after four weeks of incubation, fixed and stained for the usual haematoxylin-eosin technique of DONIACH—PELC [1], then covered with "Elvanol 51—05" [9].

The rate of DNA synthesis was estimated by the method of SAWICKI [8]. The unlabelled and autoradiographically-labelled thyroid epithelial cell nuclei were counted under the microscope in 9 fields at a magnification of 1,000 : 1, using three different sections of the gland, and

the percentual occurrence of the labelled nuclei (labelled index) was calculated. The labelled index will be considered a characteristic, although indirect, index of DNA synthesis.

The rate of protein synthesis was calculated from the number of silver grains seen over the cytoplasm of the epithelial cells and over the follicular lumen of the thyroids. The grains were counted in three different areas of five different sections of the glands of every embryo. The data of the embryos of the same age were then averaged, treating the number of granules over the cytoplasm and that over the follicular lumen separately. The counting of grains was performed at a magnification of 1,000: 1.2 × 2 mm ocular diaphragm was used.

Results

Table 1 and Fig. 1 are informative of the percentages of labelled nuclei in thyroid epithelial cells of normal and decapitated embryos of different ages. Table 1 demonstrates that the percentage of labelled cell nuclei increased with

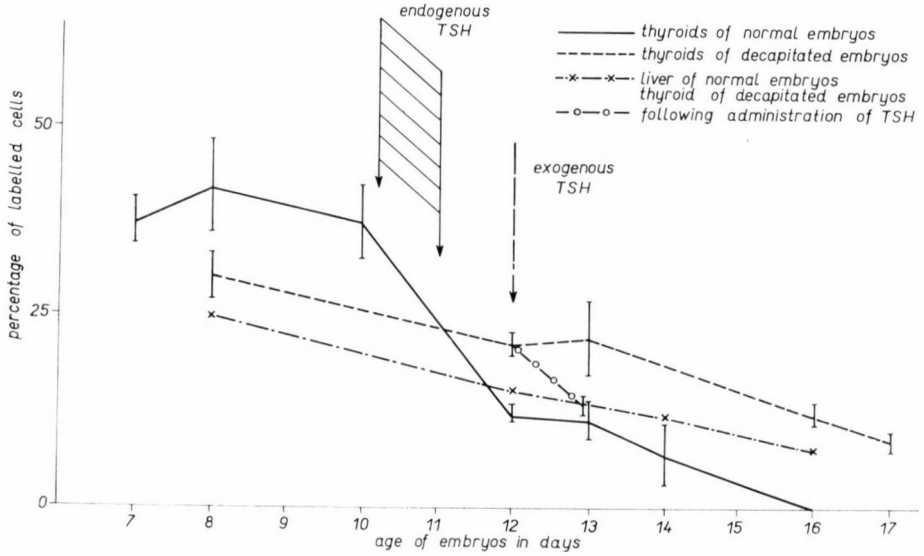


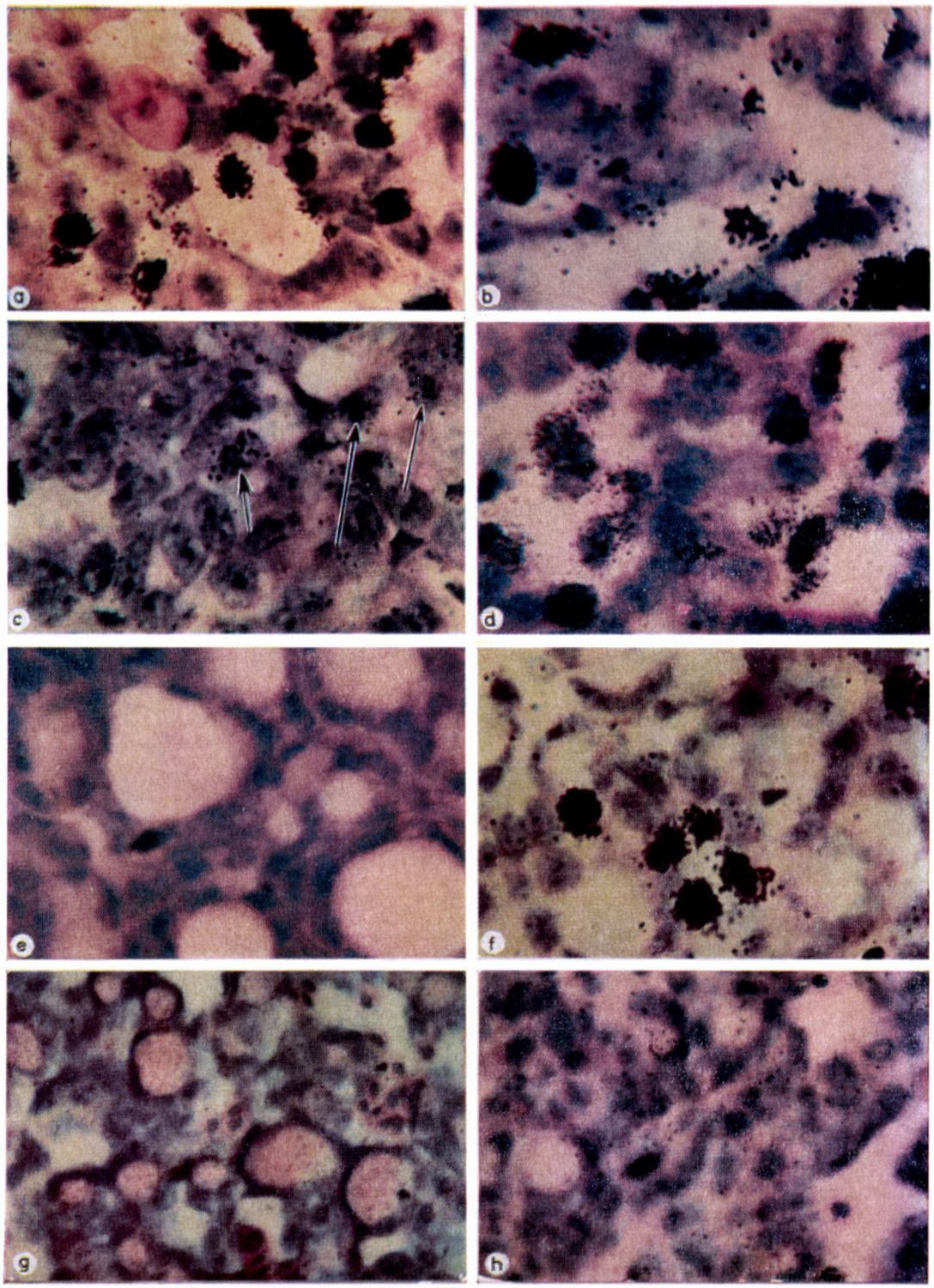
Fig. 1. Percentage of ^{3}H-thymidine-labelled thyroid epithelial cells 12 hours after thymidine injection

time following the injection of ^{3}H-thymidine. This increase was more pronounced in younger embryos than in older ones. Since 12 hours correspond approximately to two generation times of embryonic cells, this time was chosen as characteristic of the rate of DNA synthesis. Fig. 1 shows that in normal embryos between the 7th and 10th day of incubation the labelled index of the thyroid cells was fairly high 12 hours after tritiated thymidine administration. On the 12th day of incubation, i.e., one day after TSH secretion had presumably started, the labelled index critically decreased to one third of its original value. This decrease continued with less rapidity till the 16th day of incubation, when the minimum level characteristic of adult thyroids was reached.

Table 1

Percentual occurrence of ^3H-thymidine-labelled thyroid epithelial cells in normal and decapitated chicken embryos

Experimental group	Age of embryos day	Percentage of labelled cells after thymidine injection			Significant difference
		3 hours	12 hours	24 hours	
Normal embryos	7		37.4 40.1 34.9		
	8	17.5 18.4 22.2	47.0 38.8 41.4	79.6 76.2 72.5	
	10		32.6 36.5 43.3		
	12	2.3 2.0 1.8	11.2 13.5 12.8	10.6 9.1 9.0	
	13		10.8 13.2 12.7 12.5		
	14		5.5 8.3 5.5		
	16	0.0 0.0 0.0	0.0 0.0 0.0	0.0 0.0 0.0	
Decapitated embryos	8	17.2 18.6 21.6	29.9 33.0 29.3	44.4 41.9 43.2	significant diff. against 8-day-old normals $t = 8.29$ $p < 0.01$
	12	12.1 13.1 13.4	20.3 23.0 20.4	16.0 14.7 15.1	significant diff. against 12-day-old normals $t = 4.32$ $p < 0.01$
	13		20.2 23.2 25.4		
	16	10.6 9.5 0.6	11.8 13.2 11.9	11.6 10.4 11.3	
	17		7.3 6.3 6.4		
Decapitated embryos treated with TSH	13		16.6 13.1 14.3 13.6 13.6 10.8		significant diff. against 13-day-old decapitated $t = 5.57$ $p < 0.01$ and against 13-day-old normals $t = 0.97$ $0.5 > p > 0.2$



Figs 2a—h Autoradiographs of thyroid glands of normal and decapitated embryos of different ages, treated with ^{3}H-thymidine (a—f) or ^{3}H-methionine (g—h).

(a) 8-day-old normal, (b) 8-day-old decapitated embryo. Note the high incidence of mitotic activity in both embryos. (c) 12-day-old normal embryo. Only few cell nuclei are labelled (arrow). (d) 12-day-old decapitated embryo. In contrast to (c) a higher incidence of labelled cells is present. (e) 16-day-old normal embryo; (f) 16-day-old decapitated embryo. The follicular structure is well developed in both cases but labelled nuclei are present only in the thyroid of decapitated embryo. Magnification ×1,040; (g) Autoradiograph of a 16-day-old normal embryo after 3H-methionine administration. The reduced silver grains are localized mostly over the colloid. (h) 16-day-old decapitated embryo after the same treatment as (g). There are no silver grains over the follicular lumen, whereas a number of silver grains can be seen over the cytoplasm. Magnification ×630

In decapitated embryos the labelled index of the thyroids was lower at the early stages of development than in the controls of the same age and slowly decreased with time but, unlike the normal embryos, a critical fall in the labelled index failed to occur. A fairly high per cent of labelled cells (approximately 10%) still appeared on the 16th—17th days of incubation.* If exogenous TSH was injected into decapitated embryos on the 11th day of incubation, the labelled index markedly decreased almost to the level of the normal embryos of the same age. — The hepatic cell nuclei of intact embryos showed a steady gradual decrease in the percentage of labelled nuclei.

The striking differences in the number of labelled cell nuclei of normal and decapitated embryos after the 11th day of incubation are demonstrated in Figs 2a—f. There is no significant difference between the two groups on the 8th day of incubation (Figs 2a and b). However, on the 12th day a higher incidence of labelled thyroid cell nuclei is present in the decapitated embryos than in the controls (Figs 2c and d). On the 16th day of incubation, although the follicular structure has been completely developed both in the normal and decapitated embryos, practically no labelled cells are visible in the former (Fig. 2e) in contrast to a fairly high incidence in the latter (Fig. 2f).**

The results concerning ^{3}H-methionine incorporation into the cytoplasm of thyroid epithelial cells and into the intrafollicular colloid in embryos of different ages with or without decapitation are summarized in Fig. 3. In normal embryos on the 8th day of incubation silver grains indicating the incorporation of methionine were present over the cytoplasm of the cells but there were no silver grains over the lumina of the developing follicles. At this stage of development the amount of incorporated methionine in the cytoplasm increased with time, it was the highest 24 hours after isotope administration. From the 12th day of incubation tritiated methionine could be demonstrated also in the colloid. On the 16th day of incubation a considerable amount of incorporated methionine appeared in the colloid, the activity reaching its peak 12 hours after the administration of ^{3}H-methionine. In these embryos the number of silver grains over the cytoplasm was the highest 3 hours after the injection of the isotope and the cytoplasmic activity was already declining at the time when the highest activity was reached in the colloid.

In decapitated embryos, irrespective of age, tritiated methionine was incorporated exclusively into the cytoplasm of the epithelial cells. In 12- or

* Decapitated embryos cannot be kept alive longer than the 17th day of incubation.

** Some reduced silver grains could be seen over the intrafollicular colloid of thyroids of 16-day-old normal embryos. Since the rate of DNA synthesis is extremely low at this stage of development, DNA cannot be incorporated into the nuclei, but becomes decomposed. It is known that the decomposition of the exogenous thymidine runs through β-amino butyric acid, β-propionic acid, β-oxy propionate to β-alanine [14], which might probably be incorporated into thyroglobuline and then secreted into the follicular colloid. It may be supposed that in the present experiments the labelled alanine gave the observed autoradiographic effect over the colloid.

16-day-old decapitated embryos, unlike normal ones, silver grains were not found over the follicular lumen.

Fig. 2g and h demonstrate the marked differences in the localization of incorporated methionine in the thyroid glands in normal and decapitated embryos on the 16th day of incubation, 12 hours after isotope injection. In normal embryos a large number of silver grains can be seen over the colloid, whereas in decapitated embryos silver grains are only over the cytoplasm and there is no indication of isotope activity over the follicular lumen.

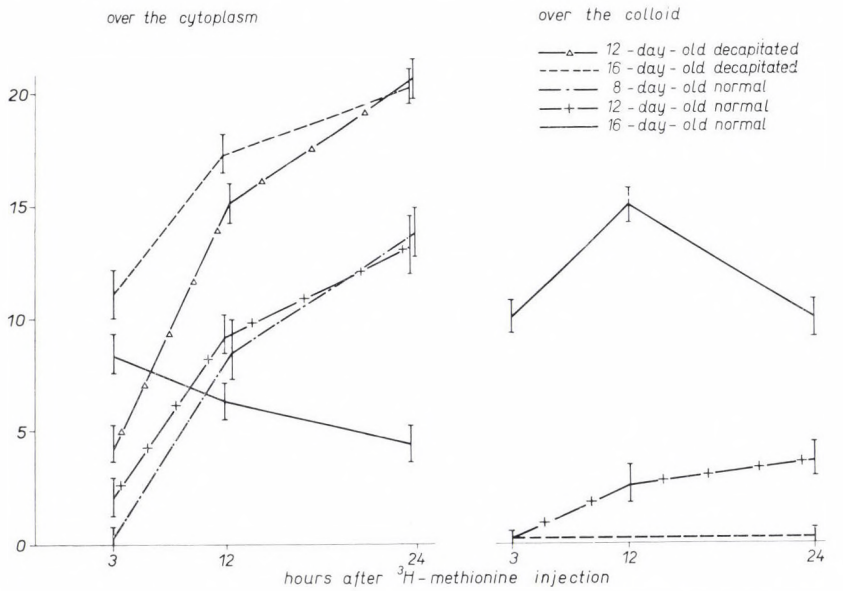


Fig. 3. The number of silver grains over the cytoplasm and over the colloid of normal and decapitated chicken thyroids

Discussion

The observations mentioned in the introduction [2, 4, 5, 6, 7, 10, 11] indicate that the structural differentiation of the thyroid epithelial cells as well as the thyroidal ^{131}I accumulation occur in chicken embryos at the time when HTS secretion and release start (10th—11th days of incubation). The present studies suggest that at the same time there are also major changes in the rate of DNA and protein synthesis of the thyroid glands. The results seem to indicate that on the 7th or 10th day of incubation there is a high rate of DNA synthesis which, however, abruptly decreases at about the 11th day of incubation. Since the rapid decrease occurred at the time of TSH secretion and release by the pituitary, it seems very likely that the marked decline is a consequence of the onset of TSH secretion. This assumption is supported by

the observation that rapid decrease of the labelled index did not occur in decapitated embryos, except when exogenous TSH had been administrated.

The marked fall in mitotic rate following the onset of TSH secretion seemingly indicates that TSH has an inhibitory effect on cell reproduction of the thyroid glands in the embryonic stage of life. It seems very likely that this is not a direct inhibitory effect but is simply related to the fact that TSH enhances cell differentiation in the thyroid glands (e.g. the differentiation of the ergastoplasm as mentioned in the introduction), and thus its action on cell reproduction would only be secondary.

Concerning the thyroidal protein synthesis, it was found that in normal embryos before the onset of TSH secretion, and in decapitated embryos irrespective of age, silver grains indicating incorporation of ^{3}H-methionine appeared only over the cytoplasm of the epithelial cells, whereas in normal embryos after the onset of TSH secretion labelled methionine was present also in the colloid. Further, it was observed that after the onset of TSH secretion the number of silver grains over the cytoplasm was the highest 3 hours after tritiated methionine administration and had already declined when it reached its peak over the colloid, whereas in 8-day-old normal embryos or in decapitated embryos of all ages, the amount of incorporated methionine increased till 24 hours following the injection of the isotope. These observations suggest that (1) in the absence of TSH (either in normal embryos, of the early stages of development, or in decapitated embryos) the rate of protein synthesis is relatively low and the proteins synthetized, being not released into the follicular lumen, are presumably cell structure proteins, (2) at the time when TSH is already secreted and released by the pituitary, the rate of protein synthesis becomes much greater than it was before and the synthetized protein being released, at least partially, into the colloid, is presumably mainly thyroglobulin.

The conclusion might be drawn that the metabolism of the embryonic thyroid gland is directed on the line of rapid self-reproduction in early stages of development and that TSH means the specific stimulus under which the metabolism of the thyroid cells "switches over" from the embryonic self-reproductive type of metabolism to the adult, i.e., to the specific hormone-productive metabolism.

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DER EINFLUSS DES THYREOTROPHEN HORMONS AUF DIE DESOXYRIBONUKLEINSÄURE- UND EIWEISS-SYNTHESE DER SCHILDDRÜSENZELLEN DES HÜHNEREMBRYOS. AUTORADIOGRAPHISCHE UNTERSUCHUNGEN MIT ^{3}H-THYMIDIN UND ^{3}H-METHIONIN

Die Desoxyribonukleinsäure- und Eiweiß-Synthese der Schilddrüsenzellen von Hühnerembryonen wurde autoradiographisch mit ^{3}H-Thymidin und ^{3}H-Methionin untersucht. Die Untersuchungen wurden an normalen und dekapitierten Embryonen verschiedenen Alters durchgeführt. Die Rate der Desoxyribonukleinsäure-Synthese wurde durch die Prozentzahl der autoradiographisch markierten Zellkerne (Markierungsindex) bestimmt, Folgerungen auf die Eiweiß-Synthese wurden von der Zahl der Silbergranula des Zytoplasmas der Epithelzellen und des Kolloids der Schilddrüse gezogen. Es wurde festgestellt, daß in normalen Embryonen der Markierungsindex bei Beginn der endogenen TSH-Sekretion (annähernd am 11. Tag der Inkubation) eine kritische Verminderung zeigt. Diese kritische Abnahme fehlte in den dekapitierten Embryonen, konnte aber durch eine einzige exogene TSH-Injektion induziert werden. In normalen Embryonen, in welchen die TSH-Sekretion noch nicht begonnen hat und in dekapitierten Embryonen — unabhängig vom Alter — wurde ^{3}H-Methionin nur im Zytoplasma der Epithelzellen der Schilddrüse inkorporiert und war im Lumen des Follikels nicht nachweisbar. In normalen Embryonen, in welchen die TSH-Sekretion schon begonnen hat, konnten hohe Werte von ^{3}H-Methionin auch im Kolloid beobachtet werden. Es wurde die Schlußfolgerung gezogen, daß das TSH der Stimulus ist, der die embryonale Schilddrüse von der rapiden Selbstreproduktion zu einer spezifischen Hormonsekretion »umschaltet«.

ДЕЙСТВИЕ ТИРЕОТРОПНОГО ГОРМОНА НА СИНТЕЗ ДЕЗОКСИРИБОНУКЛЕИНОВОЙ КИСЛОТЫ И БЕЛКОВ КЛЕТКАМИ ЩИТОВИДНОЙ ЖЕЛЕЗЫ ЗАРОДЫШЕЙ ЦЫПЛЯТ. АВТОРАДИОГРАФИЧЕСКОЕ ИССЛЕДОВАНИЕ ПРИ ПОМОЩИ МЕТИОНИНА ^{3}H И ТИМИДИНА ^{3}H

Действие тиреотропного гормона на синтез дезоксирибонуклеазы и белков клетками щитовидной железы зародышей цыплят исследовалось при помощи тимидина ^{3}H и метионина ^{3}H. Исследования проводились на нормальных и обезглавленных зародышах после различного срока инкубации. Количество синтезированной дезоксирибонуклеиновой кислоты авторы определяли на основании процентуального соотношения радиогра-

фически меченых ядер клеток (показатель мечения). Размер синтеза белков авторы установили по числу зернышек серебра, наблюдаемых в цитоплазме эпителиальных клеток и в коллоиде щитовидной железы. Было установлено, что в нормальных зародышах показатель мечения значительно понижается. К началу секреции эндогенного тиреотропного гормона (прибл. на 11. день инкубации) у обезглавленных зародышей этого критического понижения не наблюдалось, однако, однократным введением эгзогенного тиреотропного гормона удалось вызвать понижение показателя мечения. У нормальных зародышей, у которых еще не началась секреция тиреотропного гормона и у обезглавленных зародышей различного возраста метионин ^3H накоплялся лишь в эпителиальных клетках щитовидной железы. В просвете фолликулов метионина ^3H не было, в то время как в нормальных зародышах, в которых секреция тиреотропного гормона уже началась, накоплялось большое количество метионина ^3H и в коллоиде щитовидной железы. Авторы сделали вывод, что тиреотропный гормон является стимулом, переключающим эмбриональную щитовидную железу от быстрого самовоспроизведения на секрецию специфического гормона.

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RECENSIONES

M. I. PURVIS, D. C. COLLIER, D. WALLS: **Laboratory Techniques in Botany**
Second edition, Butterworths, London (1966) pp. 439, 57s 6d.

This excellent laboratory manual makes us acquainted with the techniques required for comprehensive examination of superior and inferior plants. The reader finds directives on plant anatomy and histochemistry, on the cultivation of green-house and aquarium plants, on establishing cultures of bacteria, algae and fungi, on how to make herbarium and liquid preparations, on phyto-physiological experimentation, biochemical analyses and soil researches, etc. A new enzymological chapter was included in the present edition, giving instructions about the analysis of the characteristic enzymes of bacteria, algae, fungi and floral plants. There are references at the end of each chapter, and a repertory of examining questions at the end of the book. This well-written and up-to-date technical book may be a most useful assistance for anyone engaged in botanics, including the pedagogist, the research worker and the university student.

V. FRENYÓ (Budapest)

D. KEILIN: **The History of Cell Respiration and Cytochrome**
The University Press, Cambridge (1966), pp. 415.

DAVID KEILIN had planned to write a historical review on intracellular respiration and on MACMUNN's work as soon as in 1938. Later this original frame was extended to include the early concepts of respiration together with a detailed description of the biochemical properties and function of intracellular haemoproteins. Unfortunately only eight chapters could be finished by him, the others were prepared for publication by his daughter, dr. JOAN KEILIN.

The book may be divided roughly in two parts. The first one deals with the knowledge gained from Galen until the discovery of the cytochromes by the author in 1925. KEILIN's deep interest in history paired with a thorough study and abundant quoting of the original papers make this part exceptionally valuable. The personal contributions and controversies are always presented in the scientific background of the epoch. A good example is the treatise on the cause of neglection of MACMUNN's important discovery on myohaematin and histohaematin. Was it due to the prematurity of the work; was he thought to be a dilettant; or were his contemporaries inadequate to grasp the significance of his results? The writer's answer is: no! The reason he finds in some obscure points in MACMUNN's work which baffled not only his contemporaries but himself, too. These difficulties — KEILIN believes — refrained the investigators from the repetition and comment upon these findings. In this historical part the outstanding style of the author has to be underlined, which makes this section so interesting that the reader is sorry to finish it.

The second part begins with a personal account of the first observation of the cytochromes. The presentation of further progress in this field during the following forty years is arranged around the author's own contribution. Development in the theory of cellular respiration, the properties of the different cytochrome compounds including cytochromes in microorganisms are dealt with in great detail without intending to give a complete up-to-date review. Research workers specialized in this problem will find this section very interesting.

A chronological chart of the great scientists and a large bibliography complete the book.

As a summary and recommendation it is the best to quote the author himself: ". . .the history of the development of our knowledge is very instructive to a research worker. Not

only do we learn much from past difficulties but it often helps to restore our confidence in our present work when everything in front of us appears to be wrapped in an almost impenetrable fog. It helps us to regain a sense of perspective and proportion which we frequently lose in our efforts to clarify those points of detail, without which no work is complete."

S. KERPEL-FRONIUS (Budapest)

Mössbauer Effect Methodology Vol. 1.
Ed. I. J. GRUVERMAN, Plenum Press, New York, 1965, pp. 200.

The book contains the proceedings of the First Symposium on Mössbauer Effect Methodology, held in New York on January 26, 1965. The first, for the biologists most important, part of the book reviews recent applications of Mössbauer Spectroscopy in Chemistry, Biology and Physics. In the review of HERBER, among the mentioned chemical problems, perhaps the most interesting ones are the recent Mössbauer studies on the structure of five and six coordinate organotin compounds using $^{119}SnO_2$ as source. The physicist will find a special interest in the paper of RUBY, which discusses the application of Mössbauer spectroscopy in the problems of magnetism of solids, lattice dynamics and nuclear physics.

In the review of GONSER and GRANT on the biological applications of the Mössbauer effect we find first a clear discussion of the conditions to be fulfilled by an isotope to be applicable for the Mössbauer study of biological systems. They conclude that besides the already used ^{57}Fe the isotopes ^{127}I and ^{129}I seem applicable for such investigations. Then they give an exhaustive review on the determination of the different Mössbauer parameters (isomeric shift, quadrupole splitting, nuclear Zeeman effect, resonance line width, absorption intensity) for different iron-containing biological systems (first of all for the different forms of haemoglobin). The biologist and chemist will find this paper as a very useful guide to draw conclusions from the different Mössbauer parameters on the structure of different biologically active molecules.

The second part of the book contains papers describing recent developments in Mössbauer spectrometers (feedback in electromechanical drive systems; cam-driven, constant-acceleration Mössbauer spectrometer and constant-velocity Mössbauer drive systems).

The third part of the book reviews the scattering technique measurements, describes the standardization of differential chemical shifts for ^{57}Fe, using sodium nitroprusside standard absorbers. This part of the book contains also a paper which describes three new Fortran programmes written for the purpose of computing Mössbauer spectra.

In the fourth part we find an interesting discussion of the application of superconducting magnets to Mössbauer studies. Further descriptions of new low-temperature and high-pressure techniques complete the discussion.

As it is perhaps possible to see from this brief review, this little book contains in a comprehensive form many aspects of the methods and applications of Mössbauer spectroscopy. Therefore, it fills a gap of knowledge of many biologists, chemists and physicists.

J. LADIK (Budapest)

R. E. COUPLAND: **The Natural History of the Chromaffin Cell**
Longmans, London (1965), pp. 279, 102 ills; 50s.

This excellent monograph deals with the structure and function of chromaffin cells reviewing so to say the entire pertaining literature.

The author had been constructively engaged in chromaffin tissues for 20 years, therefore able to review masterfully the results achieved in the different fields of science. In this integrating work attention is focussed on the unity of structure and function, from the macroscopic to the molecular order. The timeliness of the book is increased by many investigations, especially into the ultrastructure and innervation of chromaffin cells, not published before.

The work is divided into 9 chapters and treats the comparative anatomy, histochemistry, ultrastructure, ontogeny, innervation, blood-supply of chromaffin tissues, the secretion of catecholamines and chromaffin tumours. The text is illustrated with line diagrams, microphotographs and electron micrographs.

This work cannot be spared by anybody interested in chromaffin tissues.

I. MUNKÁCSI (Budapest)

H. R. WILSON: **Diffraction of X-rays by proteins, nucleic acids and viruses**
Edward Arnold (Publishers) Ltd., London (1966), pp. 136, 15s.

Structure research on a molecular level is an inseparable part of exact biological investigations and, particularly, of up-to-date molecular biological research. In this field the technique of X-ray diffraction has proved to be one of the most perspective methods in the last decades. Its application has already produced fine results and it has thus proved to be a suitable method for the biological, biophysical and biochemical research. The present short monograph outlines the basic principles of the diffraction of X-rays, as well as the results this method has helped to achieve in revealing the structure of proteins, nucleic acids, nucleo-proteins and viruses.

The book is divided into five chapters. Chapter I presents on a basic level the primary concepts concerning molecular structure: atomic structure, chemical bonds, molecule models. Chapter II discusses the theory of X-ray diffraction, explaining the diffraction as brought about by the crystalline system (crystal lattice) and by the variously shaped molecules (filamental, helical). Chapter III describes the results achieved with the method of X-ray diffraction in the study of protein structure, dealing with the structure of amino acids, peptides, collagen, and fibrillary and globular proteins. A special part discusses the structure of myoglobin and haemoglobin. Chapter IV describes the results achieved in structural research of nucleic acids, treating the structure of their components, such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and synthetic polynucleotids. Chapter V sums up the results achieved in examining the structure of nucleo-proteins and viruses, leaving a special part for the tobacco mosaic virus and other rod-shaped and spherical viruses.

The monograph is completed with more than 70 figures, 5 tables, 20 X-ray diagrams, pictures of some molecular models, more than 200 references and a short table of contents.

The volume deals with the question on the basic level; any beginner in this field of science may use it successfully for acquiring the basic knowledge.

A. NIEDECZKY (Pécs)

Cell electrophoresis
Ed. E. J. AMBROSE, Churchill Ltd., London (1965), pp. 204, 109 ills, 36s.

The suspended cell — either blood or somatic cell — displays a characteristic electrophoretic mobility, as a consequence of its superficial charge density. If due to identical charge density different cells cannot be separated by simple electrophoresis treatment with enzyme (e.g. neuraminidase) or chemicals or changes in pH may alter the electrophoretic mobility of the different cell types differently. In this way not only the characterization of cells is facilitated, but also information can be obtained on the structure of cell surface.

Although the electrophoretic migration of suspended cells has been well known for five decades [ELLIS, R., *Z. Phys. Chem.*, **78** (321), 1911—12], it took a long time till the technical difficulties could be overcome and the method widely accepted.

The Symposium of the British Biophysical Society in 1963 was the first meeting which dealt exclusively with the problems of cell electrophoresis. Reviewing the matter of the Symposium it turns out that the technique of cell electrophoresis may be utilized in many fields of biological and medical research.

The first part of the volume deals with methodical problems. SEAMAN discusses the theoretical and practical relations of applying the cylindric chamber used chiefly for absolute determinations. Due to its better optical qualities, the rectangular chamber demonstrated by FUHRMANN and RUHENSTROTH—BAUER is more suitable for the identification of cells and cell constituents and can be applied with both light microscope and phase contrast. LUKIEWICZ and KOROHODA explained their double chamber enabling long-term observation of single cells.

The next part presents many examples on the application of the technique. As for cell electrophoresis blood cells are most suitable, numerous authors are employed in them. The migration of normal and diseased blood and lymph-node cells of rats and mice, and of the man was studied by ARNOLD and RUHENSTROTH—BAUER, respectively, succeeding to evidence characteristic lesions in pathological cases. Similar results were achieved by FUHRMANN, who analyzed the properties of normal, proliferative and malignant liver cells, as well as by FORRESTER investigating fibroblasts transformed by polyoma virus.

Changes caused by different physical, chemical and enzymatic effects on the cell surface were investigated by SEAMAN and COOK using blood cells, by FUHRMANN using liver, and kidney cells and spermatozoa and by JAMES, who investigated the bacterial wall. DOLJANSKY and EISENBERG drew attention to the qualitative differences between free and somatic cells.

SACHTLEBEN studied the effect of antibodies, STRAUB that of viruses, on the electrophoretic mobility of erythrocytes. BEY pointed out that X-chromosome-bearing spermatozoa cannot be differentiated electrophoretically from those bearing the Y-chromosome. LUKIEWICZ and KORODA observed periodical changes in cell mobility of the *Chlorella* alga.

MUTULO and LUKIEWICZ have been the only authors trying to examine subcellular units, namely mitochondria, electrophoretically.

In the last chapter AMBROSE reviews the results of cell-electrophoretic research, in view of the structure and biological properties of cell surface.

I. RADOS (Budapest)

ILONA BANGA: **Structure and function of elastin and collagen**
Akadémiai Kiadó, Budapest (1966), pp. 225.

In the past decades great scientific interest has been devoted to the structure and function of connective tissue elements in physiology and pathology. Because of the manifold aspects of this topic, many authoritative treatises covered only selected sectors of this subject. It is a fortunate and a valuable feature of this book that, although it is mainly concerned with the macromolecular chemical-structural organization of the elastic and collagen fibres of the human vascular wall, the functional aspects of these fibrillar elements and their functional and structural interrelationships as well as the basic problems of connective tissue pathology and the pathogenetic aspects of arteriosclerosis and gerontology, are also dealt with.

The presentation is highly authoritative and based on the research work carried out in the past decade in the author's laboratory in the 1st Dept. of Pathology (headed by Prof. J. BALÓ), University of Budapest.

The first contribution to this field was the discovery by BALÓ and BANGA (1949) of the specific elastolytic enzyme of the pancreas-elastase. This discovery opened up a new line of research facilities soon adopted by numerous laboratories in several parts of the world. The discovery provided a possibility to study the chemical-structural organization of the elastic fibres, and later on, using subfractions of the enzymes of the elastase complex separated by the author and his collaborators enabled similar analyses to be made on the collagen fibres. The enzymatic effects on elastic and collagen fibres were estimated, apart from biochemical methods, with different optical methods as used in histology, polarization optics, and on the basis of physical characteristics such as strength, elasticity and heat-contraction. In this way it was possible to obtain interesting results regarding the macromolecular-structural organization of the elastic and collagen fibres as yet not accessible to other methods. The new ideas reached seem well-founded, and the limitations of present day knowledge are always pointed out. Thus the book leaves the reader with the conviction that the enzymatic approach is of great value in the study of the macromolecular structure of the elastic and collagen fibres and has provided valuable information about the ultrastructure of these structural elements.

The book is divided into two parts. Part I deals with the molecular-structural organization of the elastic fibre, Part II with that of the collagen fibre. The titles of some of the chapters may give an idea of the scope of the book: from Part I: Isolation of elastolytic enzymes; Enzymatic decomposition of elastic fibres with the elastase enzyme complex; The role of mucolysis in elastolysis; Verification of the complex structure of elastic fibres; Studies with elastase and elastomucoproteinase; Changes in arteriosclerosis and the *in vivo* effect of elastase; Changes in the chemical structure of elastic arteriae parallel with age and arteriosclerosis.

From Part II.: Soluble and insoluble collagens; The role of procollagen in the function of the fibre; Characterization of metacollagen; Interrelation between chemical contraction and peptidization; The role of polysaccharides in the construction of the collagen fibre; Effect of collagen mucoproteinase on the collagen fibre; The mechanism of collagen mucoproteinase action.

The book will be of value not only to biochemists but to those interested in connective tissue histochemistry and histopathology and especially to those who are interested in the means of study of biological ultrastructures in general.

The book is well illustrated in part with several good photos in colour. There are a subject index, and a bibliography with about 400 references. Lay out and printing are in keeping with the standards of the Acad. Press Hung.

G. ROMHÁNYI (Pécs)

A. D. HASLER: **Homing of Salmon. Underwater Guideposts**
The University of Wisconsin Press, Madison (1966), pp. 155, $6.

The author is well known by hydrobiologists. His book reported here is understandable and delightful also for the educated general public. It comprises the results of investigations conducted for decades persistently at numerous levels and in various fields of science with a complex research group.

The subject of the research was raised by the nature itself and by practical life. It is a fact established long ago that salmons (exactly, the multi-spawning species *Salmo salar* living in the Atlantic Ocean and the *Oncorhynchus* species of the Pacific, which spawns only once in its whole life) return — after staying in the sea for many years — to spawn in those rivulets, where they were born. In the centre of numerous problems connected with this fact stood the following question: which are the underwater guideposts leading back the salmons to the spawning spots where they have come into the world 5 to 7 years ago, though these places are many hundred and often even some thousand (e.g. 4,000) kilometres remote from the maritime mouth of the river. What kind of sense enables them to detect the right direction and how do they find their birth-place in the labyrinth of tributaries? The author evidences with many-sided experiments and observations carried on in laboratory and in the open space that in this period of migration salmons are guided by their olfaction. The characteristic "scent" of the birth-rivulet — originating from the soil, the vegetation and its decomposed substances, etc., — penetrates the salmons so deeply in the initial stage of their ontogeny that when their migration instinct induced by sexual hormones arises, they can "remember" that scent, even if it becomes diluted 5,000 times.

However, in the migration of salmons there is also another, more exciting and mysterious stage, to which hardly any attention was paid by research workers and fishers so far. This is the migration in the open ocean. Until the 2nd World War it was believed that during their maritime life period salmons stay on the "continental shelf" and may, therefore, find the mouth of the birth-river easily. Japanese fishers, forced to fish in the high seas after the 2nd World War, returned — to the greatest surprise of experts — with rich salmon hauls. So it has proved true — and was confirmed later by marking experiments — that salmons do not come to a standstill near the shores, but migrate even in the mid-ocean many thousand kilometres to their feeding place and from it back to the mouth of their birth-river.

The question is, how do they find the right direction leading them to the "native" shores and the mouth of the birth-river many hundred and sometimes several thousand kilometres distant from the high sea. On the basis of the results achieved by numerous many-sided experiments conducted with other fish species, the author comes to the well-founded assumption that migration is guided by the course of the sun. Though this supposed sun-compass orientation, as presented by him in virtue of his investigations, may not yet be verified, it is more than a starting work hypothesis.

This book may also convince the reader of the up-to-date state of ecological researches. It reveals that a scientist, who in his endeavour to disclose the secrets of Nature does not confine himself to one-sided, uniplanar researches, but — utilizing the possibilities of laboratory and field experiments and observations — applies the combinations of anatomical, physiological, ecological and etological methods and does not recoil from the collection of proofs nearly by bits, may get a likely answer from Nature even as to a complex of questions, the solution of which could hardly be expected so far.

E. WOYNAROVICH (Debrecen)

Experientia Supplementa

12	Neuerscheinung Proceedings of the 2nd International Symposium on Polarization Phenomena of Nucleons	Karlsruhe. September 6 – 10, 1965 Editors: P. Huber and H. Schopper (1966) 536 Seiten mit 317 Figuren. Ganzleinen Fr./DM 86,– Table of contents; I. Sources for Polarized Nuclei II. Targets of Polarized Nuclei III. Production and Detection of Polarization by Reactions and Scattering IV. Theoretical Aspects Concerning Polarization Effects V. Experiments Using Polarized Beams and/or Targets VI. Experiments on Polarization Produced in Scattering and Reactions
1	Gegenwartsprobleme der Ernährungsforschung Present Problems in Nutrition Research Les problèmes actuels de la nutrition	Symposium Basel, 1. – 4. 10. 1952. Editor: F. Verzár. 312 Seiten, 32 Figuren, Leinen Fr./DM 32,– (1953).
2	XIVe Congrès international de chimie pure et appliquée XIV. Internationaler Kongress für reine und angewandte Chemie XIVth International Congress of Pure and Applied Chemistry	Zürich, 21. – 27. 7. 1955. 5 Conférences principales et 9 Conférences de section 5 Hauptvorträge und 9 Hauptsektionsvorträge 5 Main Congress Lectures and 9 Lectures in the Sections 284 Seiten, Fr./DM 32,– (1955).
3	Acta XI Congressus Internationalis Ornithologici (Vergriffen)	Basel, 29. 5. – 5. 6. 1954. Im Auftrag des Präsidenten, Sir Landsborough Thomson, herausgegeben von Adolf Portmann und Ernst Sutter. 680 Seiten, über 100 Figuren, Fr./DM 35,– (1956).
4	Symposium über experimentelle Alternsforschung Symposium on Experimental Research on Ageing	Basel, 4. – 7. 4. 1956. Herausgeber/Editor: Prof. Dr. F. Verzár. 290 Seiten, Fr./DM 34,– (1956).
5	XVe Congrès international de chimie pure et appliquée XV. Internationaler Kongress für reine und angewandte Chemie XVth International Congress of Pure and Applied Chemistry	Lissabon, 8. – 16. 9. 1956. 6 Conférences principales et 8 Conférences de section 6 Hauptvorträge und 8 Hauptsektionsvorträge 6 Main Congress Lectures and 8 Lectures in the Sections 240 Seiten, Fr./DM 32,– (1956).
6	25 Jahre hochalpine Forschungsstation Jungfraujoch, 1931 – 1956	Herausgegeben von Prof. Dr. A. von Muralt. 86 Seiten mit zahlreichen Abbildungen, Fr./DM 8,30 (1957)
7	XVIe Congrès international de chimie pure et appliquée XVIth International Congress of Pure and Applied Chemistry XVI. Internationaler Kongress für reine und angewandte Chemie	Paris, 18. – 24. 7. 1957. 18 Conférences plénières – 18 Main Lectures – 18 Hauptvorträge. 356 Seiten, Fr./DM 36,– (1957).
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AN ENDOGENOUS FACTOR REGULATING THE LIFE CYCLE OF STREPTOMYCES GRISEUS*

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(Received June 2, 1966)

Synopsis

The regulation of the life cycle of *Streptomyces griseus* in submerged culture was studied. The vegetative and reproductive phases of growth were distinguished in the evaluation of the experiments. From the fermentation liquid of a strain with very short vegetative phase of growth a substance(s) was isolated, which was termed factor C. When this agent was added in submerged culture to the medium of a strain of *Streptomyces griseus* which do not produce conidia, the strain changed its life cycle and produced also conidia. Factor C seems to be specific and of polypeptide nature. We suppose that factor C, being an agent produced endogenously by the *Streptomyces griseus* strain itself, regulates the differentiation of the microorganism. The significance and possible use of this model to study the chemical nature of the regulatory mechanism of cell growth and differentiation is discussed.

Introduction

There have been several works published concerning the life cycle of *Streptomyces griseus* in submerged and on solid medium [1, 6, 7]. They all agree in establishing two morphologically and physiologically distinct phases during the life cycle of Streptomycetes: a vegetative and a reproductive phase of growth.

There are plenty of cytomorphological and physiological characteristics for further subdivision of these phases, but in order to study the regulatory mechanism of the switch from one phase to the other we have turned our attention only to these definitely different phases of growth.

The development of *Streptomyces griseus* is known to be influenced by exogenous factors of the physico-chemical environment [5]. In the following we have studied: were there acting any endogenous substance(s), regulating these phases of growth, produced by the Streptomyces strain itself.

* A part of this work was read at the Symposium for Microbial Genetics, Moscow, 1962.

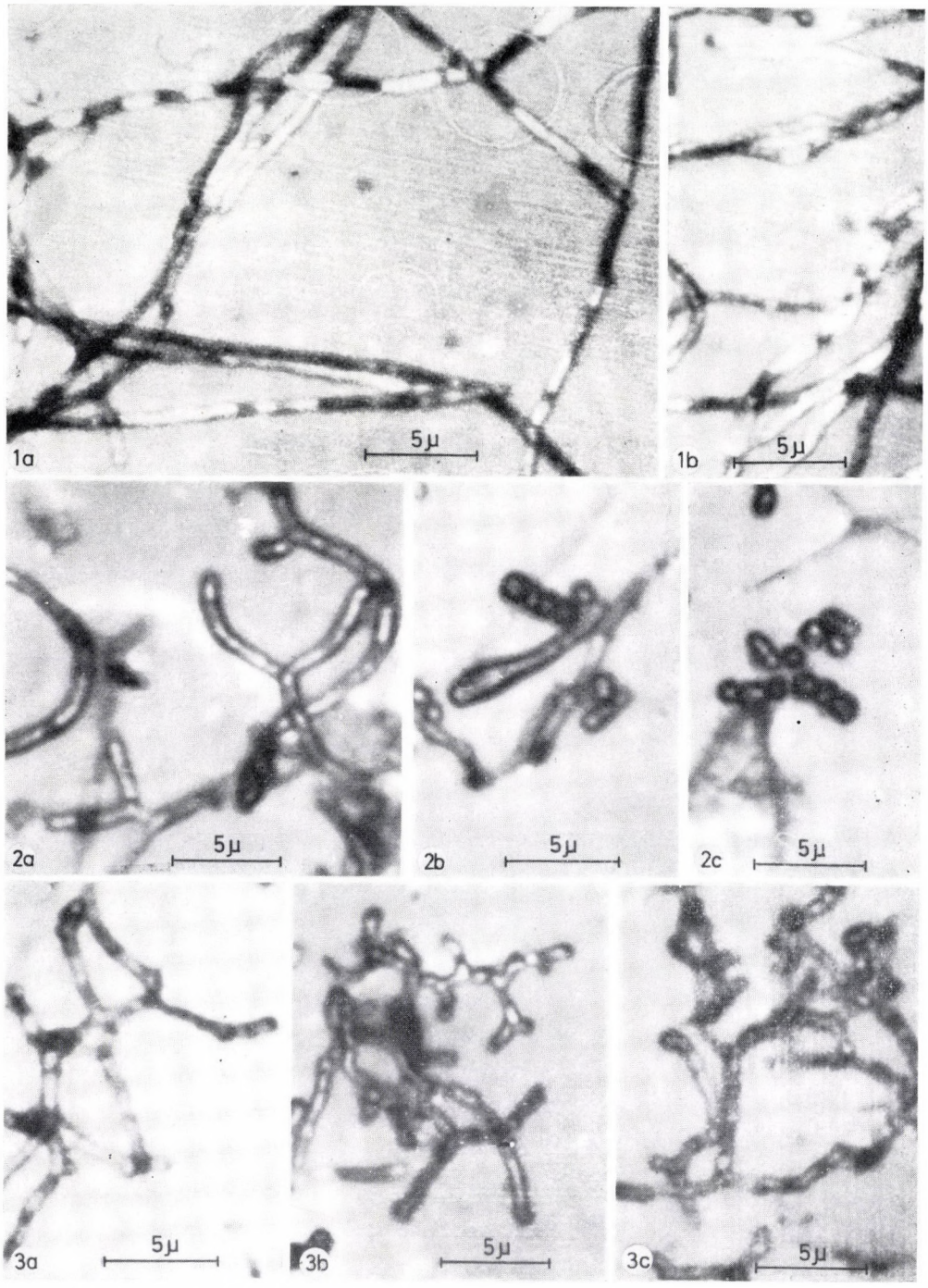

Fig. 1a, b. Streptomyces griseus No. 52—1, 72-hour submerged mycelia. Cell-wall staining
Fig. 2a, b, c. Streptomyces griseus No. 45, 72-hour submerged culture. Cell-wall staining of reproductive hyphae and conidia
Fig. 3a, b, c. Streptomyces griseus No. 52—1, 72-hour submerged culture with factor C. Cell-wall staining of reproductive hyphae

Material and method

Strains and methods of cultivation applied for *Streptomyces griseus* No. 52—1 and No. 45 were described earlier [8]. Other Streptomyces strains are from collections of Streptomyces standard strains from Prof. KURYLOWICZ, Inst. for Hygiene, Warsaw. O_2 consumption was measured in the Warburg apparatus, according to UMBREIT and co-workers [9].

Cytomorphological studies were conducted as described in a previous paper [10], with the exception of the photos, which were made in the microscope with a combination of transmitted and reflected light.

All the chemicals applied were of analytical grade unless otherwise stated.

Results

The experiments were carried out invariably under standard experimental circumstances to avoid the known exogenous influences upon the life cycle of Streptomycetes [5]. It was a good chance — in studying endogenous factors — that we found two kinds of *Streptomyces griseus* mutants, one of a long life cycle (Strain No. 52—1) and another of a short duration of life cycle (Strain No. 45). This latter one has a very short vegetative phase of growth followed by the appearance of reproductive conidiae. The former produced only filamental mycelia in submerged culture. Conidia never appeared.

Streptomyces griseus strain No. 45 during submerged cultivation always produced clumps, which was shown to be a very characteristic feature of the strain with short life cycle [8], (Figs 1 and 2).

The phenomenon. When exchanging the fermentation liquid of the two strains, the filtrate from strain No. 45, which produced conidia, remarkably changed the life cycle of strain No. 52—1, which produced only mycelial growth otherwise. Under the influence of the filtrate (its agent was called, factor C) strain No. 52—1 changed its life cycle and cytomorphology. It produced also conidia and the overall picture of its reproductive appearance became similar to the strain from which the filtrate had been taken (Fig. 3).

The undiluted factor-C-containing filtrate inhibits the growth of the mycelia of strain No. 52—1, but its effect upon the life cycle was not linked with this inhibition. The cytomorphological effect ensued also at concentrations not inhibiting growth. Growth was also inhibited but no sporocide effect could be demonstrated when the spores were mixed with concentrated culture filtrate and cultivated on solid media (Table 1).

There was no decrease in endogenous or exogenous oxygen consumption measured in the Warburg apparatus. The effectivity of factor C remained intact at pH 6.8 to 7.8. The presence of factor C proved necessary for inhibition in a particular period of the life cycle: its removal from the culture medium of the test strain even as late as in the fourth hour of cultivation abolished all its effect, and the strain grew normally.

Table 1

The effect of factor C upon the viability of the spores of Streptomyces griseus

Spore per Petri dish	Average	Incubation time in the presence of effective concentrations of factor C
44, 32, 24, 37, 42	35	0 h
56, 44, 50, 55, 57	52	2 h
29, 20, 30, 47, 32	29	4 h
44, 57, 45, 47, 59	50	6 h

The chemical composition and the physico-chemical properties of the media for cultivation are not changed significantly by factor C: if factor C was added to the medium and then inactivated at 45°C for 20 minutes, the test strain (No. 52—1) grew as if inhibitor had not been present. The factor-C-containing filtrate of the fermentation liquid of strain No. 45 was specific when tested against various kinds of Streptomyces strains. It changed only the form of growth of the *Streptomyces griseus* strains and one *Streptomyces chrysomallus*, but was inactive against the other Streptomyces strains (Table 2). Factor C is produced only in the reproductive phase of growth of *Streptomyces griseus* strain No. 45 (Table 3).

Characteristics of factor C. Factor C does not dialyse through cellophane membrane. It is thermolabile. It is inactivated quickly at a temperature of 45°C (Table 4).

Table 2

The effect of factor C upon various Streptomyces strains

Strain	Effective: + Noneffective: —
Streptomyces griseus No. 173 (3463)	+
Streptomyces griseus No. 175 (J—3475)	+
Streptomyces griseus No. 178 (6961)	+
Streptomyces griseus No. 184 (9004)	+
Streptomyces bikiniensis (11 062)	—
Streptomyces californicus (3312)	—
Streptomyces albus (3004)	—
Streptomyces chrysomallus (3657)	—
Streptomyces coelicolor (3355)	—

Table 3

Production of factor C in submerged cultures of Streptomyces strain No. 45

Time of cultivation (in hours)	Cytomorphology	Effective: + Noneffective: —
14	Germinated spores	—
18	Network of mycelia in clumps	—
24	Clumps with some mycelia	—
38	Clumps, with no or few mycelia	+
42	,,	+
62	,,	+
84	,,	+

Table 4

Effect of heat upon factor C at various temperatures

Temperature	Duration of incubation, minutes	Effective: + Inactivation: —
27°C	0—30—60—120	+
	240	+
37°C	0—30—60	+
	120	±
	240	—
45°C	0	+
	2.5	±
	5	—
	10	—
	20	—

It proved resistant to the hitherto examined enzymes except trypsin when incubated with them for a period of 4 hours at 30°C. Trypsin inactivated factor C (Table 5).

We have succeeded in isolating a little quantity of factor C in the lyophilized state (The procedure of isolation and characterizations will be published later). The almost colourless powder dissolved quickly in distilled water at a concentration of 1 mg/ml. Its UV spectrum is characteristic of a protein or peptide [2]. Factor C can be measured also with the Folin reagent, according to LOWRY and co-workers [3].

It gives a positive reaction with ninhydrine, and with the biuret reagent.

It is not precipitated with 30% TCA but it could be salted out with saturated ammonium sulphate.

Table 5

The effect of enzymes upon factor C

Enzyme 45 μg/ml	Residual activity after for 4h at 30 °C Effective: + Noneffective: —
∅	+
DNAse	±
RNAse	+
Papain	+
Trypsin	—
Pepsin	+
Lysozyme	+
Diastase	+
Hyase	+

It can be hydrolyzed with 6 N HCl and thus its amino-acid composition has been established. No phosphorus could be detected, its reducing sugar content was less than 2%.

Discussion

A switch from the vegetative phase to the reproductive phase and the length of these phases are regulated by exogenous and endogenous factors. Some similarity between the regulation of spore production of bacteria and the conidia formation of Streptomycetes may be assumed, since besides the exogenous factors which influence spore production in bacteria the existence of an endogenous regulatory substance was indicated, too [4].

In experiments with *Streptomyces griseus*, we have found an endogenous substance which may play a role in regulating the growth cycle and morphological differentiation of this organism. The substance seems to be specific, and of polypeptide nature. It is not a transforming or transducing factor. It does not contain phorphorus in a detectable amount, and the new character of producing conidia is not inherited to further generations if transferred on factor-C-free media. Investigations on the composition and the mode of action of factor C offer a remarkable opportunity to study, in a relatively simple system, the chemical nature of the regulatory mechanism of cell growth and differentiation.

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ENDOGENER FAKTOR ZU STEUERUNG DES LEBENSZYKLUS DES *STREPTOMYCES GRISEUS*

Die Steuerung des Lebenszyklus des *Streptomyces griseus* wurde in Schüttelkulturen untersucht. Bei der Auswertung der Versuchungsergebnisse wurden zwei Phasen, die sich im Wachstumsablauf deutlich unterscheiden lassen, die vegetative und die reproduktive Phase berücksichtigt. Aus dem Fermentsaft eines über eine äußerst kurze vegetative Phase verfügenden Stammes wurde ein Stoff (Stoffe) isoliert, der Faktor C. Wird dieser Wirkstoff in die Nährflüssigkeit eines solchen anderen *Streptomyces griseus*-Stammes gemischt, der bei Tiefenfermentation keine Konidien bildet, so ändert sich der Lebenszyklus dieses Stammes, er bildet Konidien. Der Faktor C ist ein spezifischer Stoff von polypeptider Natur. Es wird angenommen, daß der Faktor C als ein die Differenzierung des Stammes steuernder endogener Faktor vom *Streptomyces griseus* selbst erzeugt wird. Verfasser lenken die Aufmerksamkeit auf die vermutliche Bedeutung und die mögliche Verwendung dieses Modells zur Steuerung des Zellenwachstums und der Zelldifferenzierung.

ЭНДОГЕННЫЙ ФАКТОР, РЕГУЛИРУЮЩИЙ ЖИЗНЕННЫЙ ЦИКЛ *STREPTOMYCES GRISEUS*

В встряхиваемых культурах исследовалось регулирование жизненного цикла *Streptomyces griseus*. При оценке результатов авторы принимали во внимание две фазы, выраженно различаемые в ходе роста, вегетативную и воспроизводительную фазы. Из ферментного сока штамма с очень короткой вегетативной фазой было изолировано вещество (вещества), названное авторами фактором С. Если это действующее вещество добавить к жидкой питательной среде другого штамма *Streptomyces griseus*, при глубинной ферментации не образующего конидии, то изменяется жизненный цикл этого штамма, он образует конидия. Фактор С специфическое вещество полипептидной природы. Предполагается, что фактор С производится самым штаммом *Streptomyces griseus* в качестве эндогенного фактора, регулирующего собственную дифференциацию данного штамма. Обращается внимание на предположительное значение этой модели и на возможность ее применения для регулирования роста и дифференциации клеток.

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Acta Biol. Hung., **18**(3), 245—260 (1967)

STUDY OF TWIN ORGANISMS SPONTANEOUSLY DEVELOPED BY ADULT DUGESIA TIGRINA

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(Received June 24, 1966)

Synopsis

Mixed and clone cultures of *Dugesia tigrina* have been studied. Certain individuals of the asexually multiplying population spontaneously developed into twin organisms, a phenomenon for which the term "somatic twin formation" is suggested. The present study served to elucidate the morphogenetic process following the formation of twin organisms from the cephalic end of the adult individual as also that following the fission and regeneration of twin organisms. The phenomenon was found to be hereditary. A reduced integrative capacity of the organism and, in the first place, an inherited lability of the nervous system are presumably involved in the process under review: a spontaneous — though organized — overdevelopment of the central nervous system results in the development of double or multiple twin organisms. Histological examinations to be published in detail in a subsequent paper have confirmed this concept. A low level of nervous integration is favourable for teratogenic processes which are different from twin formation. The dorsoventral outgrowths of this kind may be benign or malignant. They will be dealt with in a later study.

Introduction

Studying the morphogenetic factors of the asexual reproduction, regeneration and somatic embryogenesis of Turbellaria, we extended our investigations, besides species indigenous to Hungary, to *Dugesia tigrina*, a planarian of American origin, which had been introduced to Hungary (presumably from a European country) some 25 to 30 years ago. Other investigators [16, 48] and ourselves [44], have observed only asexual multiplication in the Hungarian population of this species. Sexual reproduction or the formation of sexual individuals [19] has been observed in laboratory, but very rarely.

The appearance of supernumerary organs, various developmental anomalies, teratomas, and structures that may be called benign or malignant tumours have very frequently been observed in laboratory cultures of mixed or clone populations. Except for supernumerary eyes, similar phenomena are rare in other species of the genus (e. g., *Dugesia lugubris*, *Dugesia gonocephala*). Striking frequency of these phenomena in certain clones pointed to their presumably hereditary character. Detailed investigation having made it probable that primarily the morphogenetic activity of the nervous system accounts for

the observed phenomena, our recent study — in accordance with the previous ones — have been focussed on these relationships.

In the present study we investigated: (1) the formation of twin organisms from the cephalic region of the body of adult *D. tigrina* and (2) the processes following the fission and regeneration of the twin organisms, account for the formation of further double or multiple twin organisms. Although the histological findings are not disregarded in the present study, they are not discussed here in detail and will be dealt with in a subsequent paper. Certain histological observations had been reported in our previous papers [44, 48], since then other investigators [42, 43] have published similar investigations.

Material and method

We used mixed and clone populations of *Dugesia tigrina* obtained from local hot springs and aquariums, and bred forth in the laboratory. The cultures have been maintained for more than four years under the conditions described earlier. The animals were observed in vivo under the stereo-microscope. In order to be able to follow the successive phases of twin-formation more closely, we photographed total preparations. The animals were spread out in formalin, fixed with Zenker's fluid and embedded in paraffin. The sections, 4 to 5 μ in thickness, were stained with haematoxylin eosin or by some other routine method.

Results

Supernumerary (accessory) eyes

Normal individuals of *D. tigrina* develop accessory eyes (mostly two, sometimes only one or more than two) as frequently and in the same manner as other species of the genus. They appear bilaterally to the median plane of the body, before the line connecting the normal eyes, slightly closer to the median plane than the latter (Figs 1a, 1b, 1c); later they are shifted somewhat forward in the course of development. Grown to full size, these accessory eyes are equivalent to normal organs in every respect, presumably including function. As has been suggested in earlier papers [46, 47], the accessory eye, an invariable feature of the genus *Dugesia*, is no teratological phenomenon but a developing generic character. Fig. 2 presents the frontal section of the cephalic end of a specimen of *D. tigrina*, showing the normal left eye and the two supernumerary eyes.

Development of twin organisms

Supernumerary eyes and twin-formation. Supernumerary eyes developing at the level of the normal eyes as a consequence of an overdevelopment of the nervous system (cerebral ganglia) offer a morphological basis for following

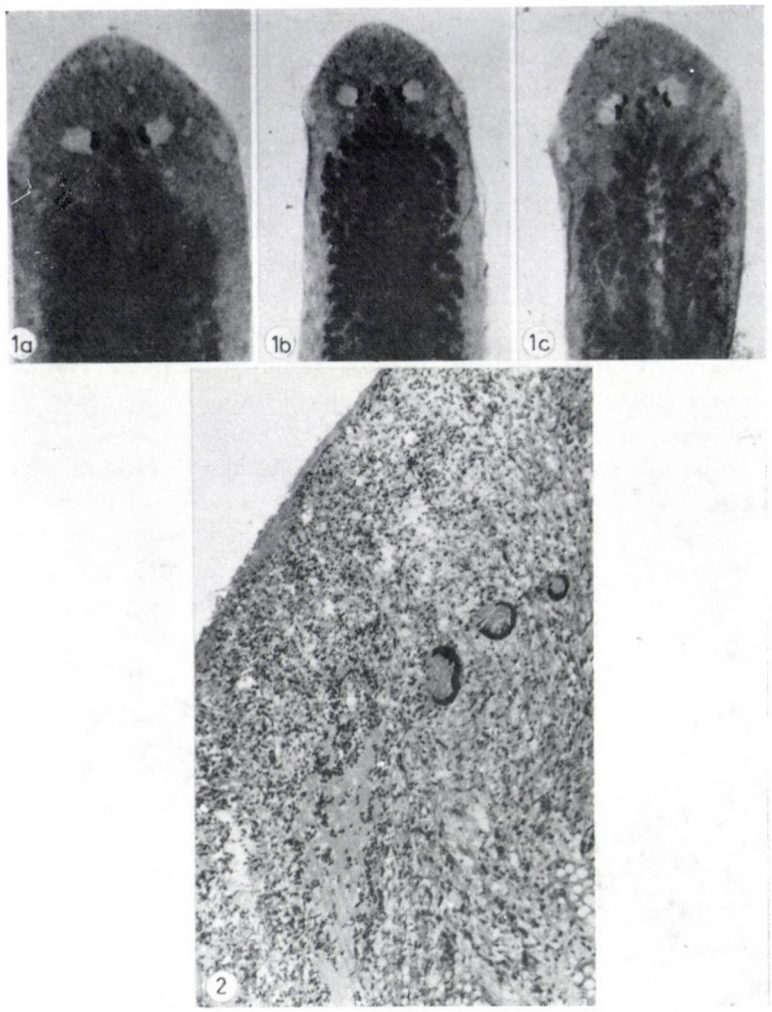

Fig. 1a—b—c. Left: normal specimen. Right: note different developmental stage of the accessory eyes in the two individuals. Total preparations, ×18
Fig. 2. Section showing normal left eye and two homolateral accessory eyes. Ehrlich's haematoxylin and eosin. ×82

the formation of twin organisms. It is, therefore, important to distinguish "normal" accessory eyes from "twin" eyes, both of them being supernumerary structures. The appearance of "twin eyes" is an important, indicative phase in the process of twin formation. Their existence enables us to select from mixed cultures those individuals which are developing into twin organisms and will produce twin progeny in clone cultures.

In contrast to supernumerary eyes, the appearance of twin eyes is preceded by an increase of the distance between the normal eyes. Their develop-

ment is essentially similar to that of the supernumerary eyes. It begins with the formation of a group of pigment cells, which gradually changes into a pigment cup; the next step is the differentiation of visual cells the distal processes whereof grow into the pigment cup, while the proximal processes grow towards the region of the cerebral ganglia. This formation of the optic apparatus is accompanied by the development of a depigmented area above the eyes. The time from the appearance of the fore-runner to the full development of twin eyes lasts six to eight weeks. Number and topography of the twin eyes depend on the organizatory conditions of the developing system, further on the structure and developmental level of the central nervous system.

Twin eyes, as supernumerary organs, are always products of superindividual twin formation, and their topographical and morphogenetic relations to the accessory eyes are the same as those of the normal eyes.

Process of twin-formation. We tried to follow the formation of double and multiple twin organisms partly by the comparison of differently developed twin organisms isolated from mixed cultures, partly by the observation of specimens in clone cultures. The initial specimens of the clone cultures were, as regards twin-formation, at different ontogenetic stages at the beginning of experiments. After their fission, we followed for some time the further development of the parent organisms as also the behaviour of the offspring. The morphogenetic picture of twin-formation was rather variable since the descendants remained capable of transverse fission even in the multiple-twin state and because fission could occur at any stage of twin-formation.

Eleven individuals were selected for clone culture. Six of them were exhibiting the first signs of the expected twin formation, having a medial twin eye and a single pharynx. Among the resulting six clones only one (clone 6) displayed a continuous tendency to form twins, while the progenies of the remaining clones (2, 4, 5, 7 and 8) appeared to be normal during the six months of observation. Clones 2, 4, 5 and 7 remained perfectly normal for a whole year, whereas in clone 8 further twin organisms and teratological phenomena were observed after 9 to 10 months.

Five adult specimens were at an advanced stage of twin formation at the beginning of experiment. Fig. 3 shows the parent organisms of the clones. Clones 1, 3, 6, 9, 10 and 11 developed towards twin-formation or produced offspring all or most of which changed into twin organisms. Clones producing apparently normal offspring were mostly descendants of adults in the 3 eyes +1 pharynx-phase of twin-formation. All descendants of parent individuals at a higher level of twin formation proved to be twin organisms.

We isolated and cultivated into clones numerous individuals of the six twin-producing clones in order to increase the number of clones derived from individuals that had been at the same stage of twin-formation when chosen for clone formation. Our attempts to elucidate the morphogenetic laws of twin-

formation were based on clones of this kind. Fig. 4 shows the number and types of subclones derived from clone 1.

With advancing time, a morphological evaluation of the clones encountered difficulties partly because the total number of individuals was steadily growing and also because of the increasing number of individuals which were at the regenerative stage following fission and, therefore, not completely suit-

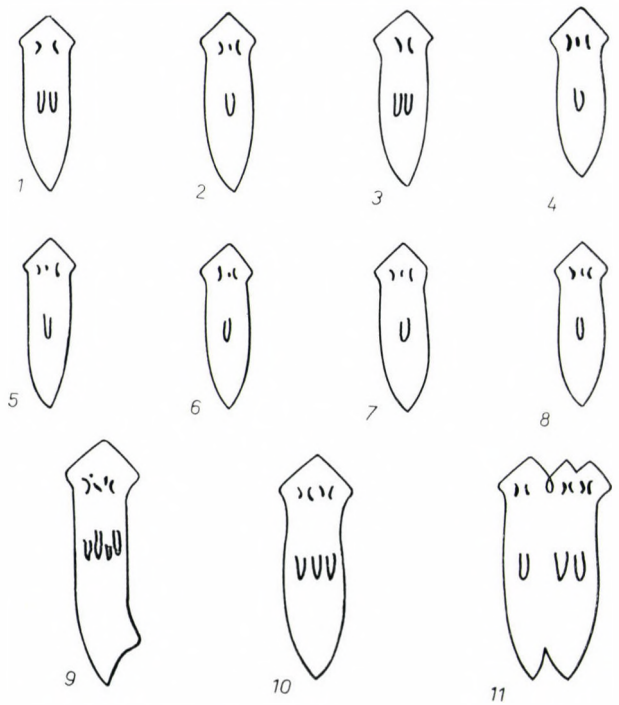

Fig. 3. Parent individuals of the initial clones. The numbers below the diagrammatic drawings indicate the clones concerned

able for evaluation. It had, moreover, to be taken into account that a certain number of individuals would be lost due to cannibalism.

Figs 5, 6 and 7 present the adult specimens of clones 6, 11 and 1d as also the distribution of types at the given time of cultivation.

We propose to describe, first, the process in which normal individuals develop into double and these into multiple twin organisms. Therefore, the following description does not include postfissional morphogenetic processes, in other words, only the normal morphogenetic process starting at the cephalic end and gradually moving in a cephalo-caudal direction will be considered. This process was found to be very slow: it may take many months and even more than a year. It should be noted that there was only a single individual in which the entire process of twin-formation could be followed uninterruptedly.

When isolated, this individual had two twin-eye fore-runners and one pharynx; it developed in the course of a year first into regular double and then into regular quadruple twins. In this early observation, we did not study the behaviour of the offspring. We restricted our investigations in connection with the other adult individuals to the different phases of twin-formation; at the

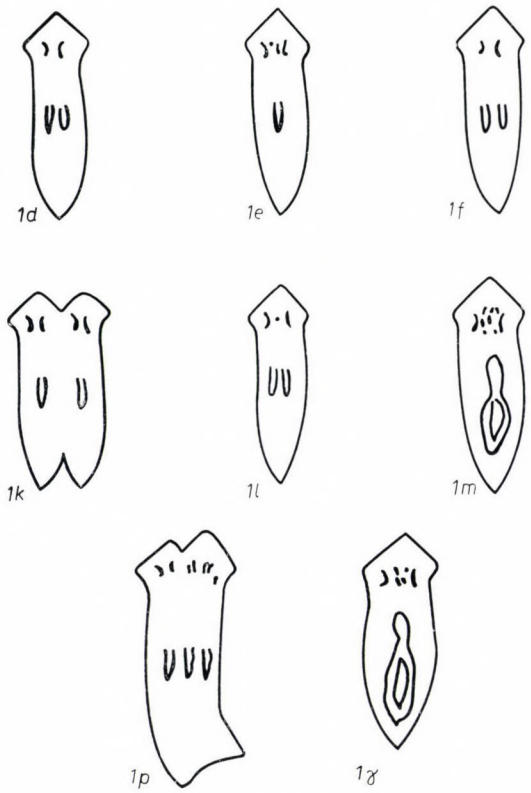

Fig. 4. Parent individuals of subclone (isolated from clone 1)

same time some individuals were prepared for histological study and their progeny was continuously observed in clone cultures.

Observations made on living animals and total preparations have shown that the formation of double twins begins with the transformation of the cephalic end and manifests itself by the appearance and development of twin eyes. In general, there appears, first, the anlage of a single twin eye (Fig. 8) followed by the appearance of a second one. Joining the normal left eye, the left twin eye develops into an eye looking to the right and vice versa (Fig. 9). At the outset, the two twin eyes have a common depigmented visual area, and it is only later that, along with the transverse widening of the head end, two

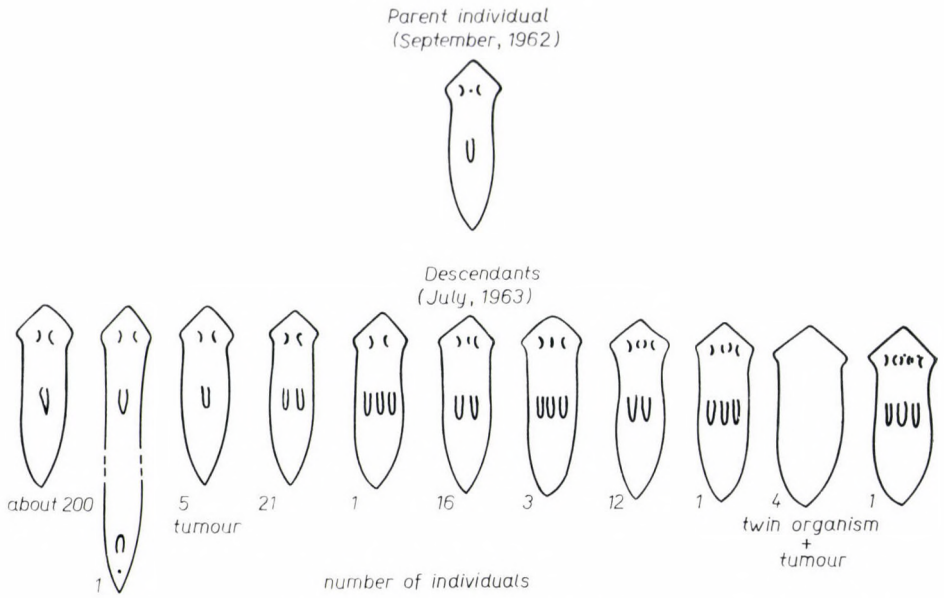

Fig. 5. Parent individual of clone 6 and its progeny. The latter display variable morphology. The second individual is conspicously elongated, and has regenerated eye pigment and a pharynx of inverted polarity

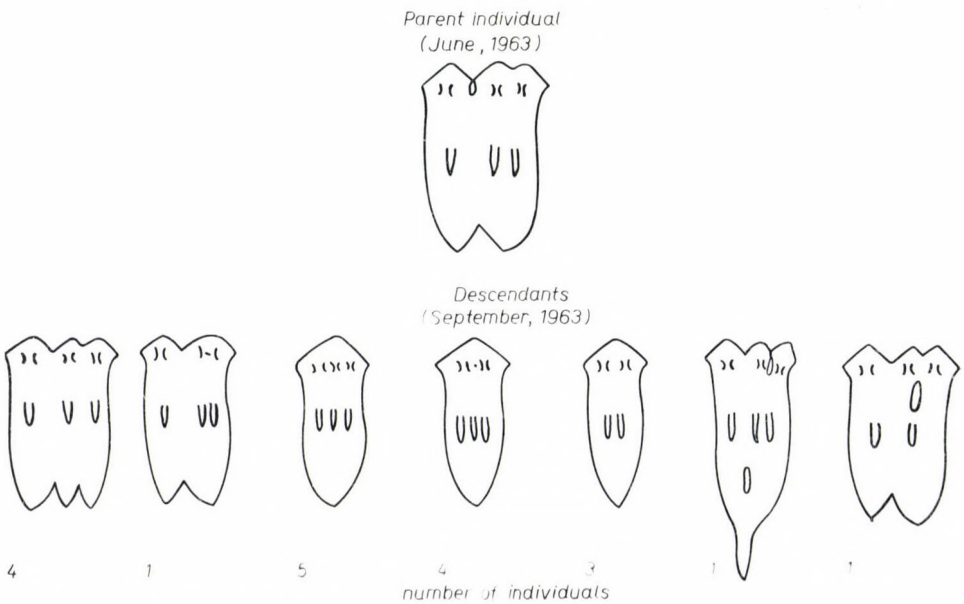

Fig. 6. Parent individual of clone 11 and its progeny. All descendants are twin organisms

separate visual areas are developed. Further morphological changes of the cephalic end consist (in addition to widening) in its transverse flattening and, finally, in the gradual development of two separate cephalic ends (Fig. 10). The width of the head end may be twice the normal size at the termination of the process. The outwardly visible changes of the post-cephalic region consist in cephalo-caudally increasing widening of the body, duplication of the caudal end and the development of a second pharynx (Fig. 11), the anlage of which was observed to appear approximately at the height of the root of the original

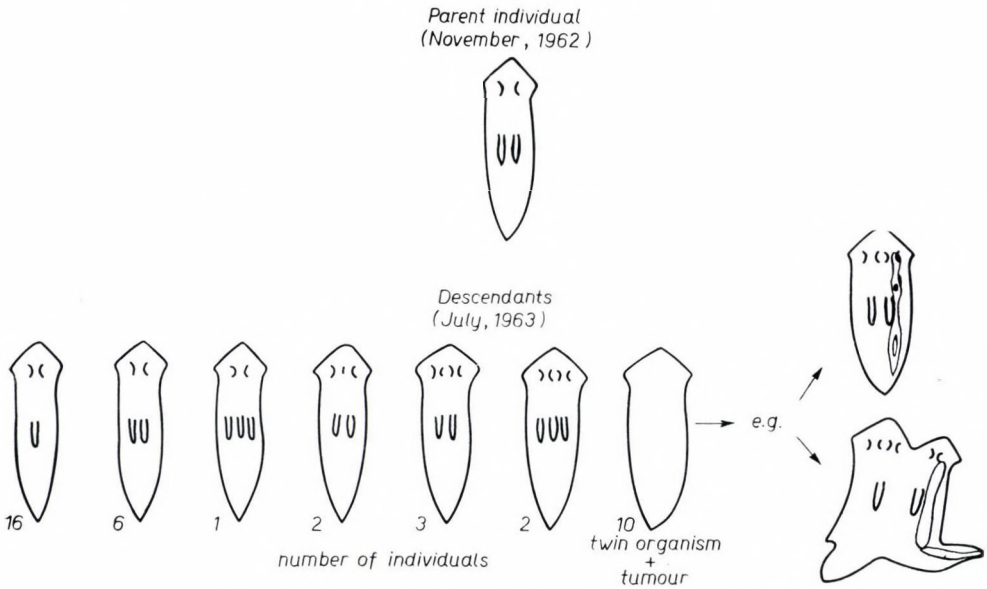

Fig. 7. Parent individual of subclone 1d and its progeny

pharynx, asymmetrically, i. e., either to the right or to the left of the original organ. A gradual displacement of the first pharynx occurs during the growth of the second so that, by the time the new organ reaches the normal size, the two pharynges are symmetrically placed on the two sides of the midline separating the twin organisms. A slowly progressing separation of the cephalic and caudal ends takes place after the complete development of the double twin organisms and results in the formation of two normal heads and the caudal region may develop similarly (Fig. 11). In the specimens observed by us each of the twin organisms developed a complete intestinal apparatus of its own and the two organisms were separated by a zone free of intestinal elements. The twins displayed signs of physiological independence, e.g., in moving nutrition and even multiplication by fission. It happened, for instance, that one of the twins underwent total or partial fission, while no such phenomenon occurred in

the other. In some cases of partial fission the caudal end developed a new head so that an entire new twin organism (of the same polarity as the original) was formed (Fig. 12).

The process of twin-formation did not stop after the double twin organism had developed; it went on, and resulted (at least in typical cases) in quadruple twin organisms. Twin-formation proceeds in such cases in both permanently

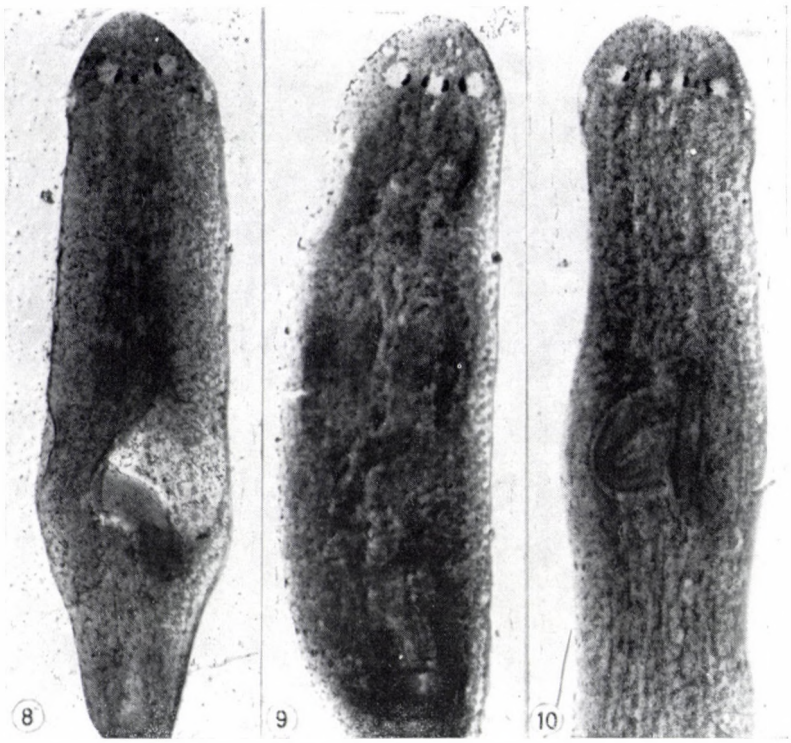

Fig. 8. Specimen with two normal eyes, one twin eye and one pharynx. Total preparation, ×14
Fig. 9. Specimen with four eyes and two pharynges. Total preparation, ×15
Fig. 10. Incipient separation of two individuals: two cephalic ends, four eyes, two pharynges. Total preparation, ×13

conjoined members of the twin organism. The end product is a twin organism with four cephalic, four caudal ends, eight eyes and four pharynges (Fig. 13). The cephalo-caudally oriented development is gradual also in this case so that the reduplication of the pharyngeal and caudal regions ensues later than that of the head end. The further development of the individuals of the double organism may occur synchronously or successively. In the latter case a temporary, or even permanent, triple twin organism may be formed (Fig. 14). The formation of regular triple twin organisms was observed only at integration from the caudal region. No development of quadruple into quintuple, sextuple,

etc., twin organisms was observed. In one of the clones an individual with a fifth pharynx was found, but this pharynx was subsequently pushed out.

As regards life functions and multiplication, multiple twins behave like double twins. There arose, for instance, a "colony" of seven individuals following partial fission (Figs 15, 16).

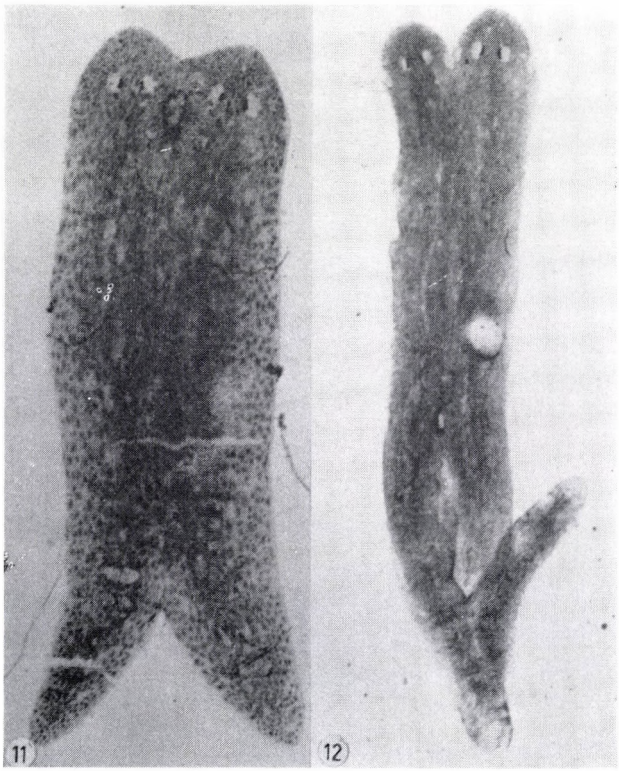

Fig. 11. Typical double twin organism. Total preparation, × 18
Fig. 12. The right-side member of the typical double twins has undergone fission and regenerated the head region independently of the other member. Total preparation, × 10

The morphogenetic processes of twin-formation eventually result in normally organised organs, normal body regions and normal twin organisms. However, some members of certain clones revealed teratogenetic and oncogenetic phenomena. Although it is not proposed to analyse these phenomena in the present study, certain significant observations made in this respect have to be noted on account of their close connection with twin-formation.

Members of the examined clones frequently evolved dorsoventrally growing structures which might be considered as benign tumours on the evidence of their development and morphology. These were not directly influenced by the above-described processes of twin-formation, although many of them contained

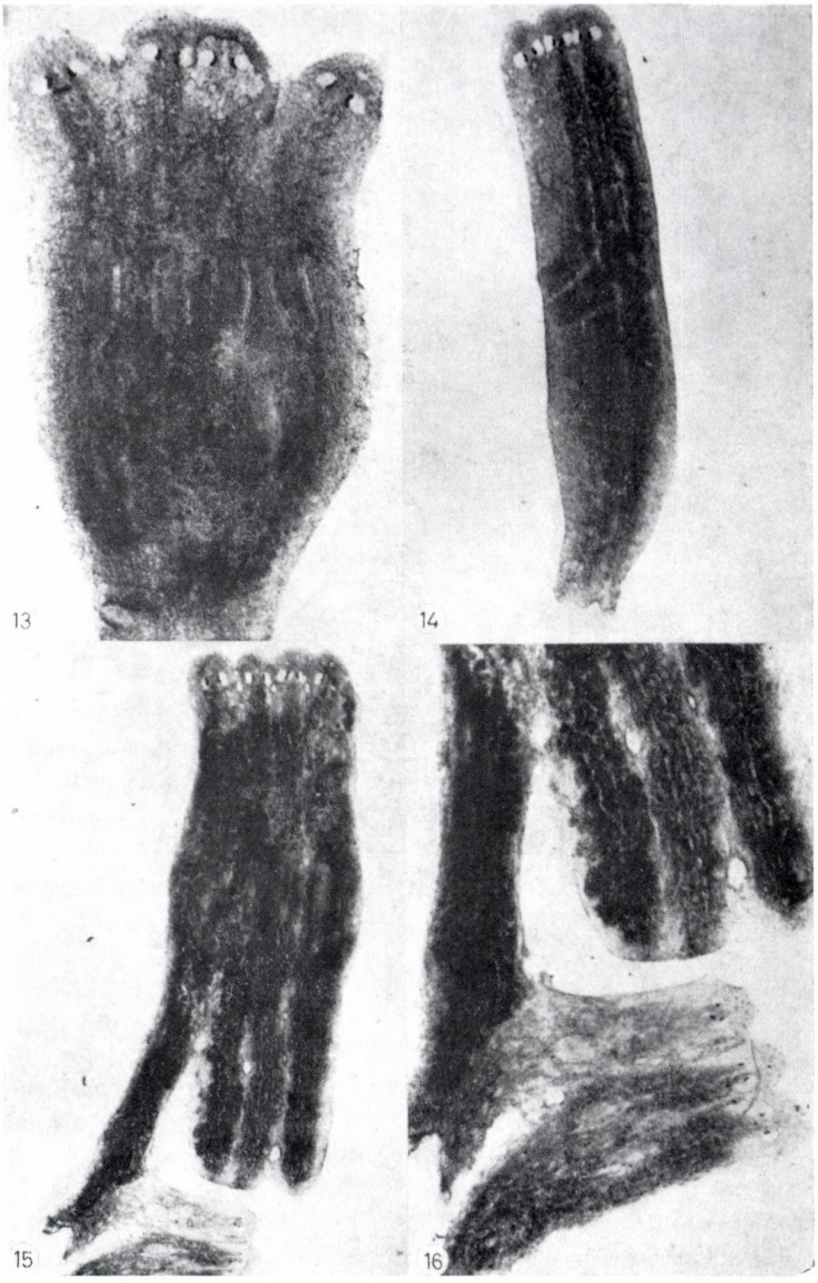

Fig. 13. Quadruple twin organism: four head ends, eight eyes, four pharynges. Total preparation, $\times 17$

Fig. 14. Triple twin organism: three head ends, six eyes, three pharynges. Total preparation, $\times 13$

Fig. 15. The three right-side members of a typical quadruple twin organism have undergone fission, and the detached posterior parts have regenerated three heads. Note the presence of six eyes and three pharynges in the regenerative blastema. Total preparation, $\times 10$

Fig. 16. Enlargement of the caudal region of the body shown in Fig. 10. Total preparation, $\times 18$

supernumerary organs, such as, eyes or pharynges. In connection with other processes, on the other hand, dorsal outgrowths growing also cephalo-caudally were observed which behaved like malignant tumours. These contrast to the "benign tumours", revealed morphogenetic processes which led to the development of atypically located central nervous system, then to the formation of a normal supernumerary individual of inverted dorsoventral polarity.

Discussion

Formation of supernumerary organs is an interesting morphogenetic feature of turbellarian flatworms. The development and regeneration of *supernumerary eyes* [2, 3, 9, 15, 20, 23, 24, 25, 46, 48], *supernumerary pharynges* [10, 26, 28, 29, 35, 36, 40, 43, 50, 51] and *supernumerary genitals* [1, 4, 5, 6, 8, 12, 13, 14, 17, 18, 22, 27, 30, 31, 32, 49] have been investigated by numerous authors. In some species, being due to developmental anomalies or posttraumatic regenerative processes, supernumerary organs cannot be considered as characteristic of the species, while in others they represent characteristics of a whole species or even higher taxonomic units.

It is known that Turbellaria show a great variety as regards number and topography of the eyes [7, 8, 11]. Supernumerary eyes of *Dugesia* have been discussed in the foregoing. Polypharyngia — attributed by some authors [21, 41] to mutation — is characteristic of certain fresh-water and terrestrial Tricladida. Multiple copulatory organs and their accessory glands may — in contrast to polypharyngia — develop independently in different orders of turbellarian flatworms, a phenomenon attributed by Ax [1] likewise to mutation.

As regards the experimental induction of supernumerary organs, Seilern-Aspang [34] produced accessory eyes in *Dendrocoelum lacteum* by cancerogenic agents, while Kishida [20] provoked the depigmentation of accessory eyes by means of dithiocarbamide; the treatment caused a specific disintegration of the pigment cells and damaged the visual cones of the retina.

Experimental twin-formation was obtained in the species *Planaria maculata (Dugesia tigrina)* by Steinmann [37, 38, 39]. He made a longitudinal cut on the cephalic part of the body. After the posttraumatic regenerative processes he got twin organisms with common caudal region. In this case the twin-stage does not refer the whole body, being only partial up to the length of the cut.

Twin organisms were gained in the species *Planaria torva* and *Dendrocoelum lacteum* by Seilern-Aspang [33]. After centrifuging differently aged cocoons of both species, he isolated blastomeres which developed into bilaterally symmetric or asymmetric twin organisms. This phenomenon was, of

course, fundamentally different from the processes discussed in this study, for SEILERN-ASPANG isolated perfectly equivalent blastomeres prior to their development was determined. It is nevertheless mentioned here because both processes lead to the growth of essentially similar twin organisms.

STÉPHAN [43] reported on the experimental regeneration of polypharyngeal individuals of *Dugesia tigrina*. Although his test animals were twin organisms, the author failed to discuss the problem of organization, did not refer to the morphogenetic role of the nervous system, nor did he correlate the development of spontaneous tumours [42] with polypharyngia.

The processes in which twin organisms developed from the caudal region of the body that had come about by fission were closely followed in our clone cultures. As has already been noted, the morphogenetic process in question is not synchronous in the entire organism but proceeds gradually in a cephalocaudal direction. Consequently, twin-formation may be differently advanced at different regions of the body. The character of the regeneration of the cephalic region from the caudal part that came about by fission mainly depends on the stage of twin-formation in the zone which develops the new cephalic region. Number and position of nerve trunks are the principal determining factors in this respect. Regeneration of the ganglia starting from the nerve trunks determines the formation of the head ends, while that of the pharynges depends on the conditions in the cerebral ganglia and the pharynx-forming zone. If, for instance, two nerve trunks are too closely packed, no pharynx is formed between them in due time. Furthermore, intensive pharyngeal growth near the new cephalic region may affect regeneration of the cerebral ganglia, while unequal regeneration of the twin organisms and a tendency of re-individualization in the formation of the head result in a cephalic region that is simpler than the expected twin-state, at least temporarily. All this goes to show why extremely variable forms and widely different phases of twin-formation may occur in clones.

During spontaneous developmental processes occurring at the cephalic end of the adult animal certain cells, escaping the control of the nervous system, participate in the integration of the new individual which remains connected with the parental organism.

The question arises how a phenomenon of this kind can occur in the adult organism. Asexual reproduction involves a disturbance of the organism's normal integration. TOKIN [45] remarks in this respect that the capacity of asexual reproduction is inversely correlated with the degree of integration. Low level of integration or a diminution of integrative power are evidently prerequisites of twin-formation. It would otherwise be inconceivable that somatic cells of a single mature individual produce two or more complete organisms which, though grown together, are independent new individuals. This peculiar phenomenon belongs to the sphere of embryogenesis rather than to

that of regeneration. We think the term "somatic twin-formation" defines it suitably.

It is not impossible that somatic twin-formation is a late manifestation of an atavistic colony-forming tendency. The present investigations regarding the integrative and morphogenetic role of the nervous system were thought to bring this problem nearer to solution.

Spontaneous overdevelopment of the nervous system is probably — at least partly — responsible for twin-formation: it gives rise to normal twin organisms in some cases, and to the development of monsters in others. Such spontaneous overdevelopment may also be due to a lack of morphogenetic autoinhibition, the cause and mechanism of which are obscure.

Hereditary lability of the nervous system and the low level of nervous integration may induce not only somatic embryogenesis, somatic twin-formation, but may also promote morphogenetic processes which belong to the sphere of teratology.

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UNTERSUCHUNG DER SPONTANEN ENTWICKLUNG VON ZWILLINGSORGANISMEN BEI DER ADULTEN *DUGESIA TIGRINA*

Die Untersuchungen wurden an Misch- bzw. Klonkulturen von *D. tigrina* durchgeführt. In den Kulturen dieser sich asexuell vermehrenden Art kann man die spontane Entwicklung einzelner Individuen zu Zwillingsorganismen beobachten. Diesen eigenartigen Vorgang bezeichnen die Verfasser »somatische Zwillingsbildung«. Es werden die Entwicklung von Zwillingsindividuen vom Kopfende der adulten Organismen und die der Teilung und Regeneration der Zwillingsorganismen folgenden morphogenetischen Prozesse beschrieben. Es konnte nachgewiesen werden, daß es sich dabei um eine Erbeigenschaft handelt. In diesen Vorgängen spielen vermutlich die Abnahme der Integrationsfähigkeit und vor allem die erbliche Labilität des Nervensystems eine Rolle. Die spontane, jedoch organisierte Hypertrophie des Nervensystems führt zur Entwicklung von regulären zwei oder mehrfachen Zwillingsorganismen. Dies wurde auch durch histologische Untersuchungen bestätigt, deren Einzelheiten in einer folgenden Arbeit veröffentlicht werden sollen. Das niedrige Niveau der neuralen Integration begünstigt die von der Zwillingsbildung abweichenden morphogenetischen Prozesse, die aber schon zu dem Begriffskreis der Teratogenese gehören. Diese dorso-ventralen Auswüchse wurden auf Grund ihrer Morphologie und Verhaltensweise zwei Gruppen: der Gruppe benigner und der Gruppe maligner Geschwülste zugezählt. Ihre detaillierte Besprechung soll ebenfalls n einer späteren Publikation erscheinen.

ИССЛЕДОВАНИЕ САМОПРОИЗВОЛЬНОГО РАЗВИТИЯ ДВОЙНИКОВЫХ ОРГАНИЗМОВ В КУЛЬТУРАХ *DUGESIA TIGRINA*

Исследования проводились на смешанных и клоновых культурах *D. tigrina* Некоторые особи этого вида, размножающегося бесполым путем, самопроизвольно развиваются в двойниковые организмы. Этот своеобразный процесс авторы называют «соматическим образованием близнецов». В настоящей работе авторы выясняют морфогенетические процессы, происходящие после деления и регенерации двойниковых особей и организмов, развивающихся из головного конца зрелых организмов. Они доказывают, что это наследственное явление, в котором, предположительно, имеет значение уменьшение интегрирующей способности организма, прежде всего наследственная лабильность нервной системы. Самопроизвольная, но организованная гипертрофия центральной нервной системы ведет к развитию регулярных двойных и многократных двойниковых организмов. Это утверждение доказывается также морфологическими исследованиями, результаты которых сообщаются в следующей работе авторов. Низкий уровень невральной интеграции благоприятствует морфогенетическим процессам, отличающимся от образования двойных организмов и относящимся уже в сферу тератогенеза. Эти дорзо-вентральные выростки авторы относят на основании их морфологии и поведения в две группы: в группу доброкачественных и в группу злокачественных опухолей. Подробное описание последних сообщается на другом месте.

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STUDIES ON THE DEVELOPMENT OF CATALASE ACTIVITY AND THE ROLE OF CATALASE IN BIOLOGICAL SYSTEMS

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Synopsis

Experiments have shown that the activation of catalase ensues at a low level of electrode potential, i.e., oxygen tension, when the enzyme is released from the structure. The reduced forms of nontoxic redox systems stop catalase activity at a low substrate concentration. But during bacterial growth the same systems stimulate the activation of the enzyme by reducing the oxygen tension. The inhibiting effect of these redox systems suggests that the H_2O_2-decomposing function of catalase must not be its only function in biological systems.

Introduction

The function of catalase in the biological system is explained generally by decomposition of the H_2O_2 produced by aerobic transhydrogenases [1]. Catalase as an oxido-reductase is in close connection with the biological respiratory system, that is, the structural elements of the cell. As to its structure, catalase consists of a thermostable nonspecific coenzyme and a thermolabile specific apoenzyme [2]. The enzyme is synthesized in ribosomes [3] and it is attached to ribonucleotides [4] in a complex form. From the point of view of localization it may be found in about equal amounts free in the cytoplasm and fixed to its particular elements [5, 6]. The distribution of the enzyme in various tissues of the animal organism is rather uneven. Moreover, a considerable fluctuation may be observed within the same individual under variable circumstances [7].

Experiments with yeast cells have shown that catalase activity significantly increases upon the application of certain surface-active agents or ultraviolet irradiation [8], suggesting that early during growth catalase is localized within the mitochondrial fraction [9], and it is dissolved from the structure under certain physico-chemical effects. This, so-called soluble, catalase is substantially more active, its activation energy is lower, and it is more rapidly destroyed by heat [10]. Thus catalase is altered at the molecular level, rather than at the cellular one [11].

In other authors' opinion the increase in enzyme activity is not due to a structural change, but the catalase occurring partly in latent form can be un-

masked and simultaneously solubilized by different treatments [12]. Besides the decomposition of H_2O_2 catalase catalyses some peroxidase-like reactions, too. Its complex with H_2O_2 can oxidize oxidizable substrates, e. g., alcohols [13]. Catalase can play a role in S-methyl group oxidation, too, which is inhibited particularly by SH compounds, sodium azide and hydroxylamine [14].

As to its stability to heat, FRASER and co-worker [10] observed that the catalase extended on intracellular interfaces is more stable to heat than that which, having separated from the interface, turns into its rolled-up, soluble, highly specific configuration.

In contrast to the tissue and horse-radish peroxidase, its activity is destroyed by boiling [15].

All these observations were made under experimental conditions which were of course rather different from biological systems. Thus the intracellular function of catalase is not yet entirely clear. The purpose of our experiments was to investigate the properties, and the changes in the activity of catalase as function of processes in biological systems and to have a better understanding of its function.

Material and method

The *Staphylococcus aureus* strain used in the first part of our experiments had been isolated from a case of pemphygoid. It is sensitive out of the antibiotics tested only to chloramphenicol, neomycin + polymyxin B and erythromycin. It is untypable with phages, being lysed only by the recently isolated phage 42 D_1. As to its biochemical reactions, it ferments mannitol and produces haemolysin, coagulase and phosphatase. For propagation we used a broth medium containing 8 g concentrated meat extract ("Lamb-Lemco" beef extract, made in England by the Oxoid Division of Oxo Ltd.), 4 g yeast extract (obtained from 100 g yeast boiled in 100 g water under 1 atm pressure and subsequently filtered), 1.6 g NaCl, 1.6 g Na_2HPO_4 and 2.4 peptone per 1 litre. The pH was adjusted to 7.0 with KOH or H_3PO_4.

Cultures were made in 100 ml Erlenmeyer flasks containing 40 ml sterile broth. For inoculation, 2 ml of a 16-hour surface-preculture was used. The flasks were closed with cotton-wool stoppers and shaken at 37°C in a water bath at 220—240 r. p. m. To follow the growth of culture the density was measured. The density was determined in red light (ML filter) by means of a UVIFOT photometer (Hungarian Optical Works). Its value was expressed in (100—T), where T means transparency.

Catalase activity was expressed in mg H_2O_2 decomposed in 5 minutes by 1 ml culture. H_2O_2 was determined iodometrically [16]. For calculating the enzyme activity we used as blank a sample in which the enzyme was inactivated by adding H_2SO_4 at zero time.

The changes of oxygen tension were followed by measuring the electrode potential [17]. A plain platinum electrode used against the saturated KCl-calomel electrode immersed in the culture.

The electrodes showed the same potential decrease when nitrogen gas was driven through the distilled water in which they were immersed. The values of measurements were converted into absolute potential and expressed in mV.

As nontoxic redox system glutathione was used. Its concentration in the medium was 5 m*M*. The control culture contained no glutathione.

The second part of the experiments was made with a two-times-crystallized catalase preparation (Reanal, Finechemical Works, Budapest). Before each experiment a "standard" catalase solution was made by dissolving the preparation in distilled water. Using 0.1 *M* H_2O_2 as substrate, 1 ml catalase solution decomposed 15—20 mg H_2O_2 within 5 minutes.

We made the measurements from 0.1 to 0.05 *M* H_2O_2 concentrations, and the $Na_2S_2O_3$ serving for titration was accordingly prepared.

To study the effect of nontoxic redox systems, ascorbic acid and glutathione were applied in a concentration of 0.1 *M*. In measuring catalase activity we used 1 ml of sodium ascorbate or glutathione. The ascorbic acid content was determined each time by dichlorophenol-indophenol titration. The oxidized ascorbic acid in mg was calculated from the difference obtained from the titrations of samples in which the enzyme activity had been stopped with H_2SO_4 at zero time and after 5 minutes incubation.

Control solutions contained H_2O_2, pH 7 phosphate buffer, and sodium ascorbate or glutathione, without catalase.

Results

For studying the function of catalase the following experiments were performed:

(1) Catalase activity, cell proliferation and electrode potential were followed during incubation of the bacterial culture;

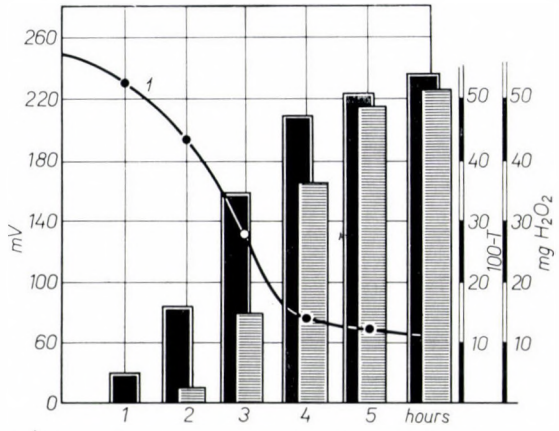

Fig. 1. Changes in the electrode potential (1), density (black columns) and catalase activity (shaded columns) in a *Staphylococcus aureus* culture

(2) The development of the catalase activity during bacterial growth was calculated for one cell;

(3) The effect of natural, nontoxic redox systems on the catalase preparation was examined at different H_2O_2 concentrations;

(4) The effect of the same redox systems on the catalase activity of the bacterium culture during growing was also examined.

(1) The electrode potential in *Staphylococcus aureus* culture fell during growth from the initial value of about 240 mV to about 60 mV (Curve 1 in Fig. 1). The decrease of potential was most intensive in the logarithmic phase and the low value of the potential remained at an approximately stable level in the stationary phase. The density given in (100—T) increased (black columns in Fig. 1) simultaneously with the fall of the electrode potential, showing a close correlation between bacterial growth and the changes of electrode potential.

Measurable catalase activity (shaded columns in Fig. 1) appeared only in the middle of the logarithmic phase and its increase became intensive in the stationary period, which represents a low electrode potential level. So the enzyme activity was delayed behind the density.

(2) The dynamics of the catalase activity per cell can be estimated by calculating the activity for a stable density, i. e., for a stable cell count; the values thus obtained are in linear relationship to the average activity per cell (Curve 1 in Fig. 2). It was found that the catalase activity of the living cell

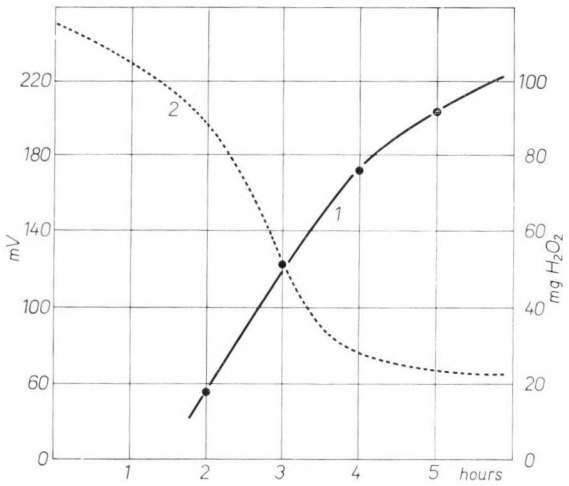

Fig. 2. The dynamics of catalase activity referred to constant density in a *Staphylococcus aureus* culture during growth

becomes intensive only from the second hour of the growth, when the electrode potential values decrease considerably (Curve 2 in Fig. 2). The activity per cell increases first linearly, but further on, with the ageing and autolysis of cells, proteolytic enzymes are liberated and inactivate catalase. Therefore, the linear increase gradually ceases, and the activity reaches a maximum.

(3) Among the nontoxic redox systems occurring in living organisms ascorbic acid and glutathione were found to inhibit the activity of the catalase preparation. The degree of inhibition depends on the substrate concentration, among others. Thus, applying 0.1 *M* H_2O_2, the activity was 85% and 72% in the presence of ascorbic acid and glutathione, respectively, as compared to the 100% control activity. At 0.05 *M* substrate concentration the respective activities were 28% and 21%. In the presence of a substrate concentration of >0.1 *M* and < 0.05 *M* H_2O_2 the inhibition gradually ceases, whereas it becomes complete when the H_2O_2 concentration is < 0.05 *M* (Fig. 3).

(4) Studying the effect of the same redox systems in the growing bacterial culture, we found that like ascorbic acid [18], glutathione speeds up

catalase production: measurable enzyme activity appeared earlier. In the glutathione-containing culture activity is demonstrable already in the first hour (black columns in Fig. 4), contrary to the control culture without glutathione (shaded columns in Fig. 4). Later catalase activity gradually increases in both

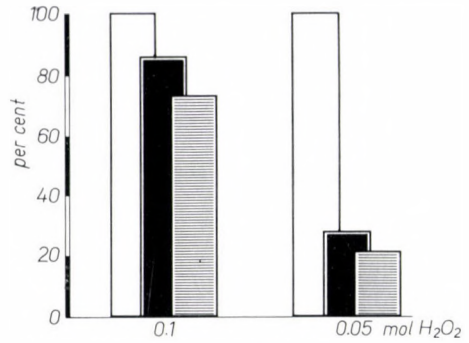

Fig. 3. The inhibiting effect of ascorbic acid (black columns) and glutathione (shaded columns) upon the catalase activity, expressed in per cent of the control value (white columns)

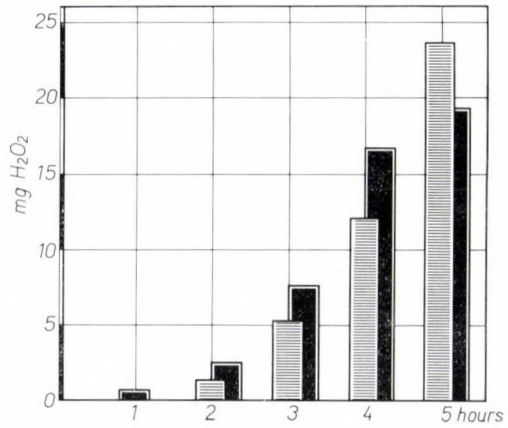

Fig. 4. Catalase activity in *Staphylococcus aureus* cultures in the presence (black columns) and absence of glutathione (white columns)

cultures, but the maximum activity of the glutathione-containing sample remains always lower than that of the control.

Discussion

It becomes ever more obvious that the intracellular localization of metabolic processes, their partial isolation and structural linkage often prevents us from obtaining an exact picture of the enzyme mechanisms even when some physical and chemical factors have been discovered. Thus, changes of the electrode potential in a biological system, e. g., microbial culture, show only the

extracellular oxidation-reduction changes, which are not identical with those occurring in the intracellular structure. Of course, the extracellular conditions have some effect upon the cell, strictly speaking, upon the fine structures within the cell, but it would be misleading to identify the changes in the electrode potential measured in a microbial culture with the changes in the intracellular free energy.

The mitochondrial localization of terminal oxidation ensures the energy supply of biological systems. So the processes demanding a greater energy supply, e. g., the proliferation consuming the energy-rich phosphate bonds, which are formed in the structures, become possible. The cell respiration in microbial cultures is primarily started by the energy demand. However, as the oxygen requirement of the cell respiration is mainly covered from the medium, the changes of the oxygen tension in the culture medium reflect the possibilities for processes in the structure.

Of the components of the culture media generally used, dissolved oxygen is the primary component which accounts for the surface-potential of a platinum electrode; a redox system more stable than O_2/OH^- would hinder the cell respiration. Being the oxygen tension the factor determining the electrode potential, changes in the oxygen tension are important in the release of free energy.

In aerobic biological systems a decrease in the electrode potential during microbial growth indicates a loss of the dissolved oxygen and, indirectly, the intensity of cell respiration [20]. The decreased oxygen content of the medium limits the amount of oxygen diffusing into the cell and, consequently, causes a potential decrease within the cell.

This fact means, on the basis of the connection between free energy and electrode potential, that the potential difference between reduced coenzymes and the O_2/OH^- system manifests itself in a change in the free energy, too. Since the direction of reactions is predetermined by the free energy content of a system, the decrease of the oxygen tension is presumably in close connection with the energy production of the oxidation mechanism and it may play a role in the structural altering of oxidative enzyme systems.

Taking the catalase activity of *Staphylococcus aureus* cultures into consideration from this viewpoint, it may be established that the H_2O_2-decomposing activity of the cultures is intensive in the stationary phase and does not run parallel with the bacterial growth; it is delayed. This delay suggests that the catalase production is "induced" [21]. But this cannot be a genetic induction, partly because H_2O_2 as an inducer cannot be detected in the system, partly because the low potential level, indicating a lowered oxygen tension, does not favour the oxidative processes resulting in H_2O_2 production. Accordingly, the H_2O_2-decomposing activity seems not to be the only role of catalase in the biological structure.

This view is further supported by investigations, suggesting that the activation of catalase is in close connection with the decrease in the electrode potential. Examination of the effects of various reducing substances upon catalase activity has uniformly shown, that in the presence of such substances H_2O_2-decomposing capacity appears in the culture earlier, in spite of the fact that the reducing substances applied are autooxidizable. Thus, catalase activation ensues sooner in an environment with low electrode potential [18].

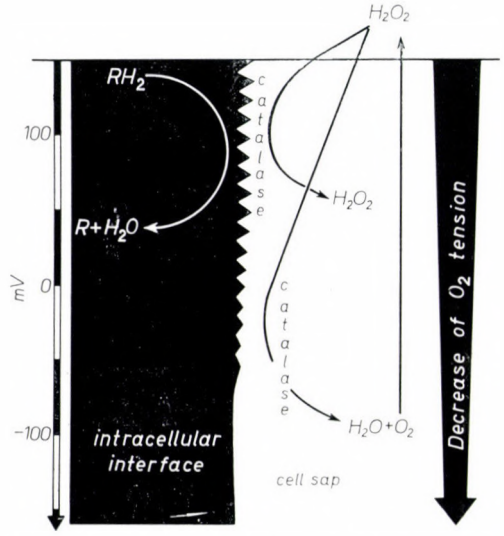

Fig. 5. The supposed mechanism of catalase activation

Also the results obtained while examining catalase induction have proved that the activation of the enzyme is gradual. The catalase activity calculated for a given density — which reflects the average activity per cell — gradually increases after the first period of growth, when the initially high electrode potential level is beginning to decline. The increase in activity is linear only in the young culture. It deviates in the stationary period, showing that the autolysis of cells results in an inactivation of catalase.

The effect of reducing compounds upon the catalase activity is double:

(1) they promote catalase induction in an earlier period of growing;

(2) they inhibit catalase activity.

These are not contradictory properties, for the inhibiting effect manifests itself only in a decrease of activity, while within the living organism a favourable condition for activation comes about. The inhibition in H_2O_2-decomposition is connected with the concentration of the reducing compound and the substrate. Among the reducing agents glutathione is an effective inhibitor at

low H_2O_2 concentrations, while in the presence a H_2O_2 excess its inhibiting effect is not significant.

This means that the natural reducing compounds, e. g., ascorbic acid, stop H_2O_2 decomposition in the presence of a low H_2O_2 concentration. This is certainly valid for biological systems where the concentration of H_2O_2 as of metabolic products in general is extraordinarily low.

Ascorbic acid, which is an inhibitor of catalase activty on the one hand, is oxidized in the presence of catalase more readily, on the other. Even catalase preparations are active in this respect. This fact points to a possible peroxidase-like activity of catalase. Supposedly, it has such a role when fixed to the intracellular structure.

The induction of catalase, its activation when the electrode potential is decreasing and the effect of reducing agents on its activity all prove that at a high level of the electrode potential, i. e., at the beginning of bacterial growth, the H_2O_2 decomposition by the structure-linked enzyme is not characteristic; it is connected rather with a peroxidase-like mechanism. With the decrease of the electrode potential, i. e., of the free energy, catalase is released from the intracellular structures and the enzyme appears in the culture as free a H_2O_2 decomposing enzyme (Fig. 5).

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UNTERSUCHUNG DER ENTSTEHUNG UND DER ROLLE DER KATALASEAKTIVITÄT IN BIOLOGISCHEN SYSTEMEN

Auf Grund der Versuchsergebnisse läßt sich feststellen, daß die Katalaseaktivierung bei niedrigem Elektrodenpotential, das heißt bei niedrigem Sauerstoffdruck erfolgt, wobei das Enzym von der Struktur abgespalten wird. Die Katalaseaktivität wird durch die reduzierten Formen der für die Zellen nichttoxischen Redoxsysteme bei niedriger Substratkonzentration vollkommen gehemmt. Während der Kultivierung bewirken jedoch diese reduzierten Formen durch die Herabsetzung des Sauerstoffdrucks eine Beschleunigung der Enzymaktivierung. Die Hemmwirkung dieser Redoxsysteme weist darauf hin, daß in biologischen Systemen die H_2O_2-Spaltung nicht die einzige Funktion der Katalase sein dürfte.

ИССЛЕДОВАНИЕ ВОЗНИКНОВЕНИЯ И РОЛИ КАТАЛАЗНОЙ АКТИВНОСТИ В БИОЛОГИЧЕСКИХ СИСТЕМАХ

На основании экспериментальных данных авторов можно установить, что активация каталазы происходит при низком электродном потенциале, то есть при низком кислородном давлении, причем энзим отщепляется от структуры. Редукционные формы окислительно-восстановительных систем, не являющихся токсическими для клетки, вызывают при низкой концентрации субстрата полную задержку каталазной активности, однако, во время культивирования они, путем понижения кислородного давления, ускоряют активацию каталазы. Ингибиторное действие этих окислительно-восстановительных систем указывает на то, что расщепление H_2O_2 не представляет собой единственную функцию каталазы в биологических системах.

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THE "NUCLEUS ORGANI PARAVENTRICULARIS", AS A NEURONAL PART OF THE PARAVENTRICULAR EPENDYMAL ORGAN OF THE HYPOTHALAMUS

COMPARATIVE MORPHOLOGICAL STUDY ON VARIOUS VERTEBRATES

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Synopsis

Using the method of comparative morphology, the paraventricular organ was examined in fishes, amphibians, reptiles, birds and mammals. We found in all the examined species and specimens a special group of nerve cells being in connection with the paraventricular organ. The cells are interpreted as constituting a neuronal part of the paraventricular organ besides the ependymal and hypendymal parts. We call this group of neurons "nucleus organi paraventricularis". Going from the vertebrates of lower organization to the higher ones, the group of nerve cells shows signs of gradual differentiation. Although a morphological relation can be observed between the neurosecretory cells and the paraventricular organ, the "nucleus organi paraventricularis" is not identical with the neurosecretory cell groups. Hypotheses based on the morphological aspect about the function of the organ are discussed.

Introduction

The paraventricular organ was described in 1921 by KAPPERS [9] as a twin ependymal organ situated on the side wall of the third cerebral ventricle. Since that time several authors have described this organ in various vertebrates ranging from fishes to mammals [1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13]. In earlier studies we have comparatively examined the Gomori-positive substance of the paraventricular organ in various vertebrates [14, 15]. Starting from the morphological aspect, most authors describe the function of the organ as secretory ("gland-like ependymal structure", KAPPERS [9]), but morphophysiological examinations of the organ are unknown so far.

Some authors point out that there are nerve cells under the ependymal part of the paraventricular organ [5, 12, 13]. In our own comparative studies we have found similar cells in numerous species. The morphological appearance suggested a group of nerve cells belonging to the organ. Since the elucidation of the question with regard to the structure and function of the paraventricular organ would be of utmost importance, in the present work we have examined the existence and the structure of these cells as well as their relationship to the ependymal part in vertebrates standing at different levels of organization.

Material and method

The paraventricular organ was examined in fishes, tailed and tailless amphibians, reptiles, birds and mammals. After paraffin-embedding and serial sectioning, the material was stained with chrome hematoxylin phloxine, aldehyde-fuchsin and hematoxylin eosin. 350 animals belonging to 28 species were examined, at an average of 10 specimens/species. The following species were studied:

Fishes: *Lebistes reticulatus, Brachydanio rerio, Xyphophorus helleri.*
Urodela: *Triturus vulgaris, Triturus cristatus, Pleurodeles waltlii, Ambystoma mexicanum.*
Anura: *Rana esculenta, Bombina bombina, Xenopus laevis.*
Reptilia: *Lacerta viridis, Lacerta agilis, Natrix tesselata, Natrix natrix.*
Birds: *Passer domesticus, Columba livia, Gallus domesticus, Anas platyrhyncha domestica, Turdus merula, Corvus frugileus, Sturnus vulgaris.*
Mammals: *Canis vulgaris, Felis domestica, Myotis myotis, Rhinolophus hypposiderus, Epimys norvegicus, Mus musculus, Erinaceus roumanicus.*

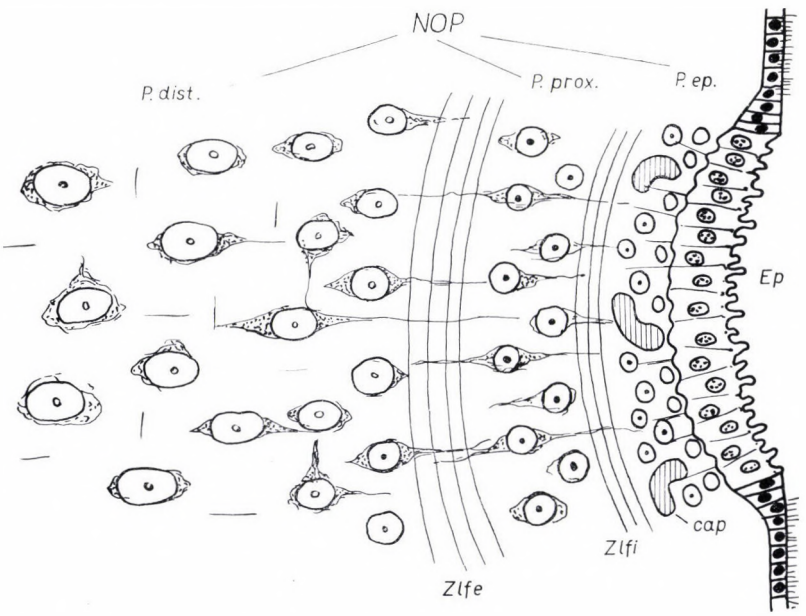


Fig. 1. Scheme of the paraventricular organ. NOP = nucleus organi paraventricularis P. dist = pars distalis, P. prox. = pars proximalis, P. ep. = Pars ependymalis, Zlfe = zona limitans fibrosa externa, Zlfi = zona limitans fibrosa interna, Cap = capillary, Ep = ependymal part

Results

Under the ependymal part of the paraventricular organ we have found in every specimen of all the examined species the specified group of nerve cells situated exclusively along the full length of the paraventricular organ. Common characteristics of the cell group, observed in every species are as follows (Fig. 1). The cells are mostly arranged in rows under the ependyma; the rows begin immediately under the ependyma and, deviating gradually, succeed one another at steadily increasing spaces. The cells next to the ependyma are small-

er than the remote ones. The cells of the external rows are less tightly arranged than the proximal ones. There are in general 5 to ten rows of cells altogether. In species of higher organization, the neurons are situated in two or three groups. The cells belong to the paraventricular part of the periventricular grey

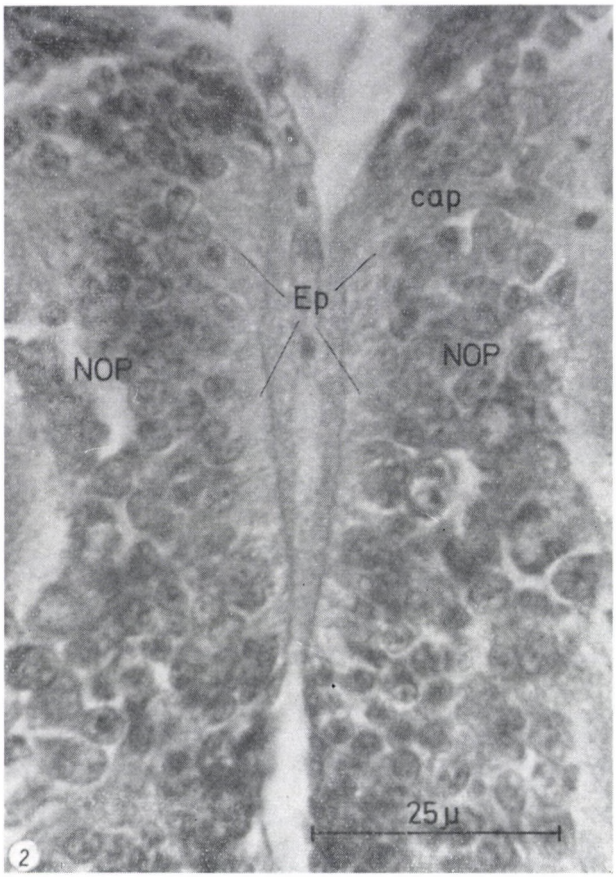


Fig. 2. Paraventricular organ of *Lebistes reticulatus*. Ep = ependyma, NOP = nucleus organi paraventricularis, Cap = capillary. Chrome hematoxilin phloxine stain

matter, and can be clearly distinguished from other periventricular nerve cells by their shape, size, localization and arrangement.

There are differences in the appearance and the degree of differentiation of the nerve cell groups in the various classes examined.

Fishes. (Fig. 2) In fishes the neurons of the paraventricular organ are arranged in 4 or 5 rows, the peripheral cells are large, those close to the ependyma are much smaller. Under the ependyma numerous capillaries are present. There is a great similarity in shape between the other neurons of the periventric-

ular grey matter and the nerve cells of the paraventricular organ which are generally small and grouped immediately around the ependymal part of the paraventricular organ.

Amphibians. In *tailed amphibians* (Fig. 3) the paraventricular organ is not well developed, its nerve cells, too, are undifferentiated. The number of cell

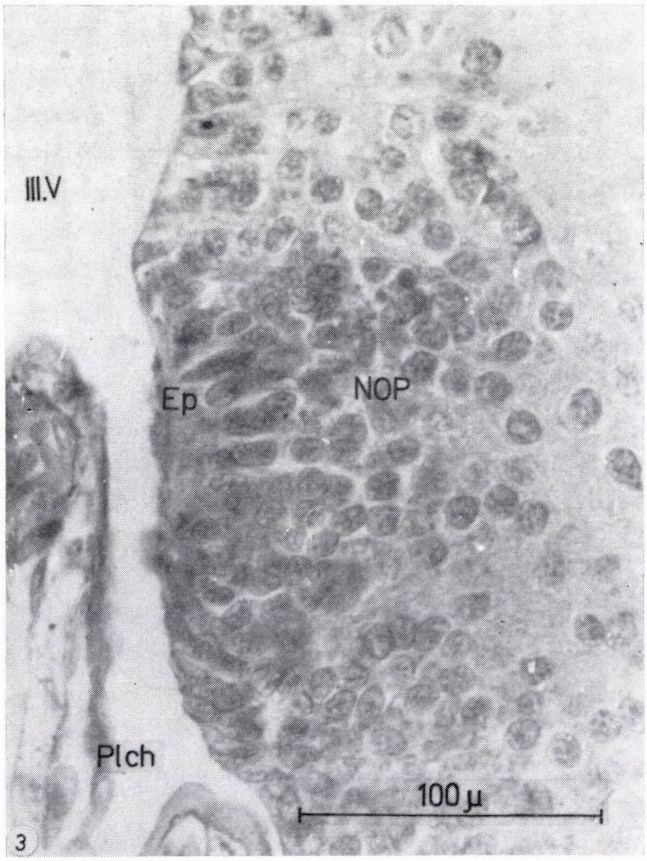


Fig. 3. Paraventricular organ of *Triturus vulgaris.* III V = IIIrd cerebral ventricle, Ep= ependymal part, NOP = nucleus organi paraventricularis, Plch = choroid plexus. Aldehyde-fuchsin stain

rows may be as many as 5 to 7. The neurons are closely pressed to one another and to the ependyma. In *Triturus* species there are no capillaries in the area of the nerve cells and of the paraventricular organ; capillary loops reach up only to the border of the periventricular grey substance. This primitive vascularization is characteristic not only of the territory of the paraventricular organ, but of the whole grey matter of the diencephalon as well.

In *Anura* (Fig. 4) the paraventricular organ is at a higher level of organization. Its neurons are arranged in 5 tight rows, separated from the ependyma by a capillary layer. There is a gradual transition of the adjacent cells to the periventricular grey matter, but the neurons of the latter are readily discernible due to their different size. While other periventricular nerve cells are large in size even next to the ventricle, those above the paraventricular organ are the smaller the nearer they are to the paraventricular ependyma. The nuclei of the neurons situated immediately under the epithelium are most similar

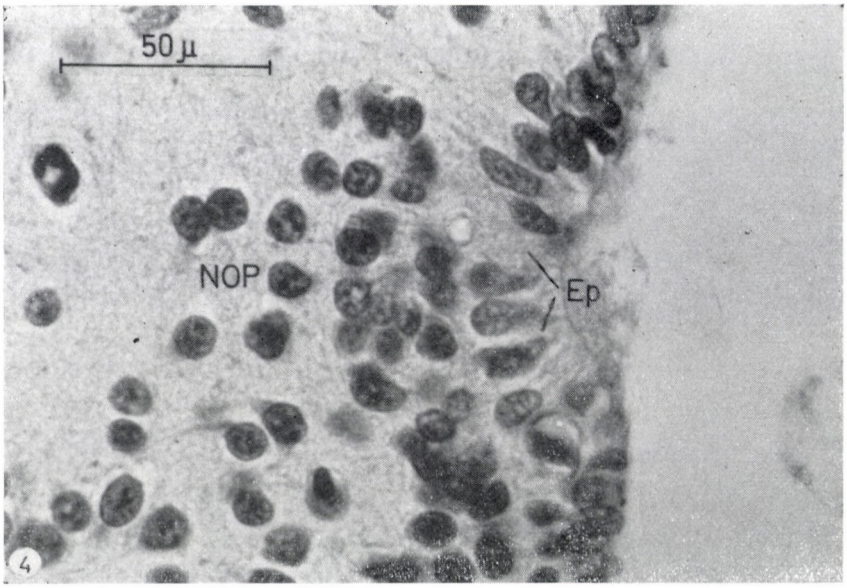
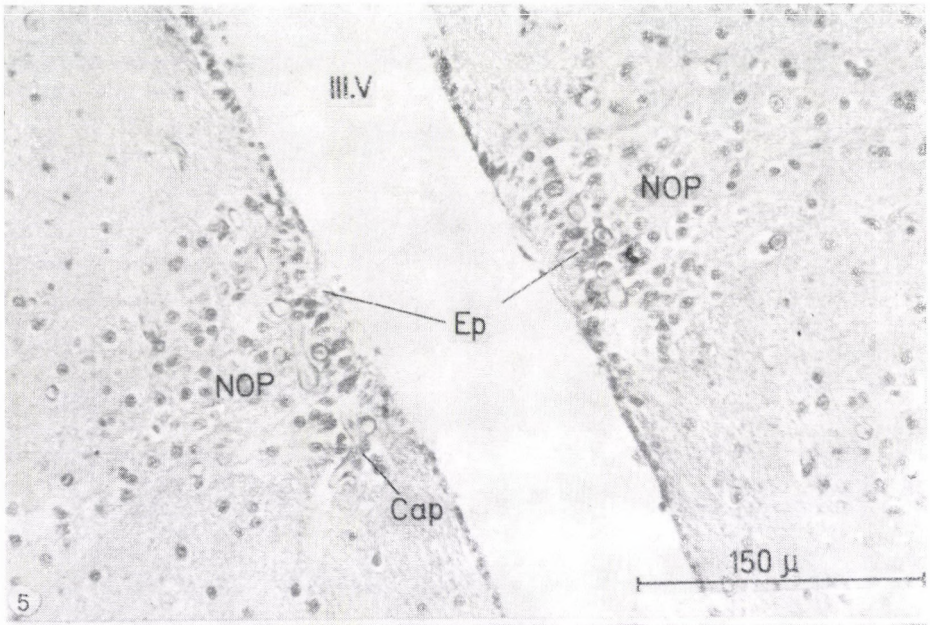


Fig. 4. Paraventricular organ of *Rana esculenta*. Ep = ependymal part, NOP = nucleus organi paraventricularis. Chrome hematoxylin phloxine stain

to those of the ependymal cells. There is no large fibrous layer between the single cell rows.

Reptiles. (Figs 5, 6, 10). The nerve cell group of the paraventricular organ is well developed in reptiles. The neurons are divided into two groups: The "pars proximalis" which is nearer to the ependyma is composed of 4 or 5 rows of rather small-sized cells. The lowest rows are adjacent to the base of the ependymal cells; by means of the histological techniques used by us, a difference between these cells and the basal ependymal cells is shown mainly in the nuclear structure: the nucleus of the nerve cells is round, larger, contains a single nucleolus, and is poorer in chromatin, whereas the basal cells of the ependyma are smaller, possess an oval nucleus containing several nucleoli and more chromatin. A large number of typical bipolar forms can be shown among the neurons with the staining techniques used by us.



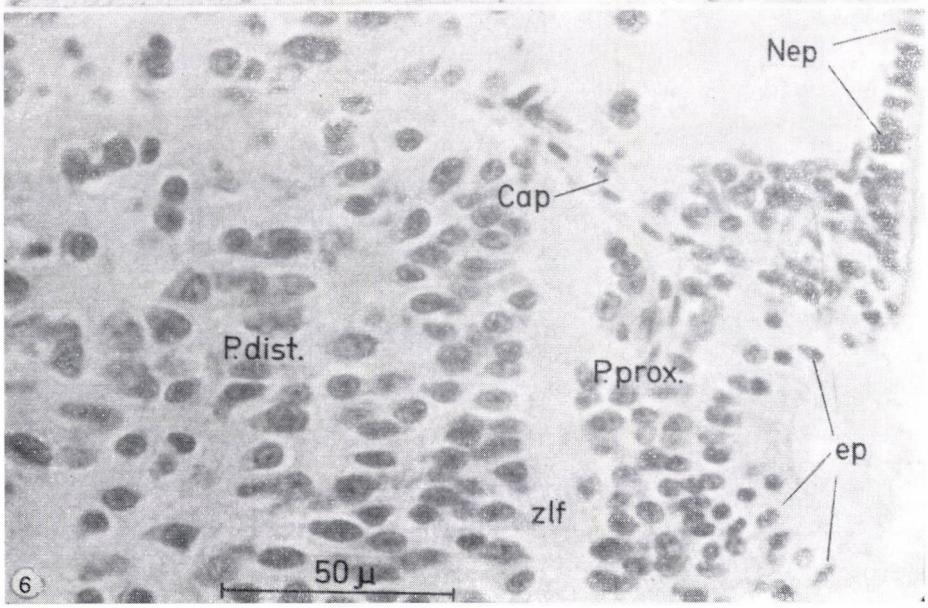


Fig. 5. Paraventricular organ of *Natrix natrix*. III. V = IIIrd cerebral ventricle, Ep = ependymal part, NOP = nucleus organi paraventricularis, Cap = capillary. Chrome hematoxylin phloxine stain

Fig. 6. Part of the paraventricular organ of *Lacerta agilis*. Nep = normal wall ependyma, Ep = ependymal part of the paraventricular organ, P. dist. and P. prox. = distal and proximal part of the nerve cell group of the paraventricular organ, Zlf = zona limitans fibrosa, Cap = capillary. Hematoxylin eosin

The distal nerve cell group ("pars distalis") is composed of 8 to 10 cell rows. Passing toward the ventricle the processes of the neurons can be followed as far as to the ependymal part of the paraventricular organ. The cells are much larger than those of the proximal group and are less tightly arranged.

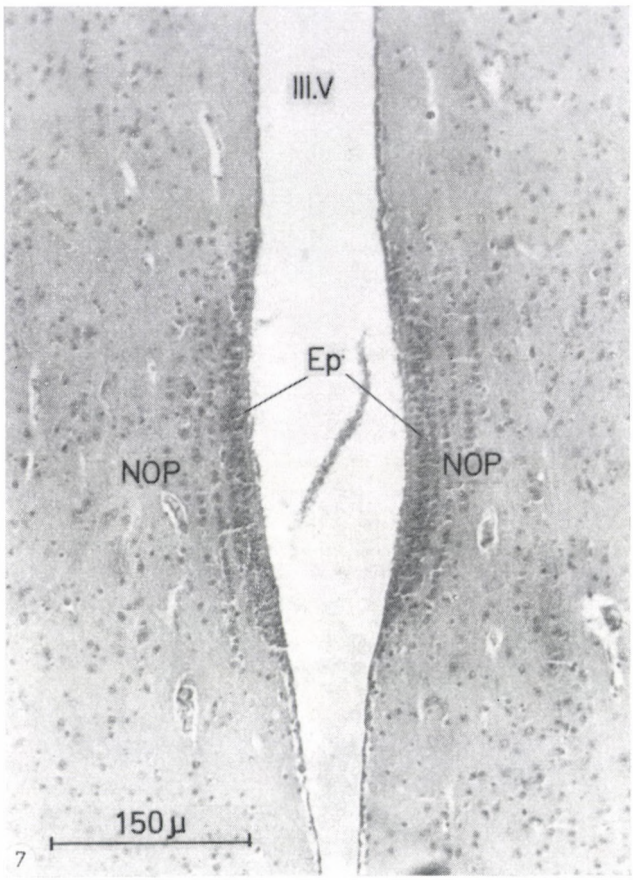
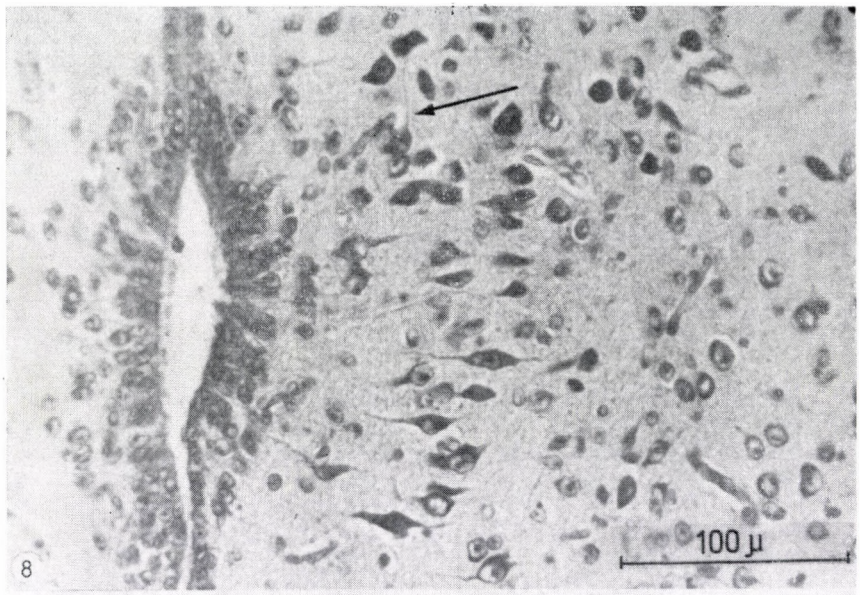


Fig. 7. Paraventricular organ of *Gallus domesticus*. III. V = IIIrd cerebral ventricle, Ep = ependymal part, NOP = nucleus organi paraventricularis, nerve cell rows. Stained with chrome hematoxylin phloxine without preliminary oxidation

The proximal cell group is separated from the distal one by a fibrous layer without neurons ("zona limitans fibrosa").

Birds. (Figs 7, 8). The paraventricular organ and the nerve cell group below it are well developed. In most of the bird species examined by us the nerve cells can be divided into three groups, i.e. the "proximal" group, the "distal" and the "ependymal" cell groups; each being composed of several cell rows. The third group originates by the separation of the proximal nerve



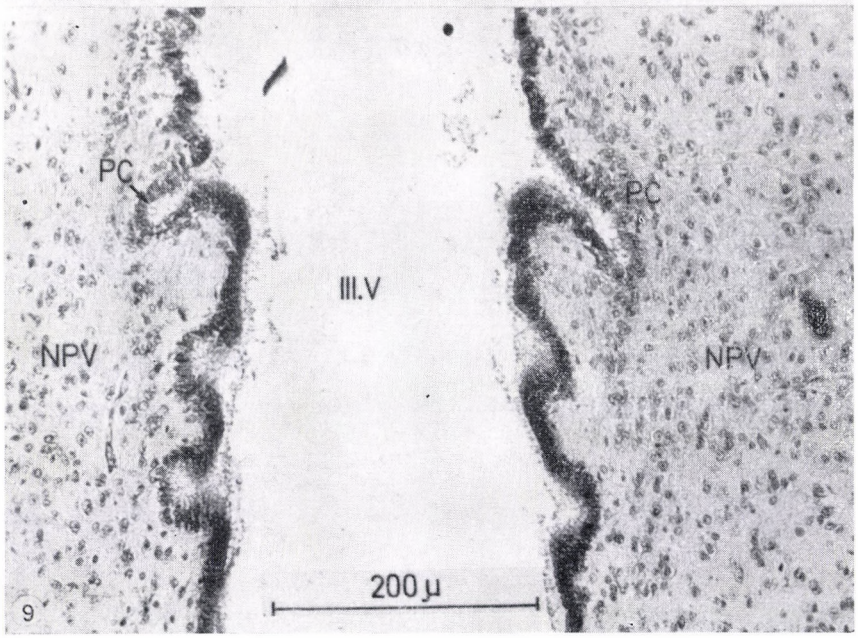


Fig. 8. Part of the paraventricular organ of chicken (white Leghorn). Numerous bipolar cells can be seen in the nucleus organi paraventricularis. At arrow, nerve cell process passing in parallel with the surface. Chrome hematoxylin phloxine

Fig. 9. Paraventricular organ of *Felis domestica*. III. V = IIIrd cerebral ventricle, PC = paraventricular ependymal crypts, NPV = circumscribed cellular areas of the periventricular grey matter located under the special ependyma. Chrome hematoxylin phloxine

cell group from the ependymal layer by means of a thin transversal fibrous zone ("zona limitans fibrosa interna"). However, small cells can be observed at the base of the ependyma, too, constituting the third neuronal group ("pars ependymalis"). Here, too, the most differentiated and largest cells can be seen in the outermost group. In this area multipolar neurons can be found. Some of their processes are passing partly perpendicularly partly in parallel to the surface (Fig. 8). The neurons are arranged in a diffuse pattern. As compared with

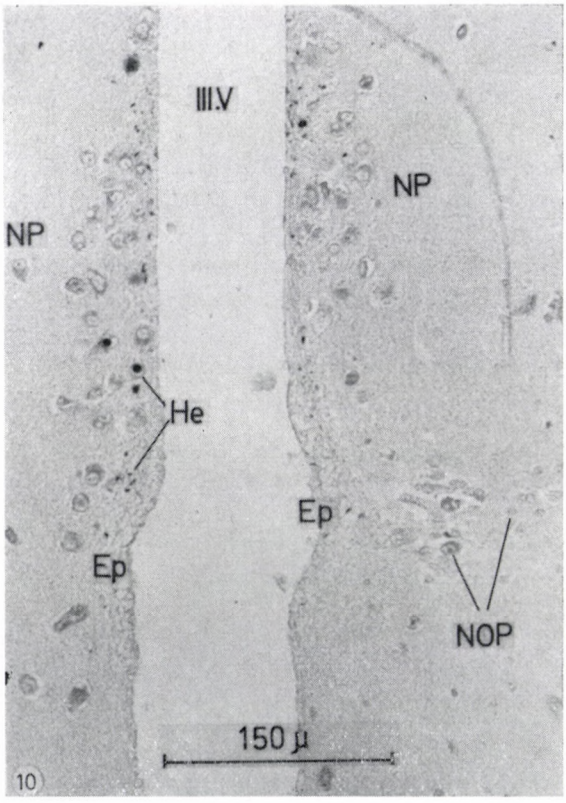


Fig. 10. Paraventricular organ of *Natrix natrix*. Ep = ependymal part of the paraventricular organ, NOP = rostral end of nucleus organi paraventricularis, NP = neurosecretory nucleus paraventricularis, III. V = IIIrd cerebral ventricle, He = Herring bodies. Aldehyde-fuchsin stain

the picture in lower vertebrates, the external or distal group has moved off rather far away from the ependyma. The distal neurons are separated from the proximal ones by a layer of unmyelinated nerve fibres ("zona limitans fibrosa externa").

Mammals. (Fig. 9). In mammals the ependymal part of the paraventricular organ differs from that of other vertebrates in so far as the surface of the

ependyma is neither straight nor arched, but it shows several protrusions and hollows, forming a semiglandular structure, "paraventricular crypts". In the species examined by us, a characteristic group of nerve cells can be observed under the paraventricular ependyma in the periventricular grey matter. These neurons are dissimilar to those of the lower vertebrates examined, their localization in rows is not strongly marked, and their relation to the ependyma is less close. The techniques used by us failed to give a detailed picture of the cell processes.

The relationship between the neurosecretory cell groups and the nerve cell groups found by us was also studied. In fishes and amphibians where the neurosecretory nerve cells are represented by the nucleus praeopticus, the neurons of the paraventricular organ are always more caudally situated than the secretory nucleus. In agreement with several authors [11, 12], we have also observed that some of the ventricular processes of the neurosecretory cells reach the area of the paraventricular organ. In reptiles and birds the rostral end of the paraventricular organ and of its nerve cell group is exactly at the level of the caudal end of the neurosecretory nucleus paraventricularis. In some sections the two groups of nuclei appear together (Fig. 10). In mammals the neurosecretory nucleus paraventricularis is situated nearer to the paraventricular organ than it is in the lower species, but it accompanies the paraventricular organ only along a short section and lies ventrally to the "proper" cell group of that organ.

In the nerve cells of the paraventricular organ the special neurosecretory stainings were consistently negative in all the vertebrates studied, and no sign indicative of neurosecretion could be observed.

Discussion

The existence of nerve cells next to the paraventricular organ was first mentioned by E. LEGAIT [12] in a dissertation written in 1942. H. LEGAIT [13] also mentioned these neurons in his work about the neurosecretory activity of the Rhode Island red hen: "En dehors des cellules épendymaires, il existe deux ou trois rangées parallèlement aux cellules épendymaires, dessinant deux ou trois arcs de cercle superposés." In 1957 FLEISCHHAUER [5] reported on nerve cells to be found in connection with the paraventricular ependyma of *Testudo graeca*. With respect to the neurons, he considers the ependyma as "matrix" ependyma, i. e., an epithelial area able to produce nerve cells in the adult, too.

Since we have found such neurons in all of the vertebrates examined, it may be suggested that, in addition to the ependymal component and the hypendymal one, also a group of nerve cells belongs to the paraventricular organ. The cell group termed by us "nucleus organi paraventricularis" can be found in lower vertebrates as well, but its degree of differentiation gradually increases

in higher species. The cell group shows the most primitive structure not in fishes but in tailed amphibians, where the "nucleus organi paraventricularis" is represented by a group of small cells lying circumscribed in the periventricular grey matter. This is in agreement with the finding that the periventricular nerve cells of the examined amphibians are in general of a less differentiated structure than those of the fishes examined by us. In reptiles the nucleus organi paraventricularis is well developed; several bipolar neurons occur among the cells. One process of the cells goes towards the ependymal part of the organ. The methods applied in the present work failed to determine the mode of connection between the ependyma and the nerve fibres, but such a connection is obvious.

The nucleus organi paraventricularis of birds is still more developed than that of reptiles. Here, the cells are arranged in three groups at different distance from the ependyma, the groups being separated from one another by a typical transversal zone of nerve fibres ("zona limitans fibrosa"). Progressive differentiation is confirmed also by the fact that in higher developed animals the nerve cells migrate farther from the ependyma.

Even in birds, some neurons remain close to the ependyma ("pars ependymalis"). These cells lie immediately at the base of the ependyma and are very small in size. A review of literature seems to show that most of the authors have considered them to be a basal row of ependymal cells. A detailed examination will show, however, that the ependyma itself is composed only of one or two rows, while the remaining rows do not consist of ependymal cells but of small nerve cells (the pars ependymalis of the paraventricular organ's nucleus). At lower magnification these neurons appear as forming a large ependymal layer composed of several rows, as described in most of the works reporting upon the paraventricular organ. There is thus a considerable difference between the ependymal part of the paraventricular organ and that of the subcommissural organ, since the ependymal part of the latter is very thick itself, consisting of several rows of plasmatic ependymal cells. On the other hand, the ependymal part of the paraventricular organ is composed only of one or two layers of ependymal cells (the other cells being neurons). All this might indicate a functional difference between the two organs.

In the mammals, the paraventricular organ shows a special morphological picture. Since the structure of the ependymal and neuronal part is somewhat different from that of lower vertebrates, it might be suggested that the ependymal formation to be found here is not quite the same as the paraventricular organ described primarily in lower animals.

The finding that the paraventricular organ has a neuronal part besides the ependymal and the hypendymal parts substantially affects any functional conception relative to that organ. In reptiles and birds, the presence of a large number of bipolar cells in the nuclear group is worthy of attention (bipolar

neurons are generally known as sensory). According to KAPPERS [9], most authors regard the function of the organ as a secretory. Secretory activity is shown mainly by the stratified plasmatic ependyma, by the resemblance to the subcommissural organ and by the accumulation of substances of variable staining properties in the ependymal cells of the paraventricular organ and, above it in the cerebral ventricle. In the present investigations, however, the demonstration of a well-developed neuronal part of the organ may suggest a significant nervous regulating or sensory function in addition to, or in correlation with the secretory function.

FLEISCHHAUER [5] expresses a special opinion, considering the paraventricular organ — mainly in lower species *(Testudo)* — as a germinative ependyma, i. e., as an epithelial area producing cells in the adult animal, too. However, some morphological facts observed especially in lower vertebrates are inconsistent with this conception. In amphibians, for instance, the whole paraventricular substance is arranged in rows, the different rows corresponding to the embryonal mitotic periods of the germinative cells and to the migration waves of the neuroblasts. So, in the periventricular grey matter, the number of divisions can be readily judged by the number of cell rows, and the differentiation of the cell rows by their distance from the ependyma. In an adult animal the number of cell rows of the nucleus organi paraventricularis is the same as that of the cell rows of the remaining periventricular grey matter. It is therefore improbable that a larger number of cell divisions should take place here than elsewhere in the periventricular grey substance. The only difference is that the cell rows in this area are smaller and lie nearer to the epithelium, and this fact permits to draw conclusion on their lower state of differentiation only.

Finally, as far as the relationship between the nucleus organi paraventricularis and the neurosecretory system is concerned, it should be mentioned that — according to our examinations — the nerve cells of the paraventricular organ are not identical with either of the neurosecretory cell groups. The possibility that the nucleus organi paraventricularis might be, for instance, an accessory part of the neurosecretory nucleus paraventricularis may be excluded by considering that we never could demonstrate any secretory phenomena in the neurons of the paraventricular organ by means of special staining methods.

Although the present comparative morphological examinations do not permit to decide unequivocally in favour of one or the other hypotheses on function, they nevertheless point out a definite direction to further morpho-physiological investigations of the paraventricular organ. We expect a progress in the field of the present morphological questions mainly from our current electron microscopic studies and our detailed investigations with impregnation techniques.

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DER »NUCLEUS ORGANI PARAVENTRICULARIS«, EIN NEURONALER TEIL DES PARAVENTRIKULÄREN EPENDYMORGANS DES HYPOTHALAMUS

Vergleichende morphologische Untersuchungen in verschiedenen Wirbeltieren

Das Paraventrikularorgan wurde in Fischen, Amphibien, Reptilien, Vögeln und Säugern mit der vergleichend-morphologischen Methode untersucht. Es wurde festgestellt, daß in den von uns untersuchten Arten und Individuen eine charakteristische Nervenzellgruppe in Verbindung mit dem Paraventrikularorgan zu finden ist. Diese Zellen wurden als ein Teil des Paraventrikularorgans aufgefaßt, der neben dem ependymalen und hypendymalen einen neuronalen Teil bildet. Zur Bezeichnung der Zellgruppe benutzten wir den Ausdruck »Nucleus organi paraventricularis«. Die Nervenzellgruppe zeigt fortschreitend von den niederen Wirbeltieren zu den höheren Vertebraten Zeichen gradueller Differenzierung. Zwischen den neurosekretorischen Zellen und dem Paraventrikularorgan kann eine morphologische Beziehung beobachtet werden, doch ist der »Nucleus organi paraventricularis« nicht mit der neurosekretorischen Zellgruppe identisch. Es wurden die Hypothesen diskutiert, die bezüglich der Funktion des Organes aus dem morphologischen Bild aufgestellt werden können.

NUCLEUS ORGANI PARAVENTRICULARIS — НЕЙРОНАЛЬНАЯ ЧАСТЬ ПАРАВЕНТРИКУЛЯРНОГО ЭПЕНДИМАЛЬНОГО ОРГАНА ГИПОТАЛАМУСА

Сравнительные морфологические исследования у различных видов позвоночных

Авторы исследовали методом сравнительной морфологии у рыб, земноводных, пресмыкающихся и млекопитающих паравентрикулярные органы. Было установлено, что у исследовавшихся видов и особей в паравентрикулярном органе обнаруживается своеобразная группа клеток. Авторы рассматривают эти клетки как часть паравентрикулярного органа, которая вместе с эпендимальной и гипендимальной частями образует нейрональную часть органа. Для обозначения этой клеточной группы авторы предлагают название **nucleus organi paraventricularis**. В направлении от низших позвоночных к высшим эта группа клеток показывает признаки постепенной дифференциации. Между нейросекреторными клетками и паравентрикулярным органом можно выявить морфологическую связь, однако, **nucleus organi paraventricularis** не является идентичным с нейросекреторными клеточными группами. Выдвигаются гипотезы о функции органа, предполагаемые на основе морфологической картины.

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EXPERIMENTAL STUDY OF THE ACHONDROPLASIA GENE EFFECTS IN THE MOUSE

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Synopsis

The autosomal recessive gene for achondroplasia (symbol cn) in homozygous condition inhibits the bone growth in the mouse. Subcutaneous implantation of long limb bones between 7- or 14-day-old cncn mice and their normal litter-mates have shown that the primary cn gene effect is manifested in the chondrocytes of the cartilaginous skeleton. On the other hand, the suppression of the bone growth is redoubled by a growth-inhibiting substance(s) which is (are) released into the blood. Electrophoresis on polyacrylamide gels showed approximately the same growth hormone content in the extracts of the anterior pituitaries of 2-, 8- and 12-week-old cncn mice as in these prepared from their phenotypically normal litter-mates. Both serum mucopolysaccharide and blood sugar concentrations are at the normal levels in cncn mice. The pathomechanisms of hereditary achondroplasia in human and animals are discussed.

Introduction

Hereditary achondroplasia is one of the serious lesions of the skeleton. It has been characterized by the inhibition of the interstitial growth of cartilage bones; the growth of the membrane bones is not disturbed. The mechanism of the achondroplasia is not clear. Studies on animals may promote the understanding of the mechanisms of action of the homologous mutant genes in human. More then seventy inheritable skeletal disorders, among them achondroplasia, have been described in animals [17]. The various methods of the experimental analysis have been widely used in the studies on the pathomechanisms of the achondroplasia in the chicken. The results of the implantation of limb primordia from 3rd-day chick embryos homozygous and heterozygous for the Creeper gene (symbol Cp) to normal embryos [18, 19] and the transplantations of prospective limb material from CpCp (incubated for 24—32 hours) to 60 hours' normal embryos [31] have shown that the primary Cp gene action is manifested directly in the chondroblasts. Similar data were found when limb bone rudiments were explanted from 6.5 to 7-day-old Cp+ embryos [38, 39]. The same data were shown in an *in vitro* study of the effects of diplopod[4] (dp^4) gene, which was localized in the X-chromosome. KIENY and ABBOTT [20] explanted chick limb bone rudiments and showed that dp^4 gene

acted in the cartilage cells and led to a disturbance of its growth and differentiation. Using organ cultures, it was shown that the brachypodism-H (bp^H) gene had similar effects in the mouse. The primary effect of the bp^H gene is manifested in the inhibition of hypertrophy of the chondrocytes in the fibula and femur primordia. The growth of the tibia is depressed secondarily in the bp^Hbp^H embryos. Apparently the bp^H gene acts in the chondroblasts arising from the postaxial mesenchyme, depressing their growth and differentiation [21]. The rat chondrodystrophy is also due to the primary action of the gene in chondrocytes. This was found by mutual transplantation of rib cartilages between affected and normal animals [10].

In *in vitro* experiments, using extracts of the Cp+ chicken embryos, it was shown that the products of Creeper gene action were present in other embryonic tissues and could suppress the growth of the limb bones of ++ embryos [39]. Similar data were obtained on another mutant in the chicken diplopod[4] [20] and on the bp^H mutant mouse. The tissue extract of 13-day bp^Hbp^H mouse embryos inhibits only the growth of the normal cartilage cells, undergoing hypertrophy [14]. A release of growth-inhibiting substance from the affected bones into the culture medium was observed in experiments with the simultaneous cultivation on the same raft of the tibiae of the Cp+ and ++ embryos. A suppression of the tibia growth of ++ embryos took place in these studies [8]. LORINCZ [23] showed that in achondroplastic cattle the concentration of the mucopolysaccharides (chondroitin sulfate B and hyaluronic acid) in urine is 5—10 times more than in the normal urine of cattle. A high mucopolysaccharide level was found also in the liver of affected animals.

DICKIE [7] discovered a mutant with hereditary disproportionate dwarfism in strain AKR/J in February 1960 at the Jackson Laboratory (Bar Harbor, Maine, USA). This anomaly is due to the autosomal recessive gene named achondroplasia (symbol cn). The foundation animals (+cn) of our mouse colony used in the present experiments were received from Dr. MARGARET M. DICKIE in 1963. In our laboratory these mice were kept in a genetically heterogeneous stock.

Material and method

The first remarkable morphological signs of abnormal development of cncn mice can be recognized from the seventh day on by retarded growth, short legs (micromelia) and some prominent forehead. Subcutaneous bone homotransplantations between 7- or 14-days sibs were made to determine the primary action of cn gene. The bones were implanted according to FELTS [11], who showed that humerus homotransplants of immature mouse grew similarly as isotransplants for 12—16 days after implantation [12]. Ulna and radius were transplanted between 7-day sibs and humeri between 14-day sibs. Ulna and radius of 7-day phenotypical normal mouse and cncn mouse were subcutaneously implanted to their phenotypically normal sibs (++ and +cn). Four series of experiments were made for humerus transplantation between 14-day sibs. The left humerus from the donor was implanted to phenotypically normal and right humerus to cncn recipients. As donors phenotypically normal mice were used for the 1st and 2nd series, cncn mice for the 3rd and 4th series. In all experiments the bones were

removed 14 days after implantation and their percentage increase in length was determined. We used only the implants surrounded by the friable connective tissue and did not use the implants which were contained in thick connective tissue capsules. Serum proteins were studied in 2-, 4-, 8- and 12-week-old cncn mice and its normal litter-mates by polyacrylamide gel electrophoresis [6, 27, 28]. The concentration of acrylamide used in the experiments was 7.5%. The somatotrophic hormone (STH) was determined in the anterior pituitary by the same method. Extracts of the anterior pituitary glands from 2-, 8- and 12-week-old mice were prepared according to LEWIS and co-workers [22], who used electrophoresis on polyacrylamide gel for the determination of STH in the extracts of pituitary glands of normal and dwarf mice. The level of blood serum mucopolysaccharides was determined as described by BADIN and co-workers [1], but the calculation was based on the glucose standard. The concentration of blood glucose was measured by antronic method [32].

Results

During the first 4 weeks after birth growth rate of cncn mice is significantly less than that of their normal litter-mates. After the 4th week weight growth rate is the same as normal. The question arose whether the inhibited growth of the cncn mouse is connected with insufficient STH secretion by the anterior pituitary, as it was shown e. g. in mice with hereditary hypopituitary dwarfism. It was found that extracts of the anterior pituitary glands of cncn mice had approximately the same content STH as their normal litter-mates (Fig. 1). Consequently, an inhibition of the growth of cncn mice is not due to an insufficient STH secretion by STH-cells. It could be suggested that the inhibited bone growth was due to the primary cn gene action in chondrocytes. For deciding this question subcutaneous implantation of ulna and radius were made between 7-day-old affected and normal sibs. Forteen days after implantation the bones were well vascularized and had their similar primary shape. There was no remarkable alteration. It was shown that ulna and radius of cncn mice implanted to their normal sibs grew much worse as compared with these bones reciprocally implanted between normal sibs (Table 1). These data were

Table 1

Percentage increase in length of ulna and radius two weeks after subcutaneous implantation between 7-day mouse litter-mates

Bone	Genotype of donor	Genotype of recipient	Number of implants	Percentage length increase	P
Ulna	cncn	++ or +cn	18	11 ± 0.7	< 0.001
	++ or +cn	++ or +cn	31	20 ± 1.1	
Radius	cncn	++ or +cn	17	14 ± 0.5	<0.001
	++ or +cn	++ or +cn	33	24 ± 1.1	

supported by reciprocal humerus implantation between 14-day sibs (Fig. 2). At the 1st series experiments the humerus was subcutaneously implanted reciprocally between normal sibs. At the 2nd series humerus of the phenotypically normal donor was transplanted to affected litter-mates. Forteen days there-

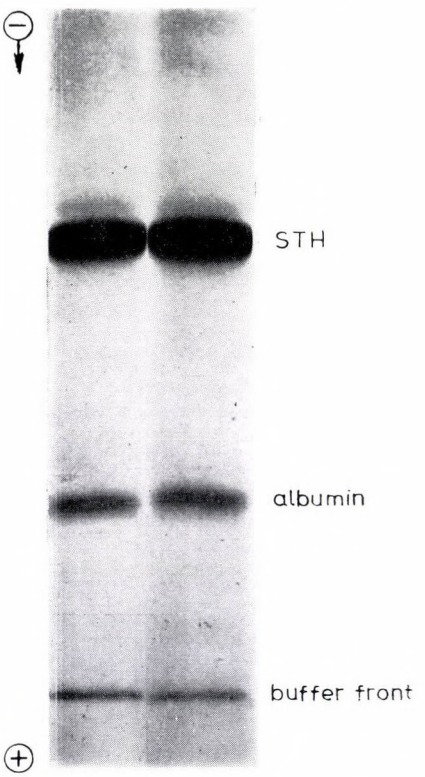

Fig. 1. Electrophoretic pattern of the anterior pituitary extracts of normal (left) and cncn (right) 8-week-old litter-mates

after bones in the 2nd series appeared like the implants of the 1st series, though their percentage increase in length was approximately one-third of that in the 1st series (Table 2). These data indicate that the humerus of phenotypically normal mice transplanted subcutaneously into cncn mice showed a retarded growth as compared with the growth of this bone implanted to phenotypically normal mice. The results of the 3rd series showed that the percentage increase in length of the cncn bone was approximately the same as in the 2nd series experiments. The inhibited growth of the implants in the 2nd and 3rd series (as compared with the 1st) may be explained by a hypothetical growth-inhibiting substance supposed to be present in the blood of cncn mice. The results of the 4th series showed that the cn gene manifested itself in chondrocytes.

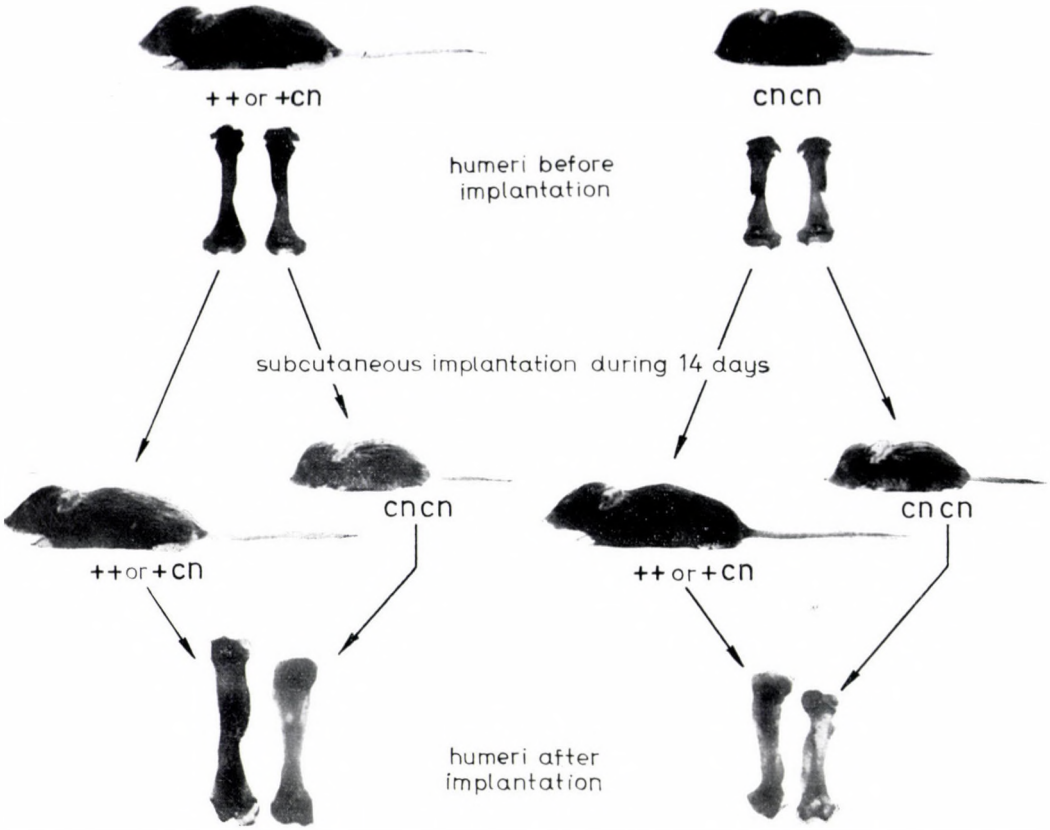

Fig. 2. Subcutaneous implantation of humeri between 14-day-old normal and cncn litter-mates

Table 2

Percentage increase in length of humerus two weeks after subcutaneous implantation between 14-days mouse sibs

N on the series	Genotype of		Number of implantt	Percentage length increasse
	donor	recipient		
1	++ or +cn	++ or +cn	36	11 ± 0.7
2	++ or +cn	cncn	21	4 ± 0.3
3	cncn	cncn	20	4 ± 0.3
4	cncn	++ or +cn	18	8 ± 0.4

Note: differences in percentage length increase of the implants between the 1st and 4th series are significant ($P < 0.01$); between the 1st and 2nd, 2nd and 4th, and between the 3rd and 4th are highly significant ($P < 0.001$).

The percentage increase in length of the cncn humerus implanted subcutaneously into their phenotypically normal sibs was twice that in the 3rd series. On the other hand it was significantly less than the percentage increase in length of the humeri of normal mice which had been implanted into normal litter-mates (1st series). Thus the results of all these series of experiments show that the growth inhibition of the cartilaginous skeleton of cncn mice is due to both the primary cn gene effect on the chondrocytes and a secondary effect through the growth-inhibiting substance in the blood.

The study of the serum proteins of 2-, 4-, 8- and 12-weeks cncn mice by the polyacrylamide gel electrophoresis did not show any difference in the quantity of the protein fractions and its electrophoretic mobility between cncn and normal litter-mates. These data show that the presence of a growth-inhibiting substance in the cncn blood does not correlate with the changes of the number of the protein fractions and their electrophoretic mobility.

The serum mucopolysaccharide concentration of cncn and phenotypically normal litter-mates is the same irrespective of the age of the animals The same is true for the blood glucose (Table 3).

Table 3

The level of mucopolysaccharides and glucose in the blood of mice

Number of animals in brackets

Genotype	Age in weeks	Body weight (gm)	Average serum mucopolysaccharides (mg per 100 ml)	Average blood glucose (mg per cent)
cncn	2—3	4.2—5.8	121 ± 14.2 (4)*	81 ± 8.0 (5)*
++ or +cn		7.0—8.9	123 ± 10.6 (6)	90 ± 7.7 (5)
cncn	5, 6, 8, 12	7.6—18.5	182 ± 13.5 (16)*	132 ± 12.4 (10)*
++ or +cn		15.8—35.2	172 ± 9.1 (20)	151 ± 14.6 (12)

* $P > 0.05$

Discussion

The results of the bone transplantations show that the inhibition of bone growth in cncn mice is due to the primary cn gene action in the chondrocytes of the cartilage bones. It should be noted that bp^H gene in the mouse has similar effects, it manifests itself by the inhibition of the limb bone growth [21]. However the bp^H gene is manifested at earlier developmental stages in the cartilaginous anlages of the limb bones. The action of the bp^H gene is limited by

the short period of the development. Products of this gene action, circulating in the blood for apparently only one day inhibit the growth of some genetical nonaffected limb bones. The short-term and local bp^H gene action leads to affection of only some limb bones and does not diminish the growth rate of bp^Hbp^H embryos [14]. The cn gene action is more prolonged and its primary effects are manifested apparently in the chondrocytes of numerous bones. Therefore into the blood of cncn mice products of cn gene action are secreted in greater amount and these may be responsible for the enhanced inhibition of the cartilaginous growth and the growth rate of body during four weeks.

DICKIE [7] reported that many of the cncn mice die between birth and weaning. In our mouse colony we observed that from the 7th to the 21st day almost 35% of all cncn mice died, 2/3 of these 35% died after the 14th day. The animals that survived this period had no differences in viability as compared with their normal litter-mates. A high mortality of calves with hereditary achondroplasia was also noted. Most of the affected calves die during the first year after birth [23]. NEEL and co-workers [26] reported that among 12 studied achondroplastic dwarfs 4 were stillbirth, 5 died at the first day after birth, two died later and only one child remained alive. GITTELSOHN and MILHAM [15] showed that most children with achondroplasia died at the first year of their life (67.6%) and the percentage of their fetal mortality was 12.9%. If achondroplastic dwarfs survive the first year, their viabilities are not reduced [30]. The data of the present paper show that cncn mice, too, have a period of high mortality. The high mortality of achondroplastic children in the first year of life also indicates a critical period of development.

For the mouse two recessive genes (dw and df) have been described. These genes lead to the hypopituitary dwarfism [33, 34, 36]. During the first days after birth the dwdw and dfdf mice do not differ remarkably from their normal litter-mates. However, soon their growth is inhibited, due to the lacking STH-cells and the decrease in number of the thyrotropic cells. Long-term STH injections to 1—4-month dwdw and dfdf mice stimulate their growth and some males even become fertile [2]. Everyday implantations of normal rat pituitary glands to dwdw mice, started from 30-days of age, led to the normalization of the growth of affected mice [35]. Electrophoresis on polyacrylamide gel showed no STH in the extracts of the pituitary gland of dwdw mice [22]. Injection of the suspension pituitary gland tissue of achondroplastic and normal bulls into dwdw mice caused a similar stimulation of growth. These data indicate that there is no difference in the STH activity of achondroplastic and normal bulls [4]. Neither the activity of ACTH and gonadotropic hormones is disturbed in achondroplastic bulls [5]. STOCKARD [37] could not find any correlation between the size of the pituitary or thyroid gland and the body type in different dog breeds. Careful histological studies of the thyroids, para-

thyroids and pituitaries showed only minor, inconsistent differences between breeds of dogs. Further, the same author failed to restore the normal form of achondroplastic dogs by implantation of normal pituitary glands or injection of growth hormone. However, STOCKARD [37] considered that the abnormal endocrine balance was due to the development of achondroplastic types of dogs; such as bassethound and dachshund breeds. Yet he could not explain ". . . if abnormal endocrine secretion is the causative agent in producing this skeletal deformation, why should one part of the skeleton suffer and other parts completely escape, while all parts are necessarily exposed to the influences of the same body fluids?" It was difficult to explain by the abnormal endocrine secretion the isolated growth disturbance of one jaw and some other bones. Therefore Stockard assumed that: "The genetic constitution of the tissues themselves determines their mode of growth and development as long as the environment is sufficiently favorable to permit the expression of the structure concerned." EVANS and co-workers [9] injected growth hormone to dachshund breed dogs from puppyhood (11 weeks of age) on until the epiphyses were closed (60 weeks of age). The weight of the injected animals increased to about twice as compared with noninjected controls while injected dogs still retained the achondroplastic form with short legs. The most prominent effect of hormone treatment was an accelerated growth of the soft tissues, particularly the skin, which showed pronounced folding. Though the overgrowth of the skin was visible even after three-week treatment, significant increase in length of limb long bones did not occur.

Using the electrophoresis on polyacrylamide gels, we could not find any remarkable difference in the STH concentration in the extracts of the anterior pituitary between cncn mice and their normal sibs. Hypopituitary dwarfism in man is treated by injections with STH [13, 16]. On other hand, the long-term treatment with using the human STH does not stimulate the growth of achondroplastic children [13, 25]. The serum STH concentration in the human nonhypopituitary dwarfs slightly exceeds the normal level [3]. Thus many data indicate that hereditary achondroplasia is not due to the affected anterior pituitary gland. It should be noted that PARROT [29] who was the first to describe achondroplasia as an anomaly, supposed that this anomaly is due to the affected cartilage. But MARIE [24] groundless rejected this hypothesis and suggested that this anomaly was connected with some functional disorders of endocrinic glands. The results of the present studies of the cn gene effects and the data of experimental studies of the primary bp^H gene action in the mouse, and genes Cp and dp^4 in the chicken lead to the conclusion that hereditary achondroplasia in different species of animals, and apparently in man, is due to the primary action of the mutant gene in the cartilage cells and also can be redoubled by its secondary effects. Evidently the main effect of these mutant genes is the inhibition of chondrocyte hypertrophy.

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EXPERIMENTELLE UNTERSUCHUNG DER EFFEKT DES ACHONDROPLASIE-GENS BEI MÄUSEN

Das autosome rezessive Achondroplasie-Gen (cn) ruft bei Mäusen im Homozygotenstadium eine Hemmung des Skelettwachstums hervor. Die subkutane Implantation von Extremitäten-Röhrenknochen in 7- oder 14tägige cncn-Mäuse oder ihre normale Verwandte zeigten, daß die primäre Wirkung des cn-Gens sich in den Chondrozyten der Knochen des Knorpelgerüsts manifestiert. Daneben wird die Hemmung des Knochenwachstums durch das Vorhandensein der Produkte dieses mutanten Gens im Blutstrom noch gesteigert. Bei cncn-Mäusen zeigt die Mukopolysacharidenkonzentration im Serum sowie die Glukosekonzentration im Blut gegenüber der Norm keinen Unterschied. Mittels Elektrophorese im Polyakrylamidgel wurde in den Extrakten des Hypophysenvorderlappens von 2-, 8- und 12wöchigen cncn-Mäusen und ihren normalen Verwandten die gleiche Wachstumshormonmenge nachgewiesen. Der Entwicklungsmechanismus der Achondroplasie bei Menschen und Tieren wird besprochen.

ЭКСПЕРИМЕНТАЛЬНОЕ ИЗУЧЕНИЕ ЭФФЕКТОВ ГЕНА ACHONDROPLASIA У МЫШИ

Аутосомный рецессивный ген achondroplasia (символ cn) в гомозиготном состоянии тормозит рост скелета мышей. Подкожные имплантации длинных костей конечностей между 7 или 14-дневными мышами cncn и их нормальными сибсами показали, что первичное действие гена cn проявляется в хондроцитах костей хрящевого скелета. Наряду с этим торможение роста костей усиливается продуктов действия этого мутантного гена в кровяное русло. У мышей cncn концентрация мукополисахаридов в сыворотке крови и глюкозы в крови не отличается от нормы. При помощи электрофореза в полиакриламидном геле в экстрактах из передней доли гипофиза 2,8 и 12-недельных мышей cncn и их нормальных сибсов обнаружено одинаковое содержание гормона роста. Обсуждаются механизмы развития наследственной ахондроплазии у животных и человека.

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LIPIDS OF THE HARDERIAN GLAND OF RABBITS

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Synopsis

(1) According to thin-layer chromatography the rich lipid contents of Harderian gland of rabbits consist of phospholipids (sphingomyelin, lecithin, cephalin), mono-, di- and triglyceride fractions and of cholesterol fractions. From the latter mainly non-esterified cholesterol and a minor esterified cholesterol fraction (esterified with an unsaturated fatty acid) could be found. (2) Lower and upper lobe (red and white lobe) showed the same lipid fractions in thin-layer chromatograms. (3) After 19-nor-androstenolon (Durabolin) treatment an increase in weight and lysozyme content of the Harderian glands was found. At the same time accumulation of a Dragendorff-positive lipid fraction could be observed.

Introduction

Connected to the nictitating membrane some rodents have a third gland in the orbita called Harderian gland, in which porphyrin synthesis [14] and storage of the bacteriolytic enzyme of tears, lysozyme [3], has been observed. As both functions may be in some connection with the rich lipid content of the gland [3, 11], the nature of lipids of the Harderian gland has been investigated by thin-layer chromatography.

As the Harderian gland of rabbits consists of two lobes, upper and lower or white and red [1], analysis of the lipids of each of the lobes was performed separately, to show if any difference exists in the nature and concentration of lipids between the two lobes. Since increase in the weight of the gland was observed after treatment with androgens [12] and an increase in the lysozyme content in the Harderian gland of rabbits was shown after treatment with anabolic steroids [3], we examined the lipids of the Harderian gland before and after anabolic steroid treatment. Simultaneously with lysozyme determinations analysis was performed partly in the two lobes separately.

Material and method

The right Harderian glands of 14 rabbits of both sexes and of 2,000—3,000 g body weights were removed under Intranarcon anaesthesia.

Thereafter, at an interval of one week, two injections of 0.025 g 19-norandrostenolone (Durabolin, Organon) were given intramuscularly. One week after the last injection the left Harderian glands were also removed.

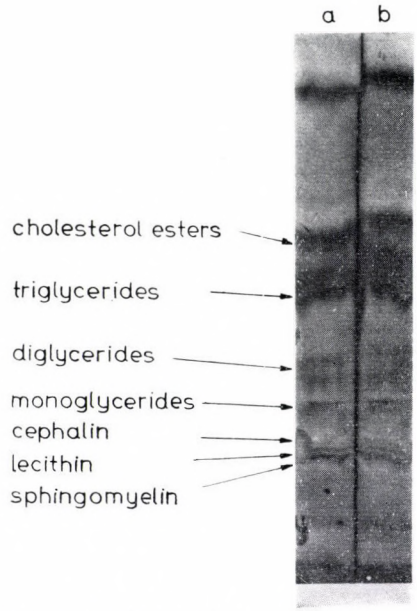

Fig. 1. Lipids of the Harderian gland of rabbits. a = red lobe, b = white lobe. Modified solvent system of SACHS and WOLFMAN. Bromthymol blue spray

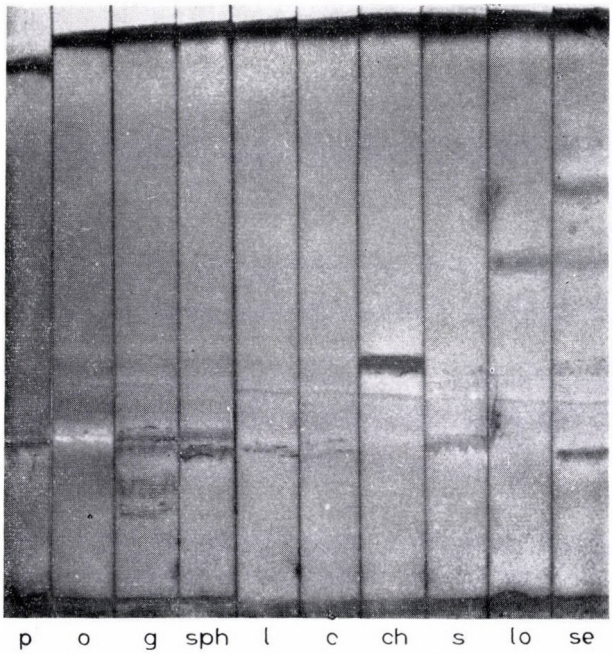

Fig. 2. Site of lipid reference substances on thin-layer chromatograms developed in the modified triple solvent system of SACHS and WOLFMAN. From left to right: p = palmitic acid, o = oleic acid, g = brain gangliosides, sph = sphingomyelin, l = lecithin, c = cephalins, d = cholesterol, s = stearic acid, lo = linseed oil, se = serum extract

The upper and lower lobes (white and red part) of the glands were weighed and homogenized separately with two volumes of saline, and aliquots of the homogenate have been used for quantitative examinations and for thin-layer chromatography. Lysozyme determinations were performed with a method described earlier [5]. The methods and results of quantitative lipid analysis will be published separately. Generally an aliquot of about 50 mg tissue was used for chromatographic purposes. The homogenized tissue had been shaken with 17 parts of Folch reagent (chloroform-methanol, 2 : 1) for 5 minutes and left at 4° C overnight, then filtered and the extraction was repeated twice. The pooled extracts were evaporated to dryness. The dry extracts could be stored without any alteration of the lipids for weeks in the refrigerator. For chromatographic purposes the extracts were dissolved in 0.5 ml of chloroform and about 0.05 ml was applied on thin-layer chromatographic plates.

Thin-layer chromatography. 20 × 20 cm glass plates were coated with a 250 μ layer of silicagel (Kieselgel G. Merck) and activated for 1 hour at 110° C.

Solvent systems: The double solvent system according to SACHS and WOLFMAN [15] modified by a third chromatography in hexane (to 15 cm height) [6] was generally used. For the investigation of cholesterol esters, the solvent system petroleum ether : ethylether (100 : 1.5) or carbon tetrachloride : chloroform (95 : 5) was used.

Bromthymol blue [16], iodine vapour [16] and oxytetracycline hydrochloride [4] were used for the detection of all lipid fractions. $SbCl_3$ was used to visualize cholesterol [16]. Zinzadze's molybdenblue reagent [2] was used as a general phospholipid spray, the Dragendorff reagent [16] for quaternary nitrogen containing compounds.

Reference substances: Lecithin was prepared from brain according to LEVENE [9], cephalins according to LEVENE [10], sphingomyelin according to LEVENE [8]; cholesterol, oleic acid, stearic acid, seed oil and stearin were commercial products. For comparison sometimes extracts of sera according to REED [13] and modified and applied by us [6] for sera, were used.

Results

According to Table 1 an increase, independent of sex, in the weight of the Harderian gland was observed in all rabbits after anabolic steroid treatment. The increase in weight was accompanied by an increase in the lysozyme content per gramm tissue. In the cases in which the two lobes were analyzed separately, increases both in weight and lysozyme content were equal in the two lobes.

According to our thin-layer chromatographic investigations using the solvent system of SACHS and WOLFMAN [15] the following main lipid fractions were found, in the sequence of migration from the start line to the front: [(Fig. 1) and see reference substances (Fig. 2)] phospholipids (sphingomyelin, lecithin, and cephalin), the first mentioned phospholipid being in the greatest amount. (With the Zinzadze reagent [2] the phospholipid fractions give only a single broad band.) Monoglycerides form the following fraction, this is much more accentuated here than in the serum. Cholesterol, di- and triglyceride fractions are in the next fractions. The triglyceride fraction is more abundant here than in the serum. The cholesterol-ester fraction is the fastest one. Investigation of the cholesterol fractions of the Harderian gland in the solvent systems carbon tetrachloride: chloroform (95 : 5) or petroleum ether : ethylether (100 : 1.5) showed, besides unesterified cholesterol (the main cholesterol fraction), also esterified cholesterol. This fraction corresponds to the cholesterol fraction esterified with twice unsaturated (dienic) fatty acids (Fig. 3), which usually dominates also in sera [6].

Table 1

The effect of 2×0.025 g 19-nor-androstenolon (Durabolin) upon the weight and lysozyme content of the Harderian gland in rabbits

The right glands were removed prior to, the left ones after Durabolin treatment

Number	Sex	Lobes	Weight mg		Lysozyme content μg/g	
			right	left	right	left
1	♀	both	1,028	1,900	0	700
2	♀	,,	916	1,300	0	2,500
3	♀	,,	739	1,320	0	2,500
4	♂	,,	420	890	0	750
5	♂	,,	613	1,100	0	800
6	♀	,,	933	1,170	0	750
7	♂	,,	685	1,000	0	650
8	♀	,,	880	930	0	670
9	♀	,,	750	840	0	650
10	♀	,,	935	1,032	0	720
11	♂	,,	1,235	1,140	0	550
Mean			830	1,147	0	1,022
12	♀	white	520	620	0	600
		red	560	550	0	600
13	♀	white	490	610	0	280
		red	725	780	0	300
14	♂	white	635	780	0	380
		red	510	740	0	460
Mean			548 598	670 690	0 0	420 453

The lipids of the upper (white) and lower (red) lobes of the Harderian gland show neither qualitative nor any marked quantitative differences (Fig. 1).

In the course of androgen treatment a significant elevation of a lipid fraction in the neighbourhood of the triglycerides, however reacting with the Dragendorff reagent, too, could be noticed (Figs 4 and 5).

Discussion

Data concerning the histological picture and morphological changes of the Harderian gland — though mostly for the rat [7, 11, 14], only few for the rabbit [1] — are available in the literature. In spite of this there are no data available on the nature of the lipids of this lipid-rich organ, one lobe being the site of porphyrin synthesis in some rodents [14] and storehouse of the bacteriolytic tear protein, the lysozyme [3].

ROHONYI and KELÉNYI [14], who investigated the rat's Harderian gland, state that the low water content of the Harderian gland is presumably due to the fact that the cytoplasm of the cells of the glandular epithelium consists largely of hydrophobic lipids. BJÖRKMAN and co-workers [1] found that both the red and the white parts contain lipid droplets and the only difference was

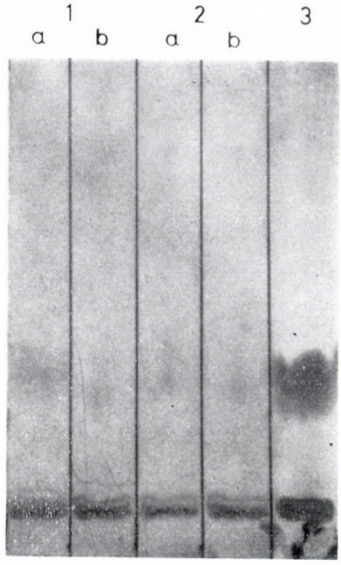

Fig. 3. Cholesterol fractions of the rabbit's Harderian glands. a = red lobe, b = white lobe. 1 = before, 2 = after Durabolin treatment. 3 = serum extract for comparison. Solvent system: petroleum ether : ethyl ether (100 : 1.5). $SbCl_3$ spray

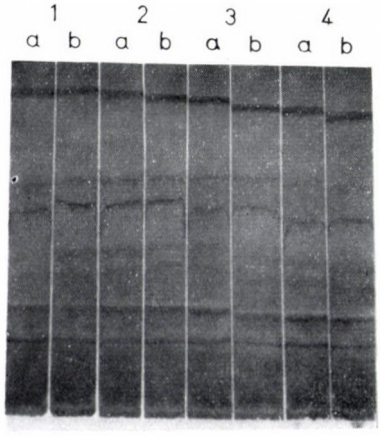

Fig. 4. Lipids of the rabbits' Harderian gland. a = X red lobe, b = X white lobe. 1, 2 = before; 3, 4 = after 19-norandrostenolon (Durabolin) treatment. Modified solvent system of SACHS and WOLFMAN. Bromthymol blue spray

observed in the size of the droplets; both lobes showed a secretion of lipid droplets. Since we found no difference in the nature of the lipids between the two lobes, it can be assumed that not their chemical nature but their site within the cytoplasm determines the size of lipid droplets.

The increase in weight of the Harderian gland after 19-nor-androstenolon (Durabolin) treatment is in agreement with literary data. The increase in lysozyme content found by us is accompanied by a change of the lipid spectrum

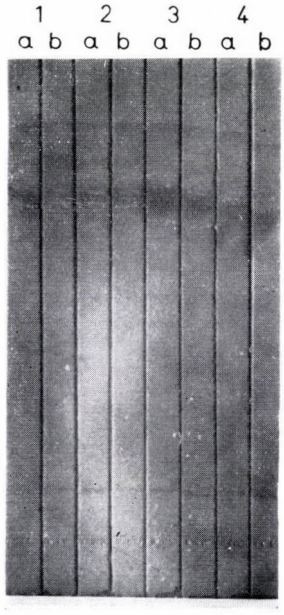

Fig. 5. Lipids of the rabbit's Harderian gland. a = red lobe, b = white lobe. 1, 2 = before; 3,4 = after 19-norandrostenolon (Durabolin) treatment. Modified solvent system of SACHS and WOLFMAN. Dragendorff reagent

manifesting in an increased intensity and in widening of the Dragendorff-positive stripe. It is still unclear whether this increase has a role in the storage of lysozyme.

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DIE LIPIDE DER HARDERSCHEN DRÜSE VON RATTEN

(1) Auf Grund der Ergebnisse der Dünnschichtchromatographie besteht der reiche Lipidgehalt der Harderschen Drüse von Ratten aus Phospholipiden (Sphingomyelin, Lezithin, Kephalin), aus Mono- und Triglyzeridfraktionen sowie aus Cholesterinfraktionen. Von den letzteren fand sich vor allem eine nicht-esterifizierte Cholesterinfraktion und eine geringere — mit einer ungesättigten Fettsäure — esterifizierte Cholesterinfraktion. (2) Die Unter- und Oberlappen (roter und weißer Lappen) zeigten bei Dünnschichtchromatographie die gleichen Lipidfraktionen. (3) Nach Behandlung mit 19-nor-Androstenolon (Durabolin) ließ sich in den Harderschen Drüsen die Zunahme des Gewichts und des Lysozymgehaltes und die Vermehrung der Dragendorff-positiven Lipidfraktion feststellen.

ЛИПОИДЫ ГАРДЕРОВСКИХ ЖЕЛЕЗ У КРЫС

(1) Согласно результатам хроматографирования на тонких слоях богатое содержание липоидов в Гардеровских железах крыс состоит из фосфолипоидов (сфингомиелина, лецитина, кефалина), из моно-, ди- и триглицеридных фракций и из холестериновых фракций. Из последних была выявлена главным образом неэтерифицированная холестероловая фракция, и меньшая фракция эстерифицированного (ненасыщенной жировой кислотой) холестерола. (2) Нижняя и верхняя (красная и белая) доли показали при хроматографировании на тонком слое одинаковые липоидные фракции. (3) После введения 19-нор-андростенолона (Дураболина) было установлено повышение веса Гардеровских желез и их содержания липоидов. Повышение веса и содержания лизозима сопровождалось увеличением Драгендорф-положительной липоидной фракции.

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ULTRASTRUCTURAL ALTERATIONS INDUCED BY ALKYLATING AGENT (R-74) IN TUMOUR CELLS*

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Synopsis

The effect of alkylating agent R-74 (1,4-bis(2'-mesyloxyethylamino)-1,4-dideoxy-mesoerythritol) was studied on NK/Ly ascites tumour cells at ultrastructural level. The agent was applied partly in a large single dose (10 mg/kg) partly in fractionated doses (4×2.5 mg/kg). Very soon after treatment (12th—24th hour) — as a sign of enhanced nucleocytoplasmic interaction — particular ultrastructural changes (cytoplasmic invaginations in the nucleus, lobulated nuclear surface, chromatin margination, nucleolar channels) appeared. At the same time segregation of the nucleolar components and appearance of numerous lysosome-like bodies were observed in the cytoplasm. Twenty-four and 48 hours after treatment — as signs of damaged cell division — "quadrilamellar" membranes, a significantly reduced electron density of the karyoplasm and outpocketing of the outer nuclear membrane into the cytoplasm were observed. At the same time in the cytoplasm vesiculation of mitochondrial cristae and occurrence of great vacuoles were noteworthy changes. Later, 120—144 hours after treatment multinuclear giant cells appeared.

In the case of fractionated application of the drug — besides the above-mentioned alterations — nuclear vacuoles, and in the cytoplasm mass appearence of lysosome-like particles were observed.

Introduction

The two important groups of alkylating agents, (a) N-mustard- and (b) methanesulphonyloxy-derivatives, show significant differences in their biological effects in spite of having active groups of similar chemical reactivity [25]. Horváth and Varga [25] attempted to produce derivatives combining the favourable characteristics of these two groups of alkylating agents. The halogen substituent of the ethylamino groups of mustard derivatives was substituted by an alkylsulphonoxy group, and thereafter numerous sugar-alcohol and alkane derivatives were prepared. According to the investigations of Csányi and co-workers [16] the owerwhelming majority of these compounds inhibited the growth of different transplantable tumours. Out of these agents the 1,4-bis(2'-mesyloxyethylamino)-1,4-dideoxy-mesoerythritol · 2Hx (x = = halogen or CH_3SO_2O) was found the most effective. This compound was

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designated R-74. Its LD_{50} for mice is 45 mg/kg body weight when administered intravenously. R-74 employed in therapeutical dose lengthened the survival time of mice bearing either NK/Ly or Ehrlich ascites tumour by at least 100%. In the case of the rats with Yoshida ascites sarcoma the R-74 therapy resulted in recovery of 70% of animals. Besides a pronounced lymphoclastic effect it hardly influenced the leucocyte number and had nearly no effect on erythropoiesis and thrombocytopoiesis [16].

The previous investigations unambiguously established the tumour growth inhibiting effect of R-74 but they had hardly told anything about the mechanism of action of this agent. The objective of the study conducted in our institute is to elucidate the mechanism of action of this drug by cytological, cytochemical, radioautographic and electron microscopic methods. In the present paper the results of the electron microscopic investigations are published with reference to data obtained by other methods [9].

Material and method

Since first of all the lymphatic tissues proved to be susceptible to the cytotoxic effect of R-74 [16], we chose the NK/Ly ascites lymphoma as test object [43, 44]. Swiss white mice were inoculated intraperitoneally with 3×10^7 NK/Ly ascites lymphoma cells. R-74 was administered at the 6th or 7th day after transplantation, when the animals already had a well-developed ascites tumour. In one series of experiments the drug was applied in a single dose of 10 mg/kg in saline intraperitoneally; in another experiment the same amount of drug was administered in fractionated doses (4×2.5 mg/kg) at 24 hours intervals. Ascites fluid samples were taken 3, 6, 9, 12, 24, 48, 72, 96, 120 and 144 hours after the single injection of R-74, whereas in the case of fractionated application the samples were taken at the 6th hour after the last treatment. About 0.3 to 0.4 ml ascites fluid was fixed for one hour at $+4°C$ in a 2 per cent osmium tetroxyde adjusted to pH 7.4 according to PALADE [45]. After dehydration in alcohol the material was embedded in Araldite. The ultrathin sections were stained with uranyl acetate and, subsequently according to KARNOVSKY [29]. The micrographs were taken by JEM 6-C electron microscope.

Results

Ultrastructure of the untreated NK/Ly ascites cell

NK/Ly ascites cells are of 10—20 μ in diameter, they have large ovoid nucleus and a narrow cytoplasmic rim (Fig. 1). There are numerous microvilli on the cell surface. The cytoplasm of the tumour cells is poor in cell organelles, particularly in endoplasmic reticulum, but is rich in free ribosomes. The rough-surfaced endoplasmic reticulum is usually present in tubular form. The free ribosomes are evenly distributed in the cytoplasm. The Golgi apparatus is of small extension and mostly vesicular type. The ovoid, elongated cylindric mitochondria possessing a medium dense matrix and well-developed cristae are spread in the cytoplasm. In the cytoplasm here and there lipid droplets and some lysosome-like bodies also occur. Sporadically there are some virus-

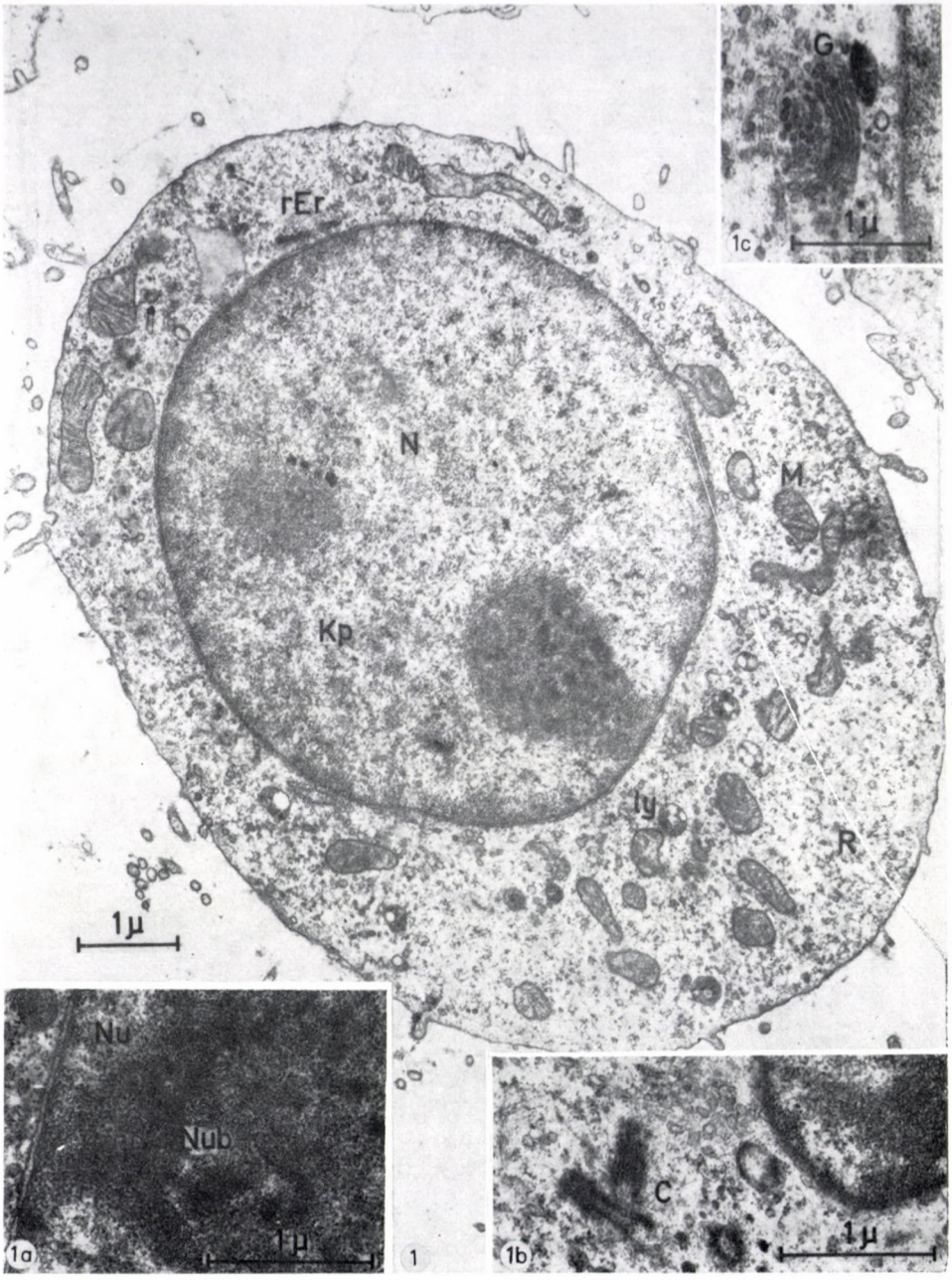


Fig. 1. NK/Ly ascites cell from 6-day-old untreated tumour. The large rounded nucleus (N) and the narrow cytoplasm are characteristic of the cells. The karyoplasm (Kp) is of medium electron density and its substance is evenly distributed. The cytoplasm is rich in free ribosomes (R), poor in lamellar endoplasmic reticulum (rEr). Numerous mitochondria (M) and some lysosome-like bodies are visible in the cytoplasm. ×18,000

Fig. 1a. In the nucleolar substance (Nu) granules approaching the order of ribosomal magnitude and smaller (70—80 Å) granules are distinguishable. Granules of both sizes occur together, embedded in a matrix of lower electron density in tight or looser distribution. The compact areas appear as tortuous, skein-like bundles (Nub) of high electron density. ×29,000

Fig. 1b. Centrioles (C) in untreated NK/Ly ascites cells. ×28,000

Fig. 1c. Golgi complex (G) in untreated NK/Ly ascites cells. ×25,000

like bodies in the cytoplasm, particularly in disintegrating cells of aged tumours. The double membrane of the oval, rounded nucleus is porous at numerous sites. The nuclear chromatin substance is of uniform distribution and medium electron density. In the karyoplasm cross-sections of deep-reaching cytoplasmic invaginations are often visible.

In the nucleus one or two relatively small nucleoli could be seen, usually in central position but occasionally they are located at the periphery, in contact with the nuclear membrane. In the nucleolar substance granules of the ribosomal size and smaller ones (70—80 Å in diameter) can be distinguished. These two kinds of granules are mingled with each other in more or less compact distribution embedded in a matrix of lower density. The compact areas appear as tortuous, skein-like bundles of higher electron density.

Changes induced by R-74 treatment

Very soon after treatment (1—12 hours) the most conspicuous change was the appearance of great number of deep cytoplasmic invaginations in the nuclear substance of moderately swollen cells. They often penetrate the nucleolus (Fig. 2). In the nucleus the distribution of the chromatin substance is altered particularly 9—12 hours after treatment it aggregates along the nuclear membrane (marginization, Fig. 3). At the same time the nuclei show signs of pronounced indentation. In the nucleolar substance of high electron density lighter fields of invaginated karyoplasm are often seen. More rarely sharp segregation of nucleolar components of different electron density can be observed (Fig. 3a). By this time the cytoplasmic organelles show no alterations.

Twenty-four hours after treatment, as a new alteration in the nucleus, heavy clumps of particular microvesicles appear beside the chromatin substance aggregated around the cytoplasmic invaginations (Fig. 4a). In the cytoplasm strongly enlarged mitochondria with light matrix and damaged cristae can be observed (Fig. 4). In addition lysosome-like bodies variable in shape, size and density, usually limited by a single membrane, are visible in a large number as compared with the controls. Already 24 hours after treatment a slight increase of rough-surfaced endoplasmic reticulum can be found in the overwhelming majority of tumour cells. The structure of Golgi apparatus shows no changes, except for a few cells in which Golgi elements are found in the peripheral part of cytoplasm, near the cell membrane, too. In some of these altered cells unusual cytoplasmic organization can be observed: along the axis of canaliculi, variable in length and width and limited by rough-surfaced endoplasmic reticulum, there is a smooth-surfaced double membrane and in this way "quadrilamellar" membranes are formed (Fig. 5). In the same cells, rough-surfaced endoplasmic reticulum of lamellar type and clumps consisting of larger (300—500 Å)

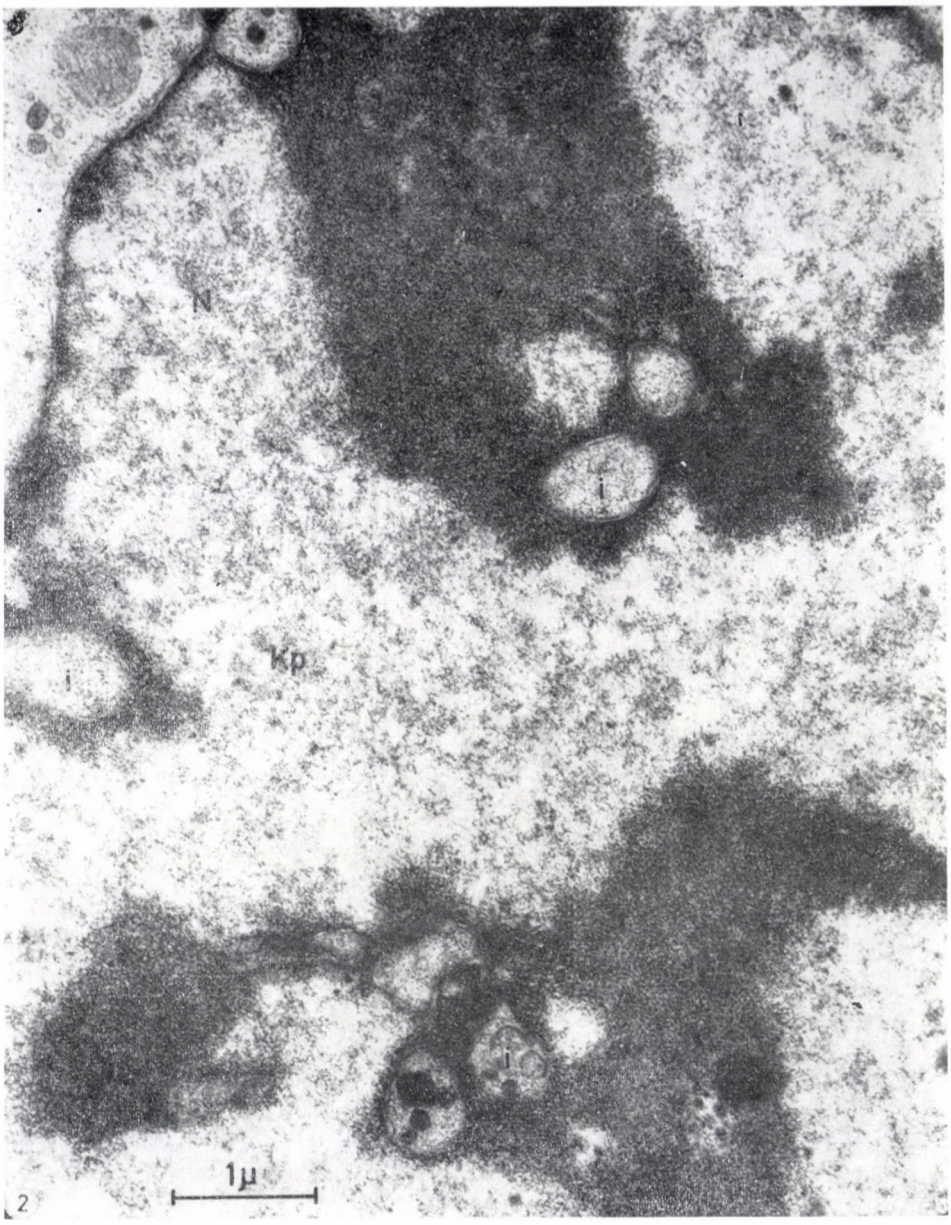
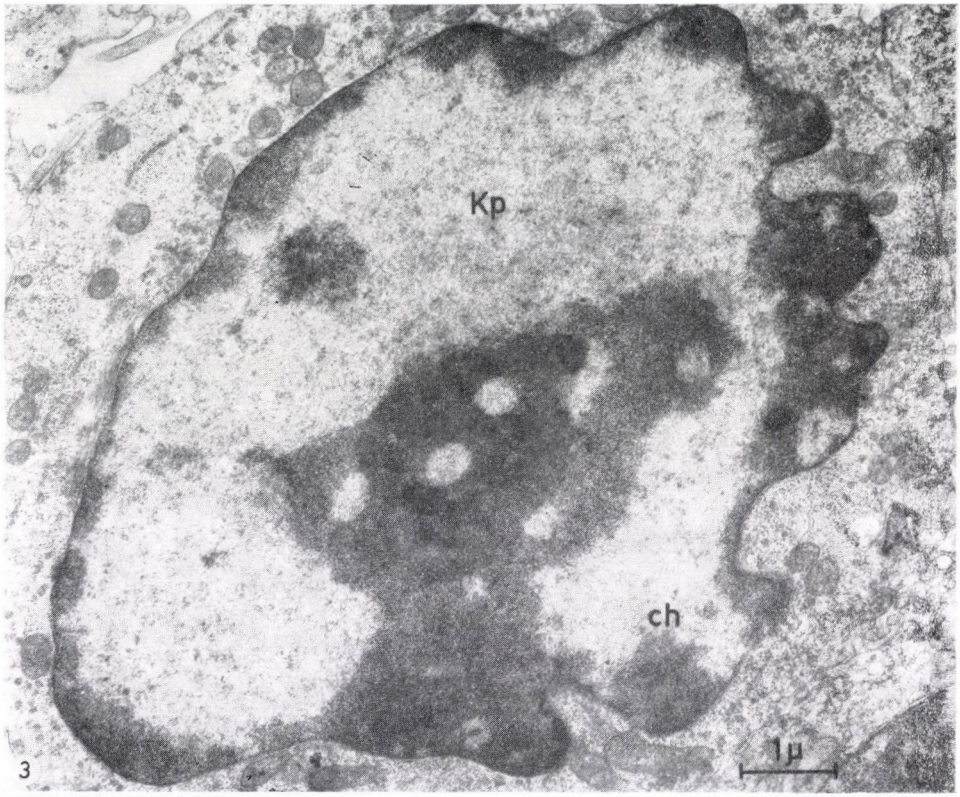


Fig. 2. An area in a nucleus (N) 12 hours after treatment with R-74. Note the deep cytoplasmic invaginations (i) in the karyoplasm (Kp). ×25,000



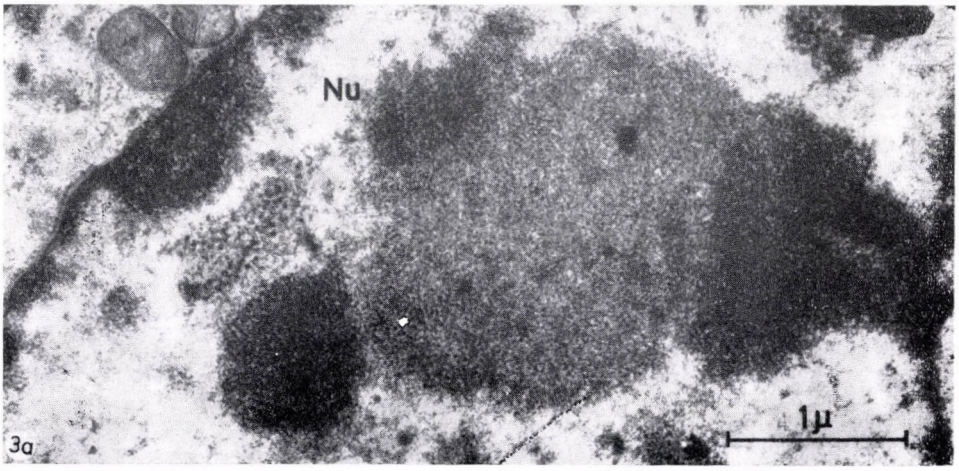


Fig. 3. The most conspicuous ultrastructural alterations 12—24 hours after treatment are the reduced electron density of karyoplasm (Kp), marginization of chromatin substance (ch) and indentation of nuclear surface. × 16,000

Fig. 3a. At the same times the segregation of nucleolar components could be observed in the nuclei of treated cells. × 31,000

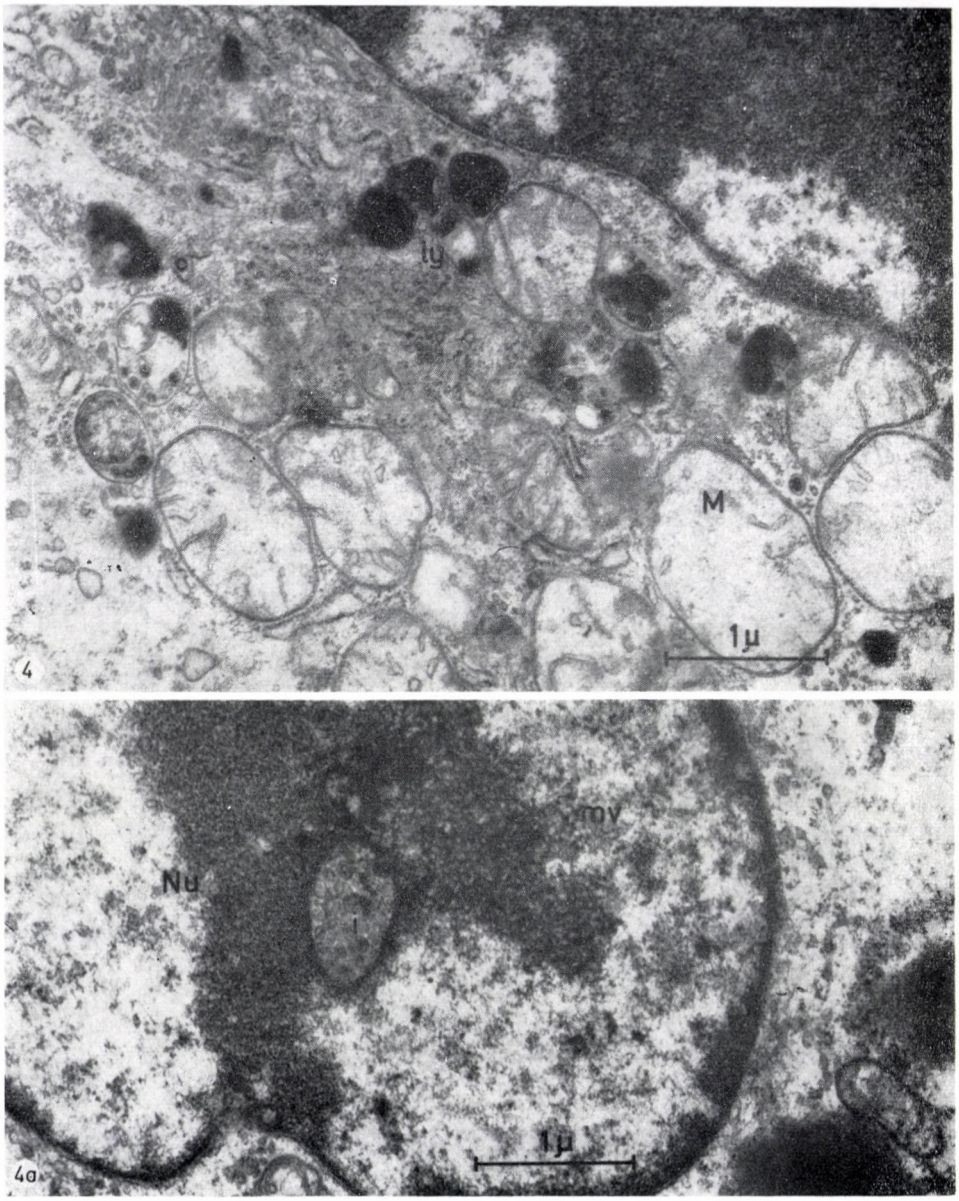


Fig. 4. Swollen mitochondria with light matrix (M) and lysosome-like bodies (ly) in a large number are visible 24 hours after treatment in the cytoplasm of treated cells. × 29,000
Fig. 4a. Compact masses of particular microvesicles (mv) near the nucleolus, and cytoplasmic fragments, (i) invaginated into the nucleus. × 29,000

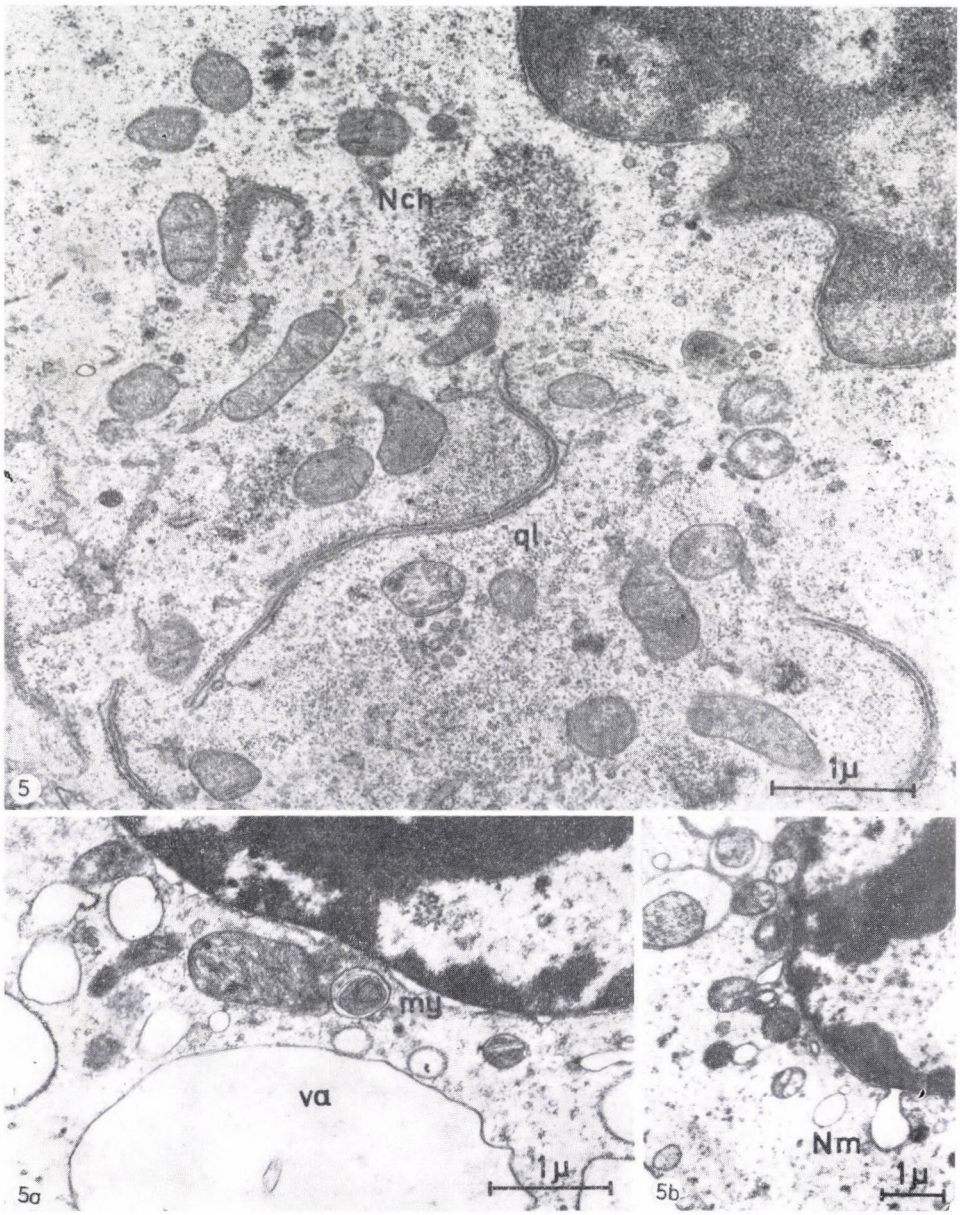


Fig. 5. Quadrilamellar membranes (ql) in the cytoplasm of treated cells, beside them disorganized nuclear substance (Nch) is visible. × 25,000

Fig. 5a. At the 24th hour after treatment large vacuoles (va) and myelin configurations (my) appear in many cells. × 20,000

Fig. 5b. Well-developed outpocketings of the outer nuclear membrane (Nm) in some tumour cells. The phenomenon reminds of "nuclear secretion". × 10,000

granules are also present in the cytoplasm. Latters resemble the interchromatic granule clumps of cell nuclei.

In conformity with the reduced Feulgen-reaction of the nucleus the reduced electron density of karyoplasm is also conspicuous soon after treatment (12—24 hours). Forty-eight hours after treatment the karyoplasm of reduced electron density, and nucleoli of unchanged high density, are characteristic of the whole cell population (Fig. 6). Widenings of the perinuclear space can be observed at the lobated nuclear surface. The outpocketing of the outer nuclear membrane forms vacuoles, sometimes 1 μ in diameter, communicating with the perinuclear space. The narrow cervical part of the vacuole facing the nucleus corresponds to the state of detachement into the cytoplasm, similarly to the mechanism of pinocytosis. Near to the nuclear membrane numerous vacuoles, variable in diameter, surrounded by rough-surfaced membranes, are visible. The vacuoles are as electron translucent as the above-mentioned widenings of the perinuclear space (Fig. 5b). In the organization of cytoplasm close perinuclear accumulation of organelles is conspicuous (Fig. 7). In the rounded mitochondria vesiculation of cristae can be observed. The lipid droplets are usually located in the indentations of nuclei. The rough-surfaced endoplasmic reticulum is represented by large, wide vesicles. Lysosomes, variable in number, are also present in the cytoplasm. Besides, in a part of the cell there are large vacuoles limited by single membrane (Fig. 6). In the vacuoles well-defined microvilli protruding into the lumen and segregated cytoplasmic fragments can be observed (Fig. 6). The peripheral part of cytoplasm is conspicuously poor in organelles due to their aggregation around the nucleus. The free ribosome stock is unchanged.

Seventy-two hours after treatment the picture is essentially identical, but in some cells as a further alteration myelin configurations can be observed (Fig. 5a).

Ninety-six hours after treatment the signs of enhanced nucleocytoplasmic interaction (lobated nuclear surface, deep cytoplasmic invaginations affecting the nucleolus) are characteristic of the cell population (Figs 8 and 8a). Peripheral localization of the nucleolus, near to, often even in contact with the nuclear membrane is common. At this time segregation of the nucleolar components is also visible in numerous cells. The cytoplasm, particularly its perinuclear part, is rich in rough-surfaced endoplasmic reticulum (Fig. 8), consisting of wide tubular elements. In the mitochondria light matrix and variably developed cristae are found. In the cytoplasm of some cells here and there virus-like particles are scattered.

At the 120th hour after treatment the cell population consists of two types of cells. The first one was earlier described as enlarged tumour cells with nuclei poor in chromatin. The other type is more rich in chromatin substance, which is evenly distributed; the nucleolus, which seems to be normal, is in

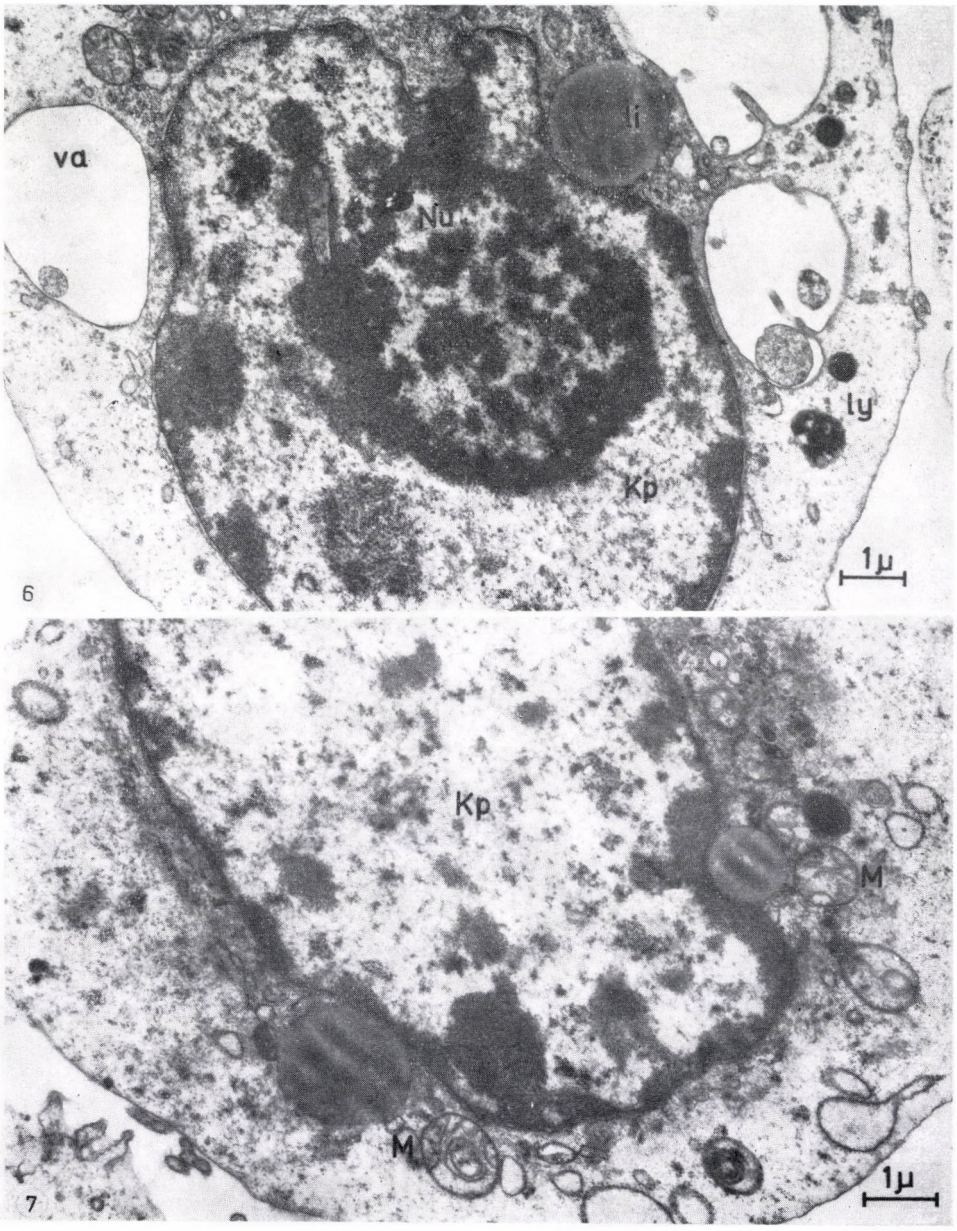


Fig. 6. Fourty-eight hours after treatment the electron density of karyoplasmic substance (Kp) is low but the nucleolus (Nu) is still highly electron dense. In the cytoplasm numerous vacuoles (va), lysosome-like bodies (ly) and here and there lipid droplets (li) are visible. ×10,500
Fig. 7. Accumulation of cytoplasmic organelles around the nucleus 48 hours after treatment with R-74. The mitochondria (M) of light matrix have disoriented cristae. The karyoplasm (Kp) is highly electron iucent. ×13,000

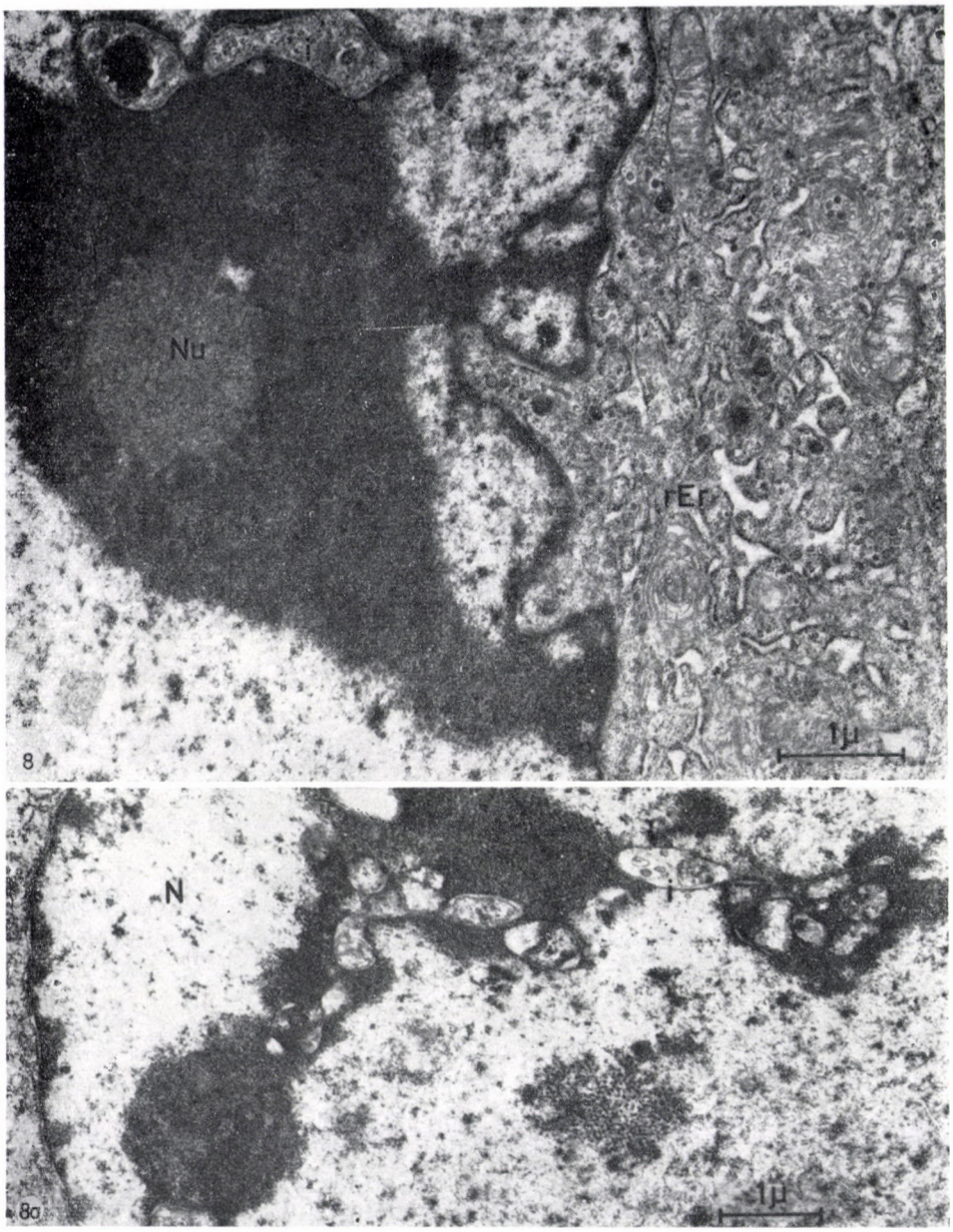


Fig. 8. Ninety-six hours after treatment. Signs of enhanced nucleocytoplasmic interaction: indented nuclear surface and cytoplasmic invaginations (i) reaching the nucleolus (Nu) are again characteristic of the cell population. In the cytoplasm: an increase in rough-surfaced endoplasmic reticulum (rEr). × 22,000

Fig. 8a. Ninety-six hours after treatment. Cytoplasmic invagination (i) of a large number in the nucleus of treated cells. × 18,500

close connection with cytoplasmic invaginations. In the cytoplasm a considerable amount of rough-surfaced tubular endoplasmic reticulum, large amount of free ribosomes, mitochondria similar to the controls and one or two lipid droplets can be observed.

Late after treatment the number of multinucleated giant cells increases (Fig. 9). It is particularly pronounced 144 hours after treatment. The giant cells have nuclei poor in chromatin and long segments of the perinuclear space significantly widened; nucleoli with normal organization can be hardly found. In the cytoplasm swollen mitochondria of light matrix and destroyed cristae are predominant. Besides, smaller vesicles limited by single or double membrane are observable, in which virus-like particles and a semiopaque material can be seen. A small amount of rough-surfaced endoplasmic reticulum of vesicular type and some lipid droplets are also apparent. Otherwise, the cell population is similar to that seen 120 hours after treatment.

Changes after treatment with repeated small doses

The effect of small doses (4×2.5 mg/kg) of R-74 applied on subsequent days was studied 6 hours after the last treatment. At this time the most conspicuous phenomenon was, besides the alterations observed after a single large dose, the occurrence of a great number of lysosome-like bodies in a considerable part of the tumorous cell population (Fig. 10). However, the tumourous cell did not show any sign characteristic of "cell destruction" and kept their ultrastructure. The lysosome-like bodies, variable in shape, size and density, were mostly located around the Golgi area near the "nuclear hof". They were usually limited by a single membrane. In their lumen light, completely electron lucent and highly electron dense granular areas alternate (Fig. 10a). Another characteristic change induced by fractionated treatment is the appearance in the karyoplasm of vacuoles varying in shape and size (Fig. 10b). They are limited by a membrane from the karyoplasm, their cavity is completely electron lucent. The vacuoles are in contact with the nucleolus, too.

Discussion

R-74 is a bifunctional alkane derivative which involves the peculiarities of two important groups of alkylating agents viz, of nitrogen mustard and alkylsulphonoxy derivatives. On the basis of their chemical properties, according to HORVÁTH and VARGHA [25], these compounds supposedly have an effect similar to the biological alkylating drugs on the tissue and cell components. According to KARNOVSKY, D. and CLARKSON [30] all of the polyfunctional alkylating agents have similar mechanism of action. According to the concord-

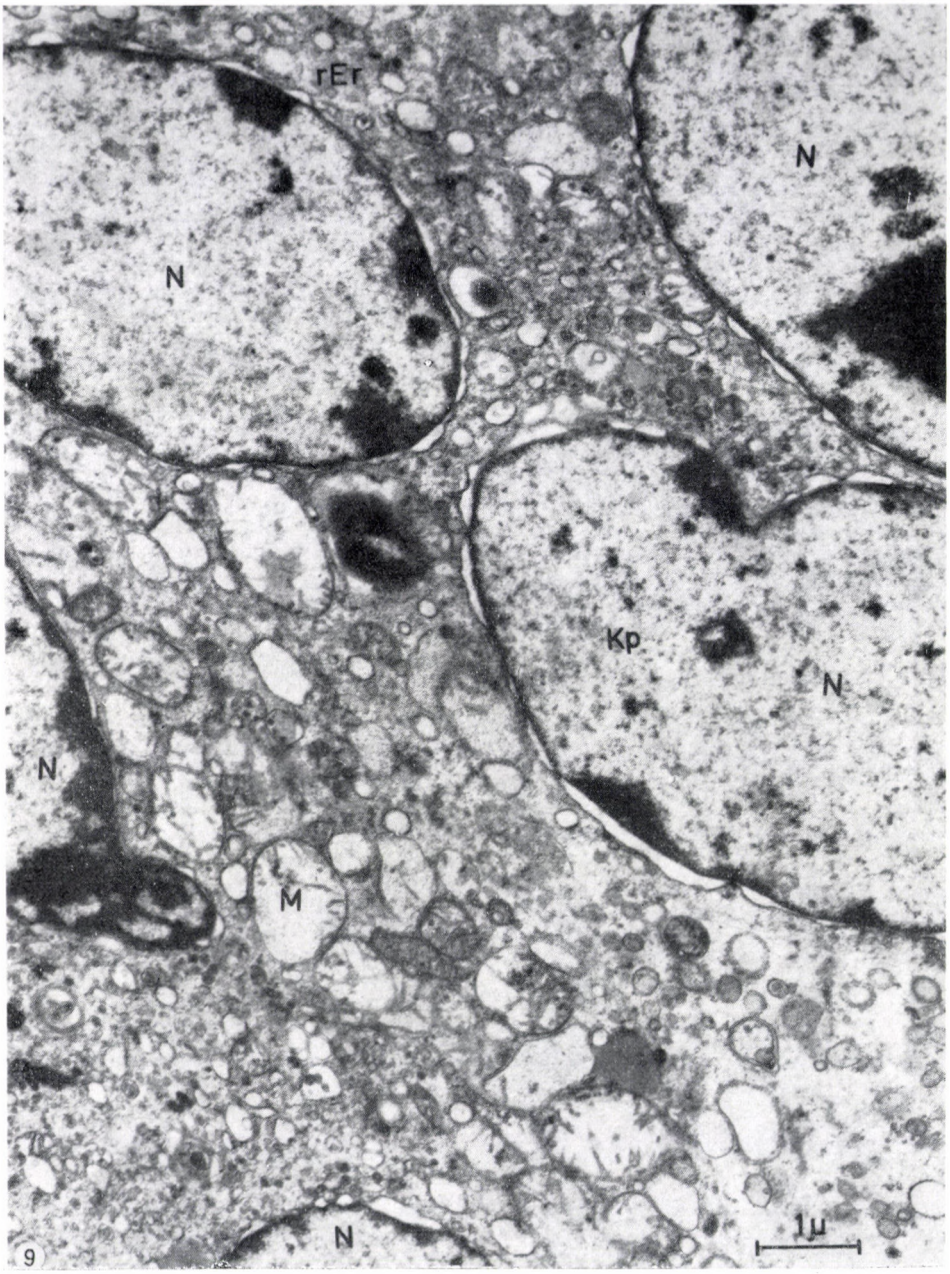


Fig. 9. Multinuclear giant cells 144 hours after R-74 treatment; the nuclei have light karyoplasm (Kp) poor in chromatin; the mitochondria (M) are heavily damaged, the ergastoplasm (rEr) is of vesicular type. ×18,000

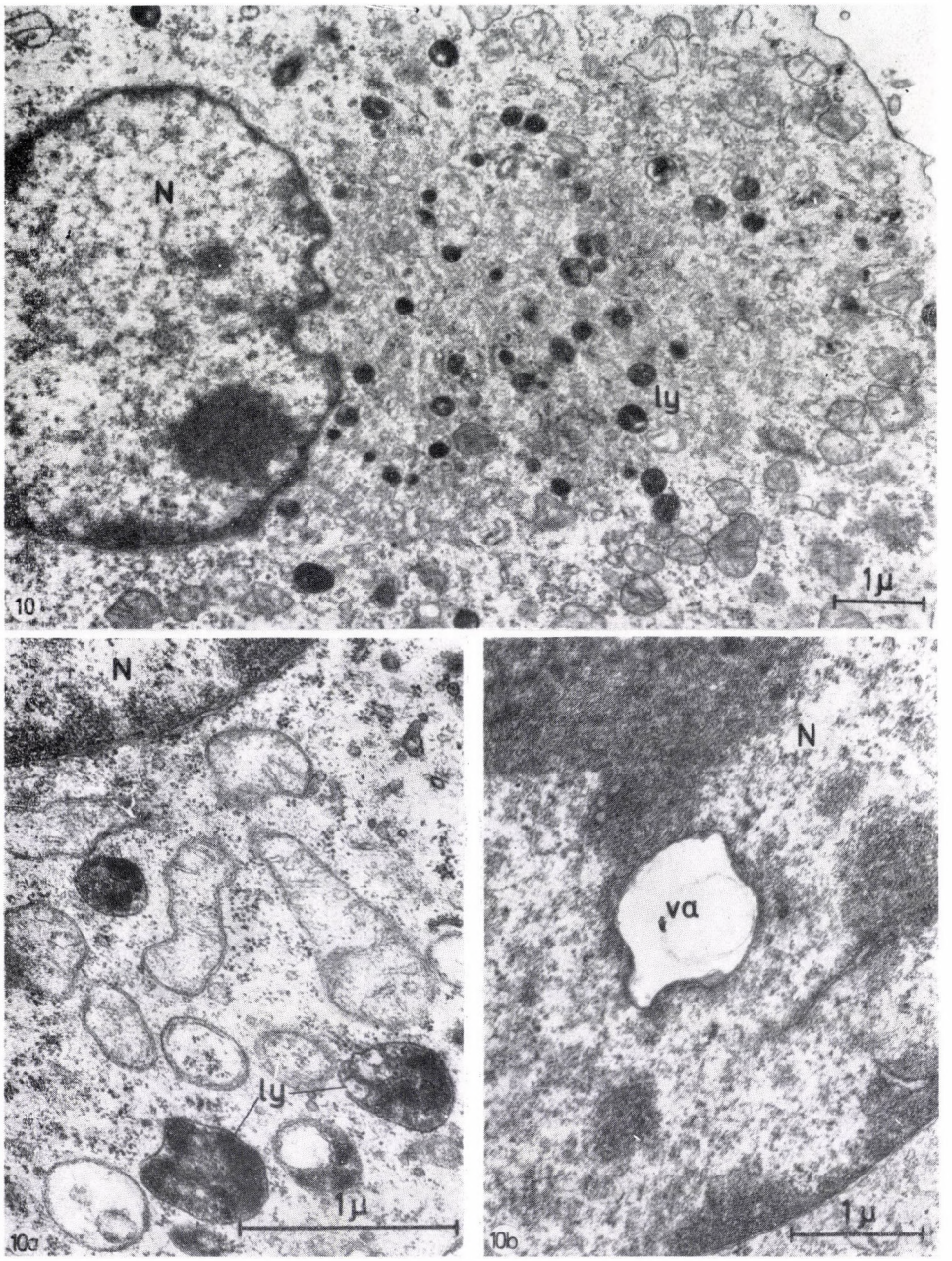


Fig. 10. After fractionated dosage of R-74 many lysosome-like bodies (ly) appeared in the tumour cells of intact structure. ×16,000

Fig. 10a. Lysosome-like bodies (ly) limited by single membrane; inside the bodies vesicles and masses of electron dense material. ×38,000

Fig. 10b. Six hours after fractionated dosage large vacuoles (va) appeared in the nucleus of some tumour cells. ×23,000

ant opinion of BROOKES and LAWLEY [10], KARNOVSKY, D. and CLARKSON [30] these agents — owing to their strong nonspecific reactivity — can intracellularly react with numerous cell components, probably without any selection. Therefore it is very important to establish the critical biochemical reactions which can heavily damage or even destroy some proliferating cells of the organism in the case of nonlethal doses for the host.

Biochemical investigations into the mechanism of action of R-74 in biological systems have not been performed, except for radioautographic studies with ^{3}H-labelled thymidine at light microscope level. For NK/Ly ascites lymphoma BÖRZSÖNYI and co-workers [9] established that a single therapeutical dose of R-74 decrease the incorporation of ^{3}H-labelled thymidine into the DNA by 25 per cent as soon as 6 hours after treatment and kept it at a low level. R-74 like other alkylating agents inhibits the DNA synthesis and this fact can be very important in formation of the cell injuries induced by the drug. Also the diminished rate of dividing cells already from 24 hours after treatment up to the 72nd—96th hours might be a consequence of the inhibited DNA synthesis.

Early after treatment, in concordance with the opinion of DAVID [17] and MOSES [42], the ultrastructural alterations observed in the nucleus are considered to be morphological manifestations of the enhanced nucleocytoplasmic interaction which makes an intense exchange of substances possible among the organelles of tumour cells.

Similar ultrastructural alterations were observed by us after 5-fluorouracil treatment [36]. Others have described similar alterations in the case of intoxications [32, 38, 48] and enhanced metabolism [15, 24], in various organs. The changed chromatin distribution in the nucleus, the marginization, can be related to the effect of the agent on nucleic acids. The accumulation of chromatin along the nuclear membrane has been described in connection with treatment with chemotherapeutics [36], ionizing radiation [19] and virus infection [8] as well. According to DAVID [17] this alteration may occur under various pathological conditions and it should not be mistaken for signs of metabolite transport toward the cytoplasm.

Segregation of the nucleolar components appeared in a relatively few tumour cells though it was consistently observed from 12 to 24 hours and again in a later period (120th—144th hours). Similar segregation of nucleolar components has been described as a result of treatment with many other drugs [27, 28, 46, 47, 49, 51, 52] and after ionizing radiation. It was observed by us [35] in KB cell cultures treated with mitomycin C; in connection with this we stressed that similar alteration had been never seen after treatment with alkylating agents. R-74 is the first alkylating agent which has been found to cause such an alterations in nucleoli. The present observation confirms the opinion of SCHOEFL [49], JÉZÉQUEL and BERNHARD [27] suggesting that the segrega-

tion of nucleolar components is not specific for some agents or effects. The cell-physiological significance of these alterations is unknown. According to BEERMANN [6], however, the lack of the normal nucleolus results in a general disorder of cell growth on the basis of disturbed protein synthesis.

Twenty-four hours after treatment with R-74 a peculiar channel system was observed in the nucleolus which had been never seen in our previous experiments related to the effect of chemotherapeutics. Similar nuclear structures in tumour cells have not been described, so far as we know. Nevertheless, CLYMAN [14], MORICARD and MORICARD [41] and TERZAKIS [50] described an entirely similar structure, designated "nucleolar channel system", in epithelial cells of the human endometrium.

Peculiarity of the highly organized channel system is the close contact with the nucleolus and the nuclear membrane delimiting the cytoplasmic invaginations. This contact can support the formation of such a direct channel system which can supply the increased transport of substances among the nucleolus, karyoplasm, nuclear membrane and cytoplasm. TERZAKIS [50] raises the idea that this channel system plays, besides its transporting activity, another role: it takes part in the metabolism of substances containing RNA. The nucleolar channel system simultaneously appeared with other alterations referring to an enhanced nucleocytoplasmic interaction. For this reason, in accordance with TERZAKIS, we suppose that the nucleolar channel system may play a role in both the transport and the metabolic cell-physiological processes.

A significant decrease of electron density of nuclei was observed. Two factors might play role in this phenomenon: (1), the increased volume of the swollen nuclei, (2), the inhibition of DNA synthesis which was demonstrated by radioautography [9]. The fall of the mitotic rate nearly to one fourth is in accordance with this [9]. The nuclear membrane is a very dynamic organelle, [7, 39] which plays an active role in the transport of both the metabolic and the genetical information between the nucleus and cytoplasm [39]. According to MOSES [42] microvesiculation of both the outer and inner nuclear membranes, reminding of pinocytosis, rarely also occurs. The nuclear membrane alteration observed by us is considered a similar formation, too. Many authors [1, 2, 5, 13, 20, 22, 23, 26, 53] consider these vesicle bulges as a morphological sign of the transport from the nucleus to the cytoplasm, i.e., of nuclear secretion. In our case, too, the occurrence in the vicinity of bulges of perinuclear vacuoles of variable size is suggestive of an intense nuclear secretion. This mechanism may play a role, besides the transport of substances, in the moderate accumulation of rough-surfaced endoplasmic reticulum observed at the same time.

Forty-eight — 72 hours after treatment peculiar quadrilamellar membrane system was observed in the cytoplasm of some tumour cells. Similar configurations had been described in Walker 256 carcinoma, as "spindle lamel-

lae" [11]. In accordance with the opinion of BUCK [11] they are considered on the basis of their submicroscopic peculiarities preformed nuclear membrane configurations which were formed in the course of the pathological cell divisions under the influence of the agent. This is supported by the fact that nuclear substance of diffuse distribution is visible in the cytoplasm near the lamellae. One of the characteristics of the cytotoxic effect of R-74 is that the alterations of cytoplasmic organelles appear later and are less pronounced than the nuclear changes. In the mitochondria alterations were not found until 24 hours after treatment. In accordance with this the moderate accumulation of lipid droplets appeared as late as at the 48th hour.

A remarkable cytoplasmic alteration is the perinuclear accumulation of the cell organelles which results in a close contact between the energy-supplying mitochondria and the nuclear membrane. Similar phenomena have been observed by many authors [3, 4, 17, 40, 42, 54] immediately before mitosis and in its early phase.

Appearance of binuclear and multinuclear cells induced by numerous other chemotherapeutics has been observed both *in vivo* and *in vitro* [12, 18, 21, 31, 33, 34]. The characteristics of multinuclear cells observed after treatment with R-74 refers to perishing giant cells.

In the case of fractionated dosage of R-74, besides the above-mentioned alterations, vacuoles appeared in the nucleus. According to DAVID [17] these alterations come about as a consequence of destruction of cytoplasmic portions first invaginated in the nucleus and then detached. The nucleus, notwithstanding the verified high exchange of substances, is not able to supply isolated cytoplasmic portion in an appropriate manner.

After R-74 treatment, especially in the case of fractionated dosage, lysosome-like bodies appear in a great number. Acid phosphatase activity was demonstrable at electron microscope level in similar lysosome-like bodies observed sporadically in the control tumour cells. On the basis of this finding we assumed that the above-mentioned bodies appearing in mass following treatment might be taken for lysosomes. To prove this assumption enzyme-histochemical investigations are in progress.

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WIRKUNG DES ALKYLIERUNGSMITTELS R-74 AUF DIE ULTRASTRUKTUR DER GESCHWULSTZELLEN

Die Wirkung des Alkylierungsmittels R-74 (1,4-bis(2'-Mesyloxyethylamino)-1,4-dideoxy-mesoerythritol) auf die Ultrastruktur der NK/Ly-Aszites-Geschwulstzellen wurde untersucht, bei einmaliger hoher Dosis (10 mg/kg) sowie auch bei fraktionierter Verabreichung (4×2,5 mg/kg) des Mittels. 12 bzw. 24 Stunden nach Verabreichung des Mittels wurden auf eine gesteigerte nukleo-zytoplasmatische Wechselwirkung hinweisende Veränderungen der Ultrastruktur beobachtet (Auftreten zytoplasmatischer Invaginationen im Zellkern, segmentierte Kernoberfläche, Chromatin-Marginisation, nukleoläre Kanälchen). Gleichzeitig ließen sich die Segregation der Nukleolus-Komponenten, ferner im Zytoplasma das Erscheinen zahlreicher lysosomartiger Gebilde aufzeigen. 24 bzw. 48 Stunden nach der Verabreichung des

R-74 wurde das Erscheinen von »quadrilamellären« Gebilden, eine bedeutende Abnahme der Elektronendichte im Karyoplasma, die Ausbuchtung der äußeren Kernmembran dem Zytoplasma zu festgestellt, was auf eine Störung der Zellteilung hinweist. Weitere bemerkenswerte Veränderungen waren die Vesikulation der Cristae der Mitochondrien und das Erscheinen großer Vakuolen im Zytoplasma. Längere Zeit nach der Behandlung (120—144 Stunden) wurden mehrkernige Riesenzellen beobachtet. Bei fraktionierter Verabreichung von R-74 wurde neben den obigen Alterationen im Zellkern das Erscheinen von Vakuolen und im Zytoplasma das massenhafte Auftreten lysosomartiger Gebilde wahrgenommen.

ДЕЙСТВИЕ АЛКИЛИРУЮЩЕГО СРЕДСТВА (R-74) НА УЛЬТРАСТРУКТУРУ ОПУХОЛЕВЫХ КЛЕТОК

Авторы исследовали действие алкилирующего средства R-74 (1,4-бис(2'-мезилоксиетиламино)-1,4-дидеоксимезоеритритол) на ультраструктуру опухолевых клеток NK/Ly асцита. Средство применялось в однократной большой дозе (10 мг/кг), а также фракционированно (4 раза по 2,5 мг/кг). Через 12 и 24 часов после введения R-74 наблюдались изменения ультраструктуры, указывающие на повышение взаимодействия между ядрами и цитоплазмой (появление цитоплазматических инвагинаций в ядрах клеток, сегментированная поверхность ядра, образование хроматинового края, нуклеолярных канальцев). Одновременно отмечалась сегрегация компонентов нуклеол и в цитоплазме — появление многочисленных лизозомоподобных образований. Через 24 и 48 часов после введения R-74 было регистрировано появление «квадриламеллярных» образований, указывающих на нарушение деления клеток, понижение густоты электронов кариоплазмы, выбухание наружной ядерной оболочки в сторону цитоплазмы. Дальнейшим, заслуживающим внимание изменением было везикуляция митохондриальных гребней и появление крупных вакуол. В поздних сроках после введения R-74 (120—144 часов) наблюдались многоядерные гигантские клетки. В случае фракционированной дачи средства помимо вышеуказанных изменений в ядрах клеток наблюдалось появление вакуол, а в цитоплазме массовое появление лизозомоподобных образований.

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ELECTRON MICROSCOPIC INVESTIGATION OF ACINO-INSULAR TRANSFORMATION IN THE RAT

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Synopsis

Transformation process of pancreatic acinar cells into beta cells was followed by electron microscopy in obese rats with hypothalamic lesion. The earliest sign of transformation was mitochondrial swelling associated with cystic enlargement of the ergastoplasm of the acinar cells. In the progress of transformation the rough-surfaced endoplasmic reticulum became more and more vesicular, and then its amount decreased gradually. Concomitantly the number of the zymogen granules decreased and that of the beta granules increased in the cytoplasm of cells under transformation, while mitochondria assumed the shape characteristic of the islet cells. The Golgi system appeared to be hypertrophic in the course of the whole process.

Introduction

Since the end of the last century many papers have dealt with the embryonic and postembryonic origin of the pancreatic islet cells. Nowadays it is generally accepted that islet cell may develop from

(a) undifferentiated cells of the embryonic pancreatic cord, before the appearance of the acinar tissue (primary islets),

(b) different parts of the duct system belonging to immature or mature acinar tissue (secondary islets),

(c) the islet cells themselves by mitosis.

As early as 1896 LAGUESSE [16, 17] suggested that islet cells may also originate from acinar cells by transformation after their dedifferentiation. Since then numerous authors, using the light microscope, have confirmed the existence of this transformation in different species [6, 10, 11, 12]. According to BENCOSME [1, 2], however, the light microscope is not suitable for deciding whether two different types of secretory granules are contained in the same cell or not. Therefore the earlier results obtained with the light microscope cannot be used as conclusive evidence in this question. For a long time it seemed that the idea of acino-insular transformation was only the result of optical misinterpretation because, in spite of the large number of investigations into this problem, not even the electron microscope could confirm it until 1959 [1, 2, 3, 5, 20].

In 1959 GUSEK and KRACHT reported an experiment [7] in which the injection of sulphonyl urea produced islet hypertrophy in rats. Electron microscopic study of the islets in these animals revealed cells which contained both the endocrine and the exocrine granules. In 1964 BJÖRKMAN and HELLMAN [4] also found cells in the "dark" islets of ducks which clearly showed the characteristics of both of these cells. The last-mentioned two investigations demonstrated the existence of acino-insular transformation conclusively.

In hypothalamic hyperphagic animals there is a very strong islet cell neogenesis, wherefore their islets seemed to lend themselves to studying the process of transformation.

Material and method

Male albino rats of our inbred strain (originally Wistar), weighing 250—300 g, were used. Bilateral electrolytic lesions were placed with a Horsley-Clarke stereotaxic apparatus in the region of the ventromedial nuclei. From the animals which became obese islet samples were removed according to the method of HELLERSTRÖM [9], under barbiturate anaesthesia in the dynamic phase of obesity. The pieces of islets were immediately fixed in cold 1 per cent OsO_4 solution, buffered at pH 7.5 for 1 hour. After dehydration the samples were embedded in araldite [18], then ultrathin sections were cut with LKB-Ultrotome, picked up on copper grids without any film coating, and stained with lead citrate [21]. Finally, micrographs were made with a TESLA BS 242D electron microscope.

Results

In most cases the islets were surrounded by a definite connective tissue, but occasionally there was a continuous transition from the endocrine to the exocrine tissue. In all cases the transitional cells were situated at the periphery of the islets. They could be observed outside as well as inside the connective tissue. In some cases, when the position of the transitional cells was fortunate, it was possible to follow, step by step, the transformation which became more and more advanced towards the centre of the islets. Thus it was possible to study the cytoplasmic organoids in the process of changing (Fig. 1).

As the first sign of transformation, some of the juxtainsular acinar cells contained a cyst-like enlarged ergastoplasm instead of the regular, closely-packed endoplasmic reticulum system of a typical exocrine cell, oriented in parallel. These cells had in them round or oval, somewhat swollen mitochondria with clear matrix and with irregular cristae of the usual density. The Golgi system was dilated. At that stage of transformation the cells contained numerous zymogen granules and only very rarely endocrine ones. At later stages these granules gradually increased in number. They differed in size and density and were surrounded by a definite membrane (Figs 2 and 3).

Fragmentation of the cyst-like rough-surfaced endoplasmic reticulum into vesicles was indicative of a more advanced stage of transformation. Numerous ribosomes were attached to the outside surface of these vesicles. Fre-

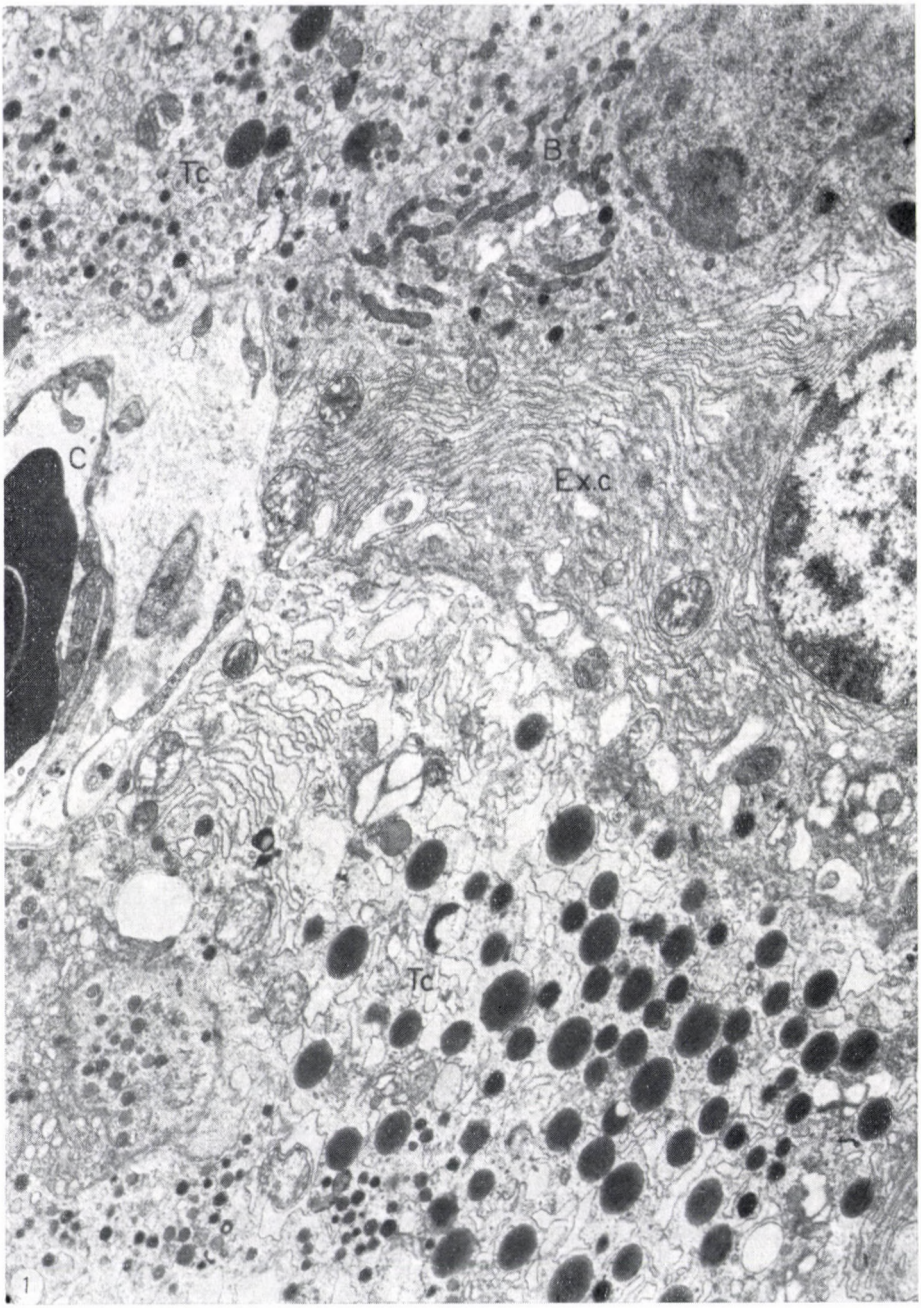

Fig. 1. Direct contact of the transforming and exocrine cells. Ex. c. = basal region of an exocrine cell; Tc = transforming cells; B = "*sui generis*" B cell; C = capillary; ×8,000

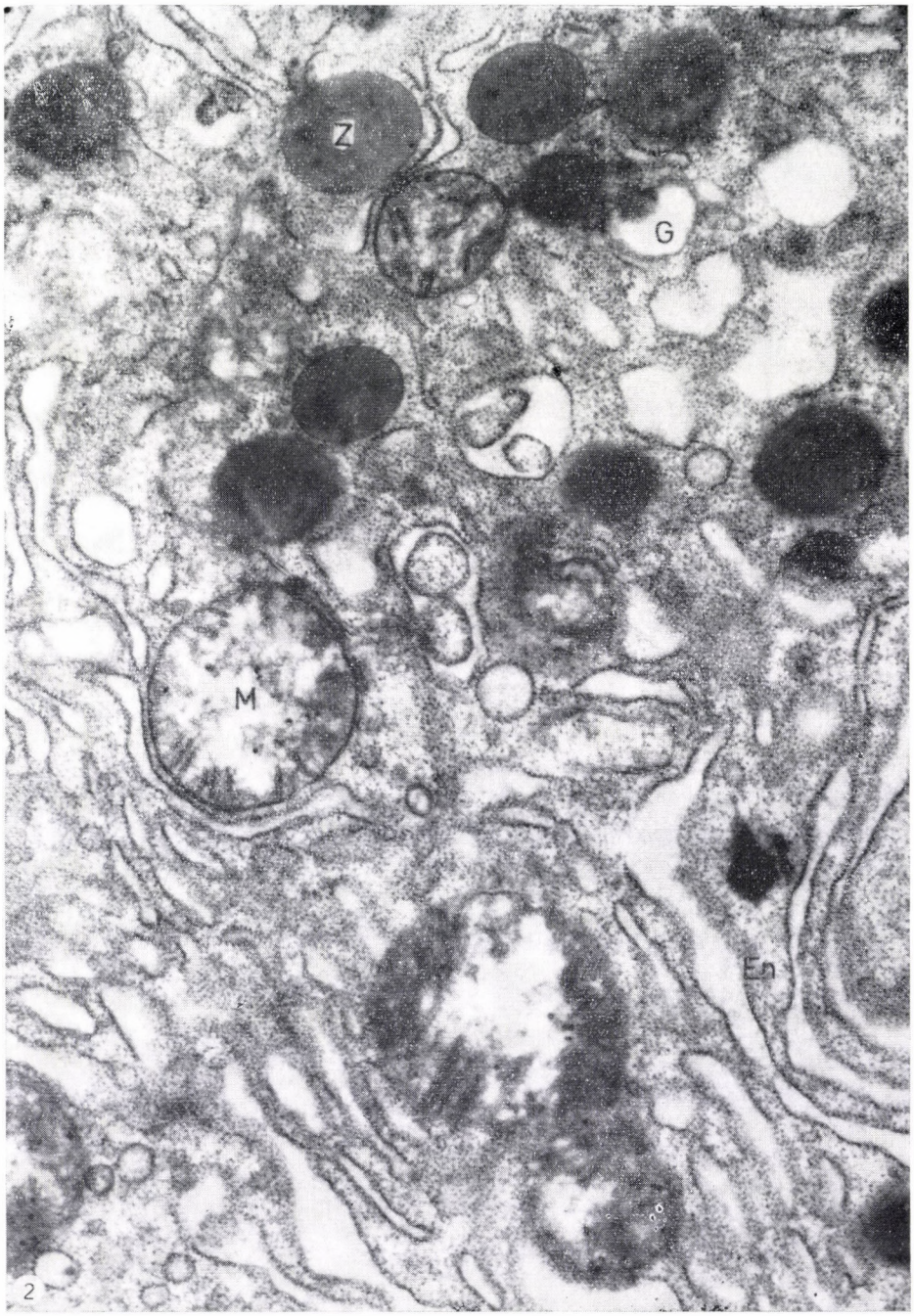

Fig. 2. Part of an acinar cell at early stage of transformation. Note the swollen mitochondria and the cyst-like endoplasmic reticulum. M = mitochondrion; En = endoplasmic reticulum; G = Golgi system; Z = zymogen granules; ×19,000

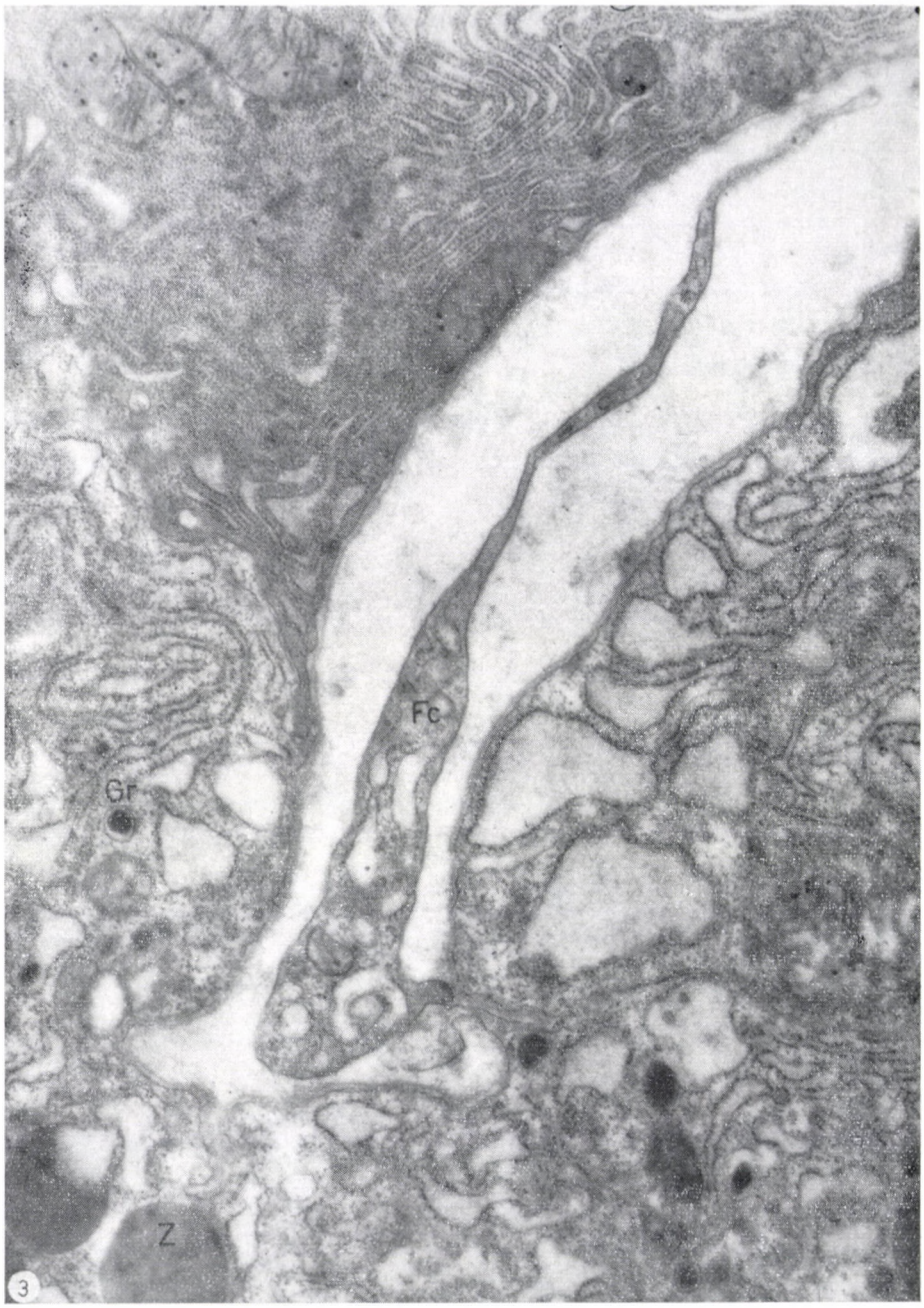

Fig. 3. Part of a pancreatic cell containing both endocrine and exocrine granules. Z = zymogen granules; Gr = endocrine granules; Fc = fibrocyte; × 19,000

quently, one or more groups of ribosomes were visible inside the vesicles. These groups probably resulted from the transsection of invaginated parts of the vesicles. It was, however, obvious that these groups of ribosomes should show different degrees of density ranging between free ribosomes and granulum-like bodies (Fig. 4). The zymogen granules were decreased in number but a few inhomogeneous prozymogen granules were indicative of an exocrine secretion, which was either still in progress or had just recently stopped [19]. Zymogen granules, with their membranes broken and their content partly disappeared, were occasionally seen (Fig. 3). In some cells dense, lipid-like materials were found surrounding a less dense, oval-shaped central substance. The endocrine granules were numerous at that stage, but they did not show the characteristics of any of the mature islet cell granule. Their density very much resembled that of the B-type granules, however, they had a tight-fitting membrane. Sometimes, however, there was a larger gap between the granules and their membranes. The mitochondria of the cells at that stage of transformation were much smaller than those of former stages.

In some cases it was possible to observe further disappearance of the cyst-like rough-surfaced endoplasmic reticulum. In a part of the cells continuous transition was seen between a rich, vesicular ergastoplasm, occupying the whole cytoplasm, and a very poor one only consisting of fragmented tubules or poorly-demarcated sacks. Simultaneously with the disappearance of the ergastoplasm the quantity of free ribosomes decreased gradually, while the endocrine granules increased in number (Fig. 5).

The origin of a fully transformed cell from an acinar cell was indicated only by the occasional presence of zymogen granules (Fig. 6). Therefore, in the absence of these granules it was difficult to establish the acinar origin of an islet cell. However, according to our observations, the final stage of acino-insular transformation might be represented by islet cells with a less-dense cytoplasm, and atypical, relatively small quantity of endocrine granules than in the *sui generis* beta cells and with somewhat swollen mitochondria.

Discussion

The question, whether pancreatic cells can be transformed or not has already been answered, also on submicroscopic level [7]. The direction of this transformation is, however, debated.

According to BJÖRKMAN and HELLMAN [4], acinar cells transformate into A cells in ducks. This was supported by the presence of these cells in the "dark" islets and also by the characteristic appearance of the endocrine granules. Though GUSEK and KRACHT [7] do not state that the transformed cells they have seen in rats treated with B cytotropic drugs are B cells, the histological pictures presented are similar to these. In hypothalamic obesity there

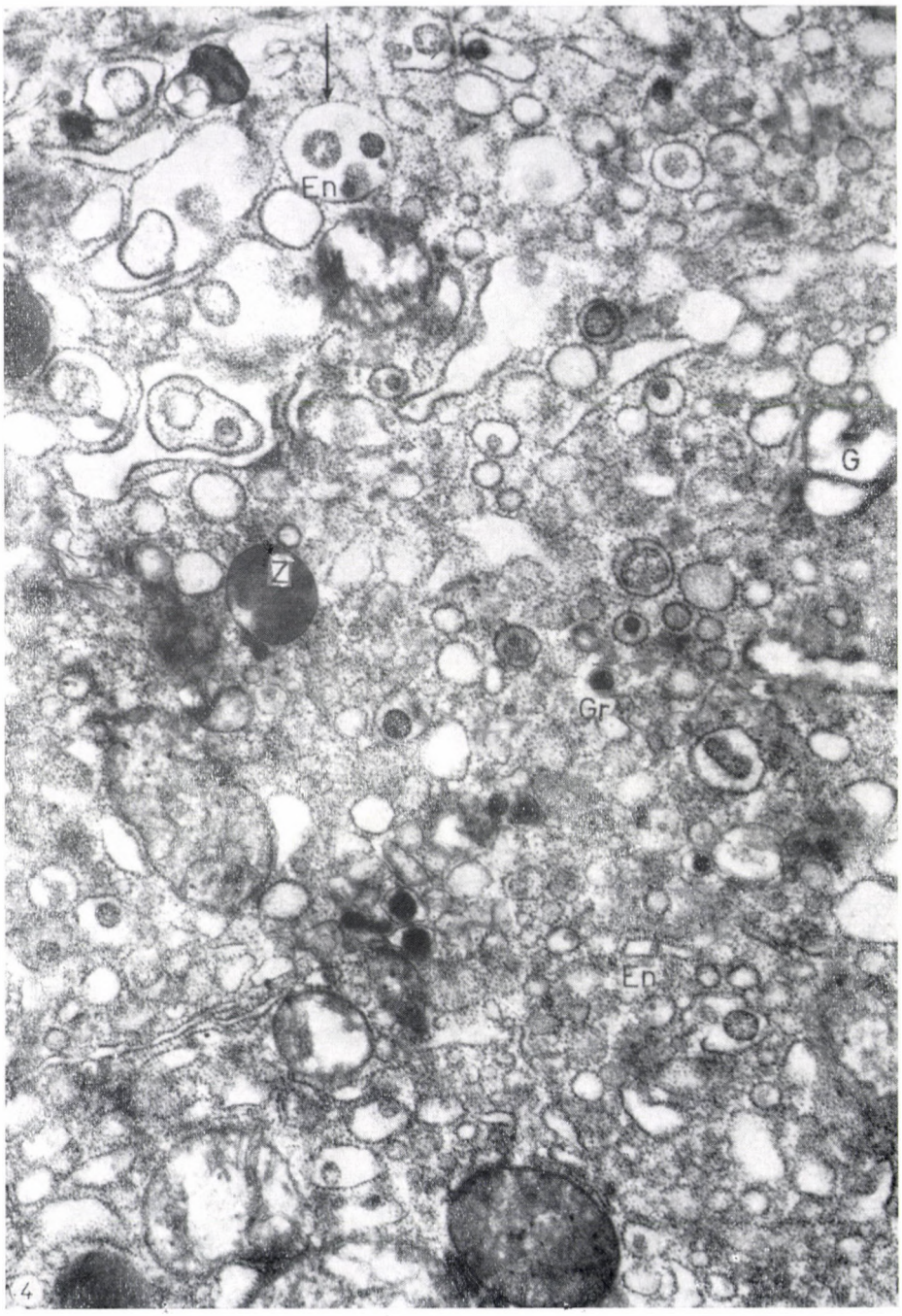

Fig. 4. Cell under transformation having vesicular endoplasmic reticulum. Note at the arrow the multiply invaginated groups of ribosomes. En = endoplasmic reticulum; Z = zymogen granules; Gr = endocrine granules; G = Golgi system; ×19,000

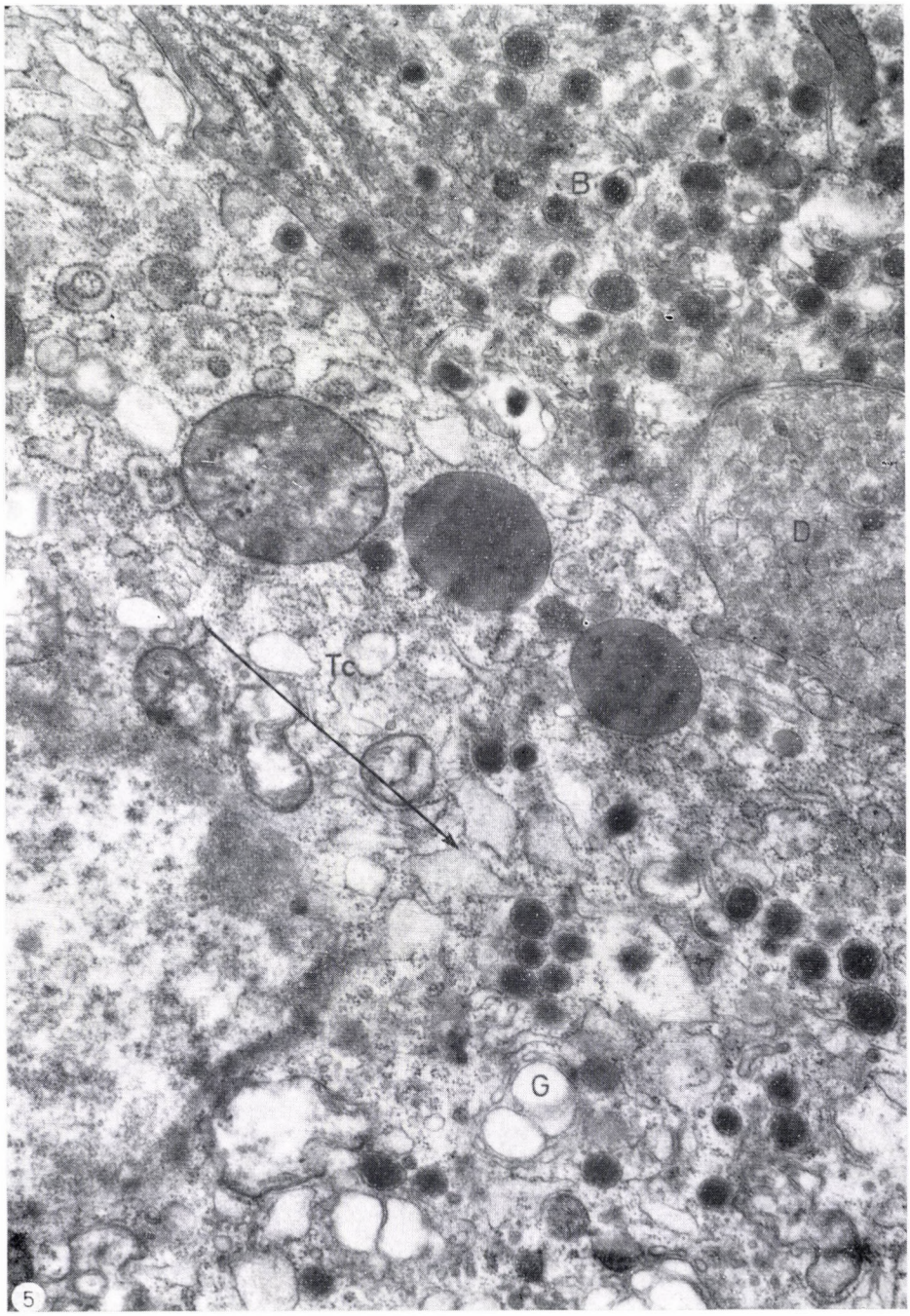

Fig. 5. Part of a transformed cell showing the disappearance of vesicular-type endoplasmic reticulum in the direction of the arrow. Tc = transformed cell; G = Golgi system; B = "*sui generis*" B cell; D = D cell; × 19,000

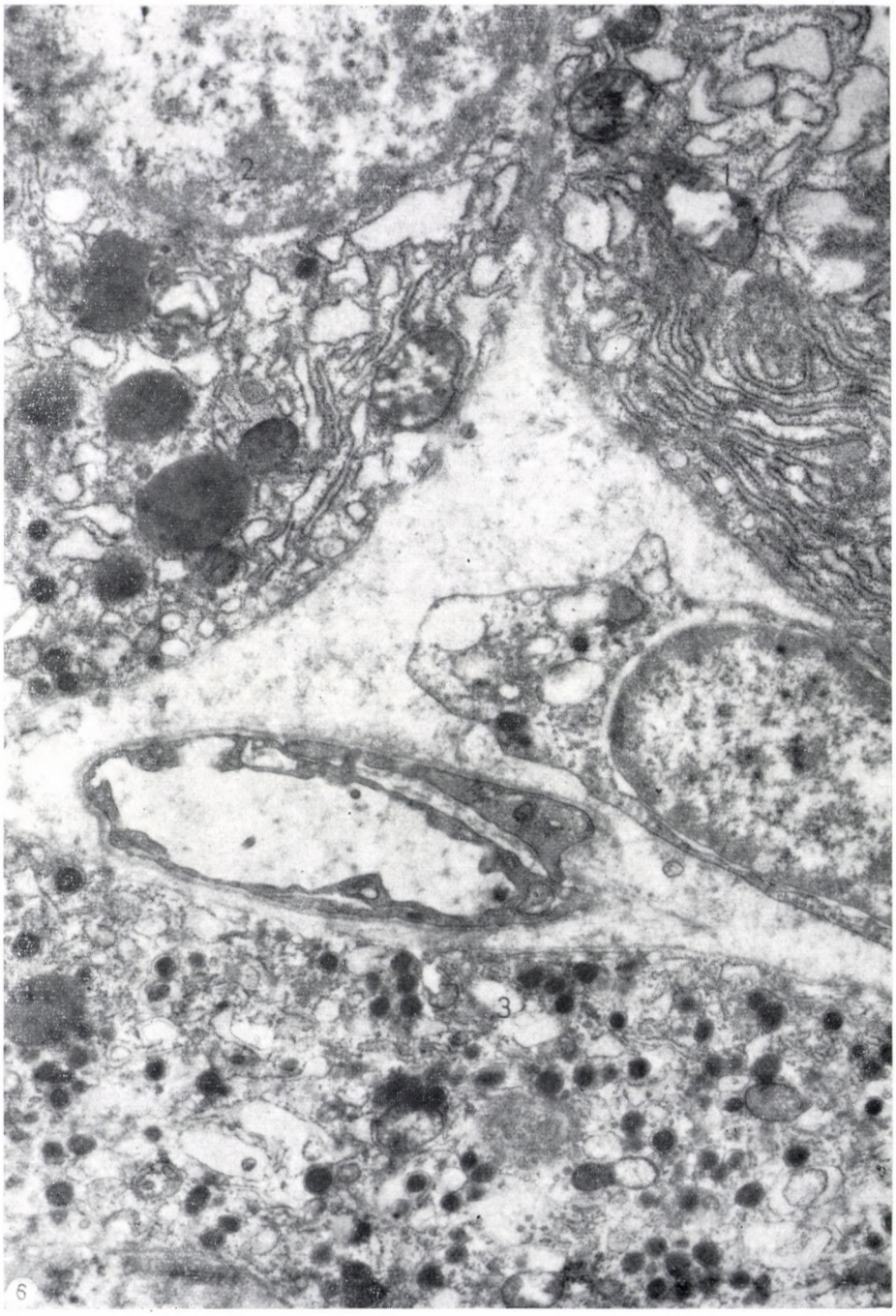

Fig. 6. The figure shows different stages of transformation in an anticlockwise direction. In cell number 1 cystic dilatation of the lamellar ergastoplasm is seen. In cell 2, besides the cystic rough-surfaced endoplasmic reticulum and several zymogen granules, already granules of endocrine character are found. In cell 3 only the single zymogen granule shows the development by transformation of the endocrine cell. $\times$ 10,800

is a pronounced hyperplasia [14, 15] and hyperfunction [8, 22] of B cells. Our observations indicate that acinar cells were transformed into B cells, since they were characterized by a gigantic Golgi system and a denser cytoplasm than any non-B-type islet cell, and the granules often resembled those of B cells. The possibility that transformation could proceed in both direction cannot be disregarded.

The most suggestive change in the course of transformation was the reorganization of the rough-surfaced endoplasmic reticulum according to the change in protein synthesis. The invagination frequently seen in our pictures points to another possibility too, namely, that the way in which the produced protein passes the membrane of the endoplasmic reticulum might be invagination.

It is very surprising that despite the great number of electron microscopic investigations of the endocrine pancreas transformation is mentioned in few papers. It was found mostly in cases with highly increased islet function or islet regeneration: in human diabetics [6, 10] in islet regeneration following alloxan injection [12, 13] after prolonged glucose infusion [24] and after administration of arylsulphonamides [7, 23]. In our obese rats B cell hyperfunction can also be assumed. Probably the replacement of islet cells takes place normally by other ways and transformation is rare and operates only when the B cells have to respond to a heavy demand like in polyphagia, regeneration or under the effect of diabetogenic agents.

Acino-insular transformation is possible not only in some species as assumed by BJÖRKMAN and HELLMAN [4] but it is a universal phenomenon independently whether the islets are isolated by connective tissue or not.

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ELEKTRONENMIKROSKOPISCHE BEOBACHTUNG DER AZINO-INSULAREN TRANSFORMATION IN DER BAUCHSPEICHELDRÜSE VON RATTEN

Im Pankreas von durch Hypothalamusläsion fettleibig gewordenen Ratten wurde elektronenmikroskopisch die Umwandlung der Azinuszellen in B-Zellen beobachtet. Als erstes Transformationszeichen trat eine Schwellung der Azinuszellenmitochondrien und die Erweiterung der Zisternen im Ergastoplasma auf. Entsprechend der Progression der Umwandlung erhielt das groboberflächige endoplasmatische Netz zunächst einen vesikulären Charakter, um dann allmählich an Menge abzunehmen. Gleichzeitig ließ sich in den sich umwandelnden Zellen die Verminderung der zymogenen Körnchen sowie die Vermehrung der Beta-Körnchen beobachten, und es erschienen die für die endokrinen Pankreaszellen kennzeichnenden Mitochondrien. Der Golgi-Apparat war während des gesamten Umwandlungsprozesses hypertrophisch.

НАБЛЮДЕНИЯ ПРИ ЭЛЕКТРОННО-МИКРОСКОПИЧЕСКОМ ИССЛЕДОВАНИИ ТРАНСФОРМАЦИИ АЦИНОЗНЫХ КЛЕТОК В ИНСУЛЯРНЫЕ В ПОДЖЕЛУДОЧНОЙ ЖЕЛЕЗЕ КРЫСЫ

Автор наблюдал при электронно-микроскопическом исследовании поджелудочной железы крыс, ожиревших вследствие повреждения гипоталамуса, преобразовывание ацинозных клеток в бета-клетки. Первым признаком трансформации было набухание митохондриев ацинозных клеток и расширение цистерн эргастоплазмы. По мере прогрессирования трансформации эндоплазматическая сетка грубой поверхности вначале приобрела везикулярный тип, а затем ее количество постепенно уменьшилось. Одновременно с этим в преобразовывающихся клетках наблюдалось уменьшение числа зимогенных зернышек, повышение числа бета-зернышек и появление митохондриев, характерных для эндокринных клеток поджелудочной железы. Для аппарата Гольджи в процессе всей трансформации была характерна гипертрофия.

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EFFECT OF CONSECUTIVE PROTEIN DEPLETION AND REPLETION ON THE GROWTH OF ALBINO RATS

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Synopsis

Ninety-two weanling female albino rats were used to study the effect of consecutive protein depletion and repletion on body weight, food consumption and the utilization and balance of nitrogen. In the first part of the study, consisting of a 77-day experiment, 80 rats were fed for alternating periods of 7, 14 and 21 days diets containing 6 and 45 per cent protein. Half of the animals were fed *ad libitum* while the remainder were kept on a restricted regime. In the second part of the study 12 rats were maintained on a similar protein supply for 73 days with alternating periods of 4, 8 and 12 days. In each case daily food consumption, faeces and urine were measured and the nitrogen content of each determined. It was observed that body weight gain was significantly affected by alternation in level of protein supply. The results appear to indicate that, in terms of growth, consecutive protein over- and undernutrition can be applied to advantage when the period of "dietary stress" is shorter. The nitrogen retention of the experimental animals depended to a significant degree upon the length of each test period, the nitrogen retention values decreasing as the protein starvation periods became longer.

Introduction

In protein metabolism the efficiency of nitrogen utilization largely depends on prior dietary conditions. There is ample indication that growing animals increase their body weights more rapidly after deficient underfeeding than those kept on an adequate regime; this is sometimes called as "catch up growth". Earlier works [1, 2, 12] have shown that during protein repletion after conditions of severe depletion, nitrogen retention is significantly increased and have been taken to indicate an increase in the apparent digestibility of the dietary nitrogen. Experiments on rats kept on a diet restricted in protein [11] showed that the absorption rate of certain amino acids from the intestine was much greater than in controls receiving standard feeding, while repletion caused a rapid and significant change in the rate of intestinal absorption to the normal level. These results emphasize the existence within the animal of certain adaptive mechanisms which play an important role in maintaining a relatively healthy condition.

In contrast to the abundance of information on body composition and biochemical changes that occur in alternately depleted and repleted animals [8], there is a lack of data on nitrogen utilization during repeated and consec-

utive periods of high and low levels of dietary protein intake. Most experiments to date deal with depletion-repletion supply of protein as a method to assess the protein quality of various food stuffs [6, 15], while others [4, 14] have either studied the utilization of food for maintenance of weight or the growth under different dietary conditions. GARCIA and RODEDRUCK [5] studied the influence of reduced protein intake on the utilization of nitrogen.

The present studies were initiated to determine the growth rate, food consumption, nitrogen utilization and retention of normally fed rats as compared with animals supplied for two months with alternating high and low levels of protein.

Material and method

Two series of experiments involving 92 female Wistar rats were carried out to determine the effect of consecutive protein under- and overfeeding on growth and nitrogen balance; levels of 6 and 45 per cent protein were chosen. In each experiment weanling animals were housed in group cages and fed a stock diet containing 20 per cent protein until they attained weights between 45 and 55 g. Those failing to make adequate weight gains with this diet were discarded.

The rats selected for the first experiment were divided into 8 groups (A_1, A_2, A_3, A_4 and B_5, B_6, B_7, B_8), each of ten animals. The rats of the first four groups were fed *ad libitum* and are referred to as the main group "A". The animals of the "B" series, referred to as the "restricted main group" ("B"), received a predetermined and measured amount of daily food, the quantity of which was increased stepwise as the animals increased in size. The low and high level of daily protein intake was alternated in the periods of 7, 14 and 21 days for groups A_2 and B_6, A_3 and B_7, and A_4 and B_8, respectively. Groups A_1 and B_5 served controls. The non-restricted animals had access to food at all times while the restricted animals were fed weighed quantities of food every day as follows: at the beginning of the experimental period 22 g per 100 g body weight, and at the end of the experimental period 13.4 g per 100 g body weight (average 14.3 g per 100 g body weight). The weight of food was calculated to be just

Table 1

Composition of diets

Diet "N"* % ingredients	Diet "P" % ingredients	Diet "R" % ingredients
15.0 Corn	50.7 Starch	34.8 Casein
30.0 Barley	10.0 Corn	10.0 Corn
25.0 Oat	10.0 Barley	14.0 Barley
10.0 Sunflower cake (extracted)	20.0 Oat	10.0 Oat
12.0 Meat meal	3.0 Yeast brewers' (dried)	3.0 Yeast brewers' (dried)
3.2 Yeast brewers' (dried)	4.3 Sunflower oil	10.0 Sunflower cake (extracted)
2.8 Sunflower oil	1.0 $CaCO_3$	12.0 Meat meal
1.0 $CaCO_3$	1.0 NaCl	4.2 Sunflower oil
1.0 NaCl		1.0 $CaCO_3$
		1.0 NaCl
Total 100.0	100.0	100.0
Diet contained:		
20.01% protein	5.57% protein	45.24% protein
6.01% fat	6.02% fat	6.06% fat
349.90 Cal	376.30 Cal	355.10 Cal

* Normal

sufficient for the requirements for normal growth of the animals. Daily records were kept of food consumption and the animals were weighed individually at weekly intervals.

In the second experiment 12 young rats were divided into 4 equal groups. The first group served as control and were supplied with a normal stock diet containing 20 per cent protein, while the other three groups were fed alternating on low and high levels of dietary protein at intervals of 4, 8 and 12 days. All the animals were kept in metabolic cages providing continuous access to food and water while collection of faeces and urine was facilitated by screen bottoms fitted to the cages. Daily records were kept of food intake, weight gains and output of urine and faeces.

The composition of the experimental diets used in both studies is shown in Table 1. Diet "P" provided the low level of protein and diet "R" the high level of protein; the contents of casein and starch were adjusted to yield the desired protein level. Efforts had been made to approach the dietary conditions which may often occur in practice, i.e., the high protein diets contain protein of high quality while the low protein diets have protein low in quality.

The nitrogen contents of the feaces, urine and the residual food as well as that of the diet were determined by the Kjeldahl procedure.

Results and discussion

Information on the mean weight of the rats at the beginning and at the end of the experiment for each group in the first experiment are shown in Table 2 and Figs 1 and 2. From a comparison of group "A" with group "B" it is evi-

Table 2

Body weight of animals at the begining and at the end of the first experiment; average daily food consumption per rat

Group	Body wt at the beginning g	Amount of food g	Calories	Protein g	Body wt at the end g
A_1	45.0 ± 3.2[1]	15.6	54.6	3.1	158.9 ± 12.9
A_2	45.0 ± 3.4	19.3	70.3	5.3	157.0 ± 12.5
A_3	45.0 ± 3.4	22.1	80.5	6.2	145.9 ± 15.1
A_4	45.1 ± 3.2	19.4	70.8	5.2	155.5 ± 20.3
B_5	45.0 ± 3.3	10.3	36.2	2.0	157.0 ± 10.6
B_6	45.0 ± 3.3	13.8	50.2	3.9	165.6 ± 9.7
B_7	45.1 ± 3.4	13.4	48.9	3.7	160.9 ± 18.0
B_8	45.1 ± 3.1	13.4	48.9	3.6	164.6 ± 15.0

[1] Average ±SE of mean

dent that those animals who showed the greater body weight were those whose feeding had been limited during each test period. It was noted that the final average body weight of the experimental "restricted" main group, including the control animals, was 5 per cent greater than that of the "*ad libitum* group" ($P > 0.05$). With regard to the experimental animals alone there was a consider-

able difference in the final average body weights between the two main groups (152.8 g compared with 163.7 g; $P < 0.05$).

The rats that had been maintained on a restricted diet grew more rapidly than those kept on an *ad libitum* diet as shown by the body weights expressed

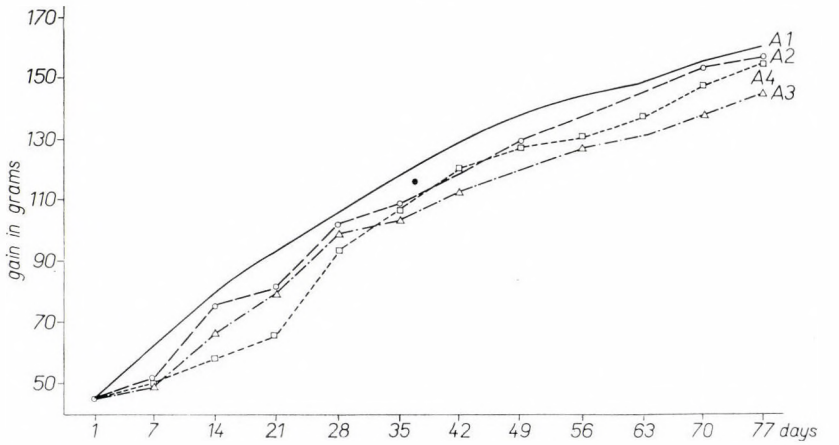

Fig. 1. Weight gain of rats (main group "A") fed alternating diets containing high and low levels of protein. A_1 = Control group; A_2 = 7-day group; A_3 = 14-day group; A_4 = 21-day group

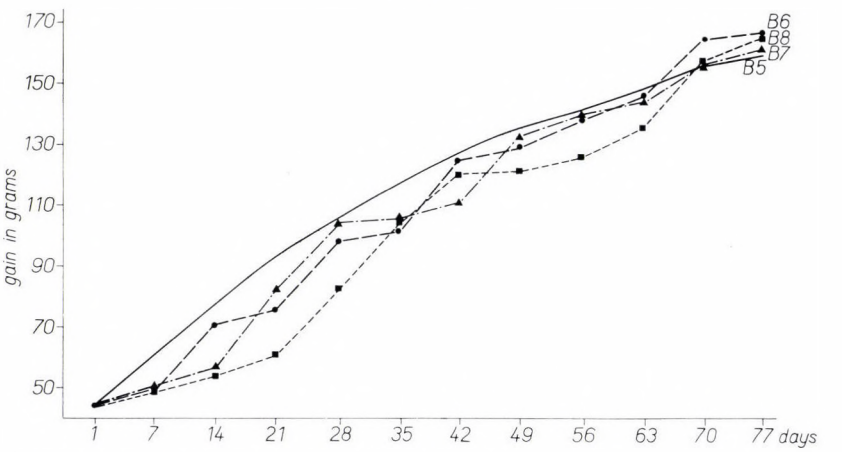

Fig. 2. Weight gain of rats (main group "B") fed alternating diets containing high and low levels of protein. B_5 = Control group; B_6 = 7-day group; B_7 = 14-day group; B_8 = 21-day group

on a percentage basis: $A_1 = 100\%$, $A_2 = 99\%$, $A_3 = 92\%$, $A_4 = 98\%$ and $B_5 = 100\%$, $B_6 = 105\%$, $B_7 = 102\%$, $B_8 = 105\%$; however, no significant difference could be shown between the two main groups.

The food consumption by groups "A" and "B" was markedly different from each other, the daily food consumption by group "A" having been significantly higher ($P < 0.01$) than that by the "restricted" main group "B".

Due to the restricted food intake of group "B" it was also noted that the differences in consumption of calories and protein showed the same significance ($P < 0.01$). In group "A" the daily food consumption showed more variation during the first two thirds of the 77-day experiment than the last third, when the food intake become more uniform.

Inspection of the data for the second experiment in Fig. 3 reveals that, in general, a variation in the level of protein within the range of 6 to 45 per cent and fed for consecutive periods over 73 days, produced the same marked effect

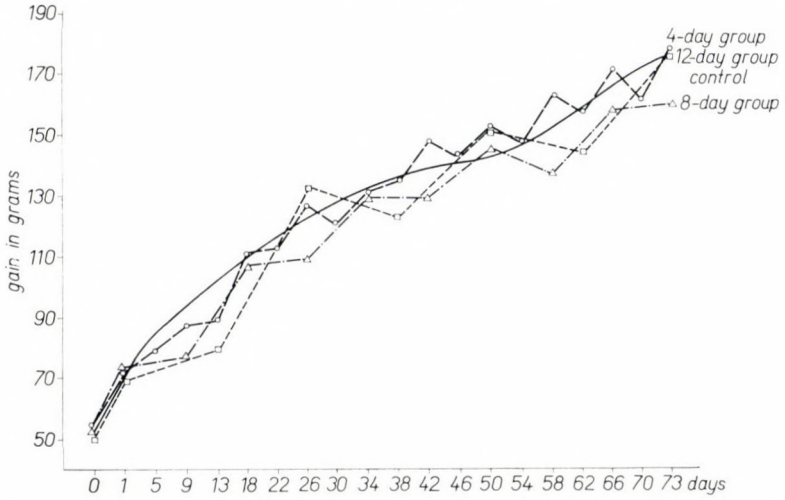

Fig. 3. Weight gain of rats used in nitrogen balance experiment and fed alternating diets containing high and low levels of protein

upon growth response as in the first experiment. It should be noted that, like in the first experiment, the most favourable weight gains were obtained when the dietary protein level was alternated most frequently (i. e. every 4 days). Since no significant variation could be shown to exist between the growth curves of the two series of experiment, it seemed permissible to combine the results of the 92 rats used "*in toto*".

Results for dietary nitrogen utilization (Table 3) show that there was no obvious difference between the average daily consumption for each group although, of course, the nitrogen intake was markedly influenced by the difference in diet given during each test period. Thus, during protein depletion, the animals consumed only 110—118 mg of nitrogen, while the total nitrogen intake on repletion was 901—1024 mg daily. Although the period of consecutive protein depletion and repletion differed among the groups, similar apparent digestibility coefficients were observed for each group and period (depletion gave values of 43—51 per cent and repletion values of 91—92 per cent). The values obtained agree with those of COFFEY, MANN and BOLMANN [3], who

Table 3

Average daily food consumption, nitrogen intake and apparent digestibility coefficients of proteindepleted and -repleted rats

Protein in diet %	Control group			4 day-group			8 day-group			12 day-group		
	Food consumption g	Nitrogen intake g	Coeff.[1] %	Food consumption g	Nitrogen intake g	Coeff. %	Food consumption g	Nitrogen intake	Coeff. %	Food consumption %	Nitrogen intake %	Coeff. %
6	—	—	—	12.7	117.74	46.9	12.6	109.87	51.0	12.6	112.49	43.3
20	12.1	460.91	85.1	—	—	—	—	—	—	—	—	—
45	—	—	—	13.5	1023.66	92.2	12.4	919.20	91.8	12.4	901.10	91.2

[1] Apparent digestibility coefficient $= \frac{N_{food} - N_{in\ faeces}}{N_{food}} \cdot 100$

showed that the protein absorptive efficiency was at a higher level in dogs given a protein-rich diet than in those kept on a definitely protein deficient diet.

Continuous nitrogen balances based on the average data for a 4-day period (Table 4) showed a marked increase in nitrogen retention when the nitrogen intake was normal (control group gave values of 41 per cent of the nitrogen intake) while lowered retention values were observed in those groups undergoing periods of "protein starvation"; the longer the period of starvation, the lower the retention values (alternating periods of 4, 8 and 12 days gave retention values of 34, 30 and 22 per cent, respectively). The effects of periods of protein depletion and repletion were most marked in the values obtained for the daily average retention of nitrogen. A comparison of the 12-day group receiving overnutrition with the corresponding 8- or 4-day group reveals that the former animals showed a marked sparing of nitrogen in terms of nitrogen output; however, in terms of nitrogen utilization or retention the converse was observed. The differences between the retention values of the 4-day group and the controls during periods of both depletion and repletion were highly significant ($P < 0.01$). The length of the test period had a marked effect upon the differences obtained. The mathematical evaluations indicate that the nitrogen retention of the animals depended to a significant degree upon the length of the period of protein depletion and repletion.

The effects of protein overnutrition were also evident in the urinary nitrogen excretion when greater values were obtained with diets rich in protein and a corresponding decrease with starvation. This may be a reflection of a direct effect upon body stores, which have the ability to lose more or less nitrogen when submitted to different dietary restrictions [2, 13]. These observations seem to confirm the results of HOLT, HALAC and KAJDI [7], who stated that during early periods of total nitrogen deprivation, the rats that had been kept

Table 4

Nitrogen balance data of the control and experimental groups
(mg N/rat/day)

Experimental days	4-day group		8-day group		12-day group	
	Control	Experimental	Control	Experimental	Control	Experimental
1—4*	205.8 ± 24.9[2]	50.1 ± 14.0				
5—8	121.0 ± 43.1	226.2 ± 58.1	163.4 ± 55.3	40.8 ± 16.8		
9—12*	165.2 ± 48.0	—2.5 ± 38.1			164.0 ± 52.3	27.1 ± 21.8
13—17[1]	202.2 ± 60.0	404.6 ± 106.1	183.7 ± 60.5	299.4 ± 164.2		
18—21*	189.5 ± 42.0	23.4 ± 38.2				
22—25	240.0 ± 101.8	519.8 ± 102.5	214.7 ± 80.6	—5.5 ± 36.6	210.8 ± 71.4	321.6 ± 149.1
26—29*	279.8 ± 41.9	13.4 ± 36.9				
30—33	143.3 ± 64.1	340.0 ± 75.3	211.5 ± 87.6	367.5 ± 158.1		
34—37*	217.7 ± 99.8	—2.1 ± 34.1			213.6 ± 90.4	—9.5 ± 19.0
38—41	228.5 ± 114.5	464.1 ± 93.3	223.1 ± 105.2	—0.02 ± 21.9		
42—45*	231.7 ± 86.4	—50.9 ± 76.3				
46—49	212.5 ± 66.6	347.7 ± 172.8	222.1 ± 76.1	356.0 ± 207.2	224.3 ± 89.0	214.2 ± 167.8
50—53*	119.9 ± 119.1	—49.5 ± 51.6				
54—57	106,1 ± 43.9	301.3 ± 159.9	113.0 ± 88.1	—23.0 ± 38.7		
58—61*	186.4 ± 55.8	—80.1 ± 42.8			137.5 ± 85.5	—25.8 ± 48.4
62—65	163.2 ± 44.5	232.6 ± 217.5	174.8 ± 50.7	198.2 ± 188.0		
66—69*	174.4 ± 51.4	—56.2 ± 51.1				
70—73	176.5 ± 69.0	352.4 ± 105.6			171.4 ± 54.6	150.8 ± 141.8
Daily N intake, mg	460.91	576.91		520.76		512.20
Daily N excretion, mg	274.04	408.33		366.60		400.63
Daily N retention, mg	186.87	168.58		154.16		111.57

* Depletion period
[1] 5-day period
[2] Averages ±SE of mean

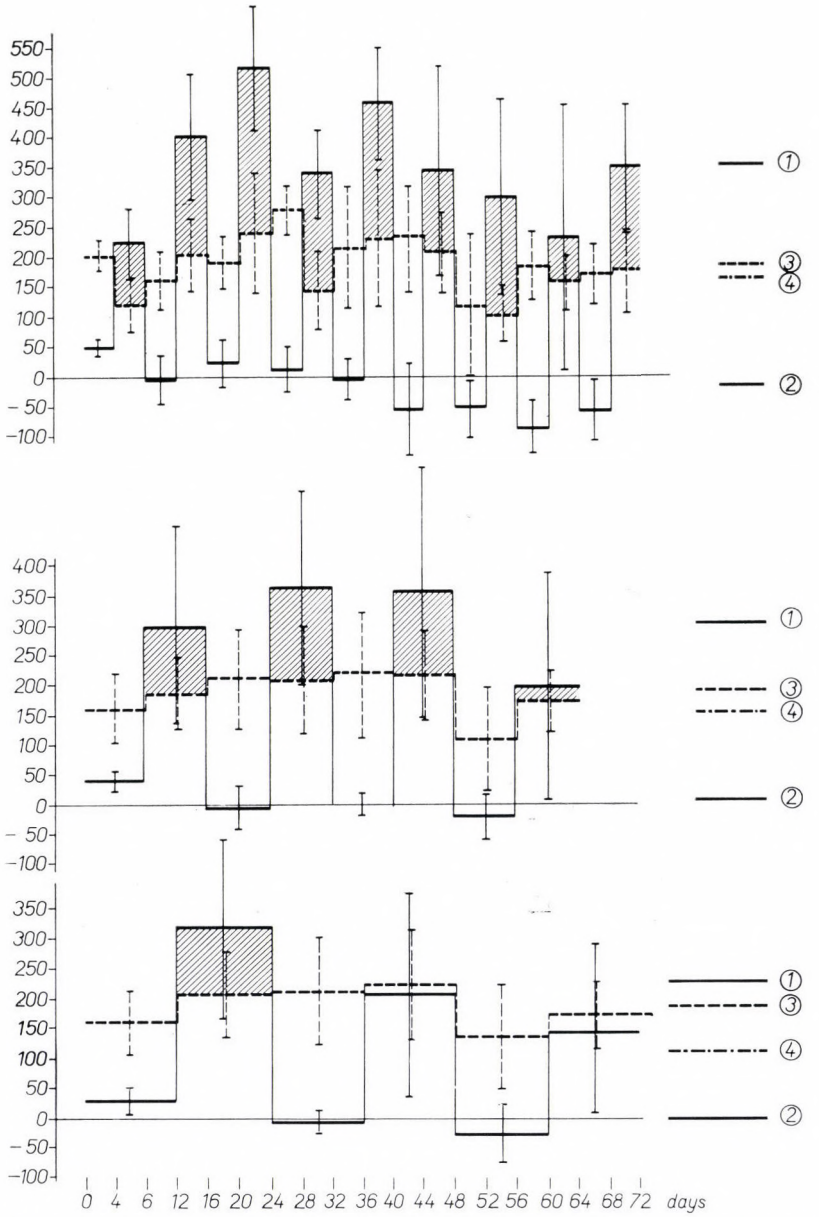

Fig. 4. Changes of nitrogen retention (mg N/rat/day) in the first, second and third experimental group during depletion and repletion periods
1 = Average nitrogen retention during the period of protein overfeeding; 2 = Average nitrogen retention during the period of protein undernutrition; 3 = Average nitrogen retention of the control group; 4 = Average nitrogen retention during the experiment. Vertical lines represent the SE of mean

on a high level of protein catabolized their protein stores more intensively than those that had been maintained on a "normal" diet.

From these data it appears possible that the feature of a "narrowing" in nitrogen retention as the periods of protein over- and undernutrition advanced (Fig. 4) may account for the decreased capacity of the organism to adapt itself to a changed nutritional status.

It should be noted that there was no significant difference in the final average body weights between the controls and the groups fed for alternating periods of 4 and 12 days. It is probable that the final body weight responses were obscured partly because of the gut fill, partly, the 4- and 12-day groups were in "catch up state" due to the refeeding period at the end of this experiment.

The data presented here suggest that nitrogen utilization may be favoured when the organism is recovering from protein starvation and particularly when the protein intake is in great excess of that required for standard growth. The results indicate that consecutive periods of protein over- and undernutrition can be applied to better advantage when the "dietary stress" (in terms described here) is of short duration. In a consideration of these results, it must be kept in mind that a change in the frequency of the dietary conditions may have a further detrimental effect, i. e., occasional anorexia; this may in fact be a consequence of dietary change.

Finally, it should be pointed out that the importance of the duration of the protein depletion period must be strictly related to requirements for the maintenance of animal body weight and size [9, 10].

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DIE WIRKUNG ABWECHSELNDER FÜTTERUNG MIT EIWEIßARMEN BZW. -REICHEN FUTTER AUF DAS WACHSTUM WEIßER RATTEN

Die Wirkung abwechselnder eiweißarmer bzw. -reicher Nahrung auf die Gewichtszunahme, den Nährstoffverbrauch und die Stickstoffutilisation wurde an weißen Ratten untersucht. Im ersten, 77 Tage dauernden Teil des Experiments erhielten 80 Tiere eine eiweißarme (6%ige) bzw. -reiche (45%ige) Ernährung, abwechselnd in 7, 14 bzw. 21tägigen Intervallen. Die Hälfte der Tiere erhielt das Futter ad libitum, die andere Hälfte erhielt bestimmte Futterrationen. Im zweiten Teil des Experiments wurden 12 Tiere 73 Tage lang ähnlich ernährt, nur daß die eiweißarme bzw. -reiche Diät in 4, 8 bzw. 12tägigen Intervallen gewechselt wurde. In sämtlichen Fällen wurde der tägliche Nährstoffverbrauch, das Ausmaß der Stickstoffaufnahme sowie die Menge des mit dem Harn und dem Kot ausgeschiedenen Stickstoffs gemessen. Aus den Versuchsergebnissen ist ersichtlich, daß die Gewichtszunahme in erheblichen Maße von der Eiweißzufuhr abhing. Die eventuelle günstige Wirkung des abwechselnden eiweißarmen bzw. -reichen Futters manifestiert sich nur in dem Falle, wenn der sog. „diätetische Stress" von kürzerer Dauer ist. Die Stickstoffretention der Versuchstiere hing in sämtlichen Fällen in erheblichem Maße von der Versuchsdauer ab: das Ausmaß der Stickstoffretention verminderte sich proportional der Eiweißstarvation.

ДЕЙСТВИЕ ПОСЛЕДОВАТЕЛЬНОГО ПРИМЕНЕНИЯ БЕДНОГО БЕЛКАМИ И БОГАТОГО БЕЛКАМИ РЕЖИМА ПИТАНИЯ НА РОСТ КРЫС АЛЬБИНОСОВ

Автор исследовал у крыс альбиносов действие последовательного применения бедного белками и богатого белками режимов питания на прибавление в весе, потребление питательных веществ и использование азота. В первой части опыта автор чередовал богатый белками (45%-ый) и бедный белками (6%-ый) режимы питания у 80 животных с 7, 14 и 21-дневными интервалами. Половина подопытных животных получила питание по желанию, а другая половина — определенное количество пищи. Во второй части эксперимента 12 животных получили подобный режим питания, в течение 73 дней, с той лишь разницей, что богатый и бедный белками режимы питания чередовались 4, 8 и 12-дневными интервалами. У каждого животного было определено суточное потребление питательных веществ, был проведен анализ величин приема азота и количества азота, выделенного с мочой и с калом. Из результатов эксперимента следует, что прибавление в весе в значительной мере зависит от содержания белка в применяемой диете. Предположительное благоприятное действие чередования бедного и богатого белками режимов питания проявляется в том случае, если так наз. «диететический стресс» продолжается короткое время. Задержка азота организмом подопытных животных во всех случаях в значительной мере зависила от продолжительности периода исследования: по мере белкового голодания понизился и размер задержки азота.

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EFFECT OF GLUCOCORTICOIDS ON THE THYMUS

I. HISTOCHEMICAL AND ELECTRON-MICROSCOPIC INVESTIGATION OF MAST CELL FORMATION

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Synopsis

Formation of mast cells was induced in the thymus of adult rats by short-term and prolonged treatment with cortisone. It is concluded from histochemical and electron-microscopic examinations that mastocytopoiesis does occur in the thymus. The light- and electron-microscopic picture of the developing forms of mast cells is described. A hitherto unknown crystalline structure was detected in the granules of developing mast cells.

Introduction

The existence of correlations between thymus and adrenals has long been known [2, 11, 12, 14, 26, 33, 34]. Reaction to stresses or treatment with cortisone induces accidental thymic involution: the organ may lose 70 to 75 per cent of its weight in one or two days [2, 12], a phenomenon mainly due to an emigration of thymocytes. Recent investigations seem to prove that the connection between glucocorticoids and the thymus is mutual, and closer than formerly supposed. It was found that glucocorticoids affect the metabolism of thymocytes also in vitro [28, 29]; the experiments of Dougherty and co-workers [15] have made it furthermore probable that a cortisol-cortisone conversion from the effective to the ineffective form, and presumably vice versa, takes place in the lymphatic organs, and that during long-term treatment the thymus plays a decisive role in these processes.

Simultaneously with the emigration of thymocytes, glucocorticoids induce a large-scale production of mast cells in the thymus. It appears that — at least as far as rats and mice are concerned — the thymus is one of the main sites of mastocytopoiesis and that cortisone is a prime mover in this process [11, 12, 14]. Cortisone promotes mast cell formation in other parts of the organism, too [13, 14], but less intensively than in the thymus. We demonstrated in earlier studies [4, 5, 7, 9, 10, 40, 41] that the process begins with production of PAS-positive cells which develop subsequently into metachromic (i. e. mast) cells.

The present experiments had a double purpose (1). We wanted to study the histochemical changes which characterize the incipient phase of cortisone-

induced mastocytopoiesis and to collate these phenomena with ultrastructural findings. It was hoped to identify in this way the developing forms of mast cells, regarding which some theories have been developed [35], but no precise data are available (2). We wanted furthermore to observe those ultrastructural changes which occur in the thymus under the influence of short-term or prolonged treatment with glucocorticoids. This first article deals mainly with the formation of mast cells; the general thymic reaction is only touched upon where it is indispensable.

Material and method

Sixty-seven adult male rats of the Wistar strain from our laboratory stock were used.

Histochemical analyses. Forty animals, divided in unequal groups, were treated with prednisolone (Di-Adreson F-aquosum, ORGANON) or cortisone (Adreson, ORGANON). The reason we employed prednisolone was that its intravenous injection was expected to produce an acute but essentially cortisone-like effect. The single intravenous dose of prednisolone was 5 mg/100 g body weight, and the thymus was removed 30, 60 minutes or 6 hours after the injection. The long-term treatment with cortisone lasted 3, 6 or 8 days with daily doses of 5 mg per 100 g body weight; the thymus was removed 24 hours after the last injection.

After being removed, the thymuses were placed in Bouin's fixative and embedded in paraffin. Sections, made in two planes, were stained with toluidine blue (pH 6.0), azure A (adjusted to pH 3.0 with citrate puffer), alcian blue [22], PAS, Hale-PAS, Giemsa and Gomori's paraldehyde fuchsin (with and without oxidation).

When applying Gomori's staining method to certain selected sections we employed as oxidant potassium permanganate; in this reaction sulphuric acid was replaced by hydrochloric acid; the molarity and pH value of the reagent agreed with those of the reagent containing sulphuric acid. Other sections were oxidized with potassium periodate combined as usual with sulphuric acid.

Other selected preparations were subjected to digestion with diastase or lipid extraction with chloroform-methanol; still other samples were fixed in formalin, frozen, and — after extraction with chloroform-methanol, or without extraction — stained with Oil red O.

Electron-microscopy. Twenty-seven animals were divided into groups of 3 or 5 members. Chest was opened under ether anaesthesia at 10, 30 or 60 minutes following single intravenous administration of prednisolone (5 mg per 100 g); at 6 hours following single dose of Adreson (5 mg/100 g); at 24 hours after the last daily dose of Adreson administered for three or six days. The apex of the heart was cut off and a cannula inserted in the aorta. To remove the blood, the blood vessels were perfused for one or two minutes with dextran (Plasmodex, HUMAN) and pre-heated to 37°C whereafter the thymus was perfused (likewise through the cannula) for 15 minutes with a 4.5% solution of glutaraldehyde (Fluka) in 0.1 M buffer of pH 7.2. Subsequently the thymus was removed and cut into fragments of 1 mm^3. The fragments were then kept for two more hours in the fixative rinsed with phosphate buffer postfixed in a 1% solution of osmium tetraoxide, dehydrated with ethanol and transferred through propylene oxide to be embedded in Araldite. The sections were contrast-stained with uranyl-acetate dissolved 50% methanol [38] and lead citrate [32], and studied in an electron microscope, type JEM 6-C.

Results

Histochemical analyses

Results are shown in Tables 1, 2, 3.

Since neither the test animals nor the controls had medullary cells that seemed to be in relationship with mastocytopoiesis, phenomena observed in the medulla of thymus are not dealt in this study.

Chronology

As can be seen in Table 1, all methods employed by us failed to reveal specifically staining cells in the thymic cortex of the control animals. This applies also to the test animals examined 10 and 30 minutes after the injection of prednisolone. It was at 60 minutes that a few PAS- and Gomori-positive cells began to appear, but most of these had disappeared by 6 hours. A great number of PAS-, Gomori- and Hale-positive cells were observed after three-day treatment, and there was a vast amount of Gomori- and Hale-positive cells after a treatment of six days. By the eighth day, also the number of PAS-positive cells had reached that of Gomori- and Hale positive cells.

Table 1

Staining reactions of cells in the cortex of the thymus at different times following treatment with prednisolone or cortisone

Group	Chloroform methanol + PAS	Diastase digestion + PAS	Paraldehyde-fuchsin	Paraldehyde-fuchsin after oxidation	Hale	Alcian blue	Azure A	Toluidine blue	PAS
30 min.	—	—	—	—	—	—	—	—	—
60 min.	+	+	—	+	—	—	—	—	+
6 hrs	—	—	—	+	—	—	—	—	—
3 days	++	++	—	++	++	—	—	—	++
6 days	++	++	—	++++	++++	—	—	—	++
8 days	++++	++++	—	++++	++++	—	—	—	++++
Controls	—	—	—	—	—	—	—	—	—

Cell types

Histochemical examinations revealed two dominant types of the Hale-, PAS- and Gomori-positive cells: large and smaller cells. The large ones, probably reticular cells, showed shortly after the treatment, mild vacuolation, which became very pronounced after prolonged treatment. The said reactions remained positive after lipid extraction with chloroform-methanol.

Frozen sections, stained with Oil red O, showed large cells rich in lipids in the Gomori-positive zone. Oil red O-reaction was negative after extraction with chloroform-methanol.

The smaller cells, giving positive reaction with Gomori, Hale and PAS, were still larger than the small lymphocytes (Fig. 1). They formed groups either in the cortical and capsular areas where mast cells were present, or independently. They failed to become metachromic when stained with toluidine blue and azure A, and did not stain with alcian blue.

Since it was only after oxidation that large and small cells assumed the characteristic violet colour, the suspicion arose that we were dealing with sulphuration (and not oxidation), considering that the oxidant contained sulphuric acid. Therefore, we modified paraldehyde-fuchsin staining (Table 2). The

Table 2

Reactions of small (C_b) cells (multiplying) under the effect of cortisone) after treatment with different oxidants and stains

	Toluidine blue	Azure A	Alcian blue	Toluidine + diastase	Azure A + diastase	Alcian + diastase	Toluidin + lipid extraction	Azure A + lipid extraction	Alcian + lipid extraction	Paraldehyde-fuchsin
$KMnO_4 + H_2SO_4$	+	+	—	+	+	—	+	+	—	+
$KMnO_4 + HCl$	+	+	—	+	+	—	+	+	—	+
$KJO_3 + H_2SO_4$	—	—	—	—	—	—	—	—	—	—

Gomori reaction was found positive even when hydrochloric acid had been substituted for sulphuric acid, indicating that the violet colour resulted from oxidation. It was moreover found that small cells which did not stain with toluidine blue and azure A prior to oxidation, became slightly metachromic thereafter (Figs 2, 3, 4). Extraction with chloroformmethanol and digestion with diastase had no effect on the metachromasia.

There were, thus, two characteristic cell types encountered in the thymus after cortisone treatment of 3, 6 or 8 days. One type, found in the cortex and medulla alike, consisted in reticular cells containing large amounts of lipid and filled with Gomori-, Hale- and PAS-positive matter in the intervacuolar space. These will be termed C_a cells in the following (C meaning evoked by cortisone). Although the dimensions of the other type were suggestive of lymphoid cells, their origin could not be determined on the evidence of light microscopic histochemical examinations. The light microscope revealed them to contain a Gomori-, Hale- and PAS-positive matter. Their cytoplasm became slightly metachromic after oxidation. These cells will be termed C_b in the following.

We paid particular attention to the appearance of mature mast cells (Table 3). In the controls, and in the test animals 30, 60 minutes and 6 hours after prednisolone treatment mast cells were not found except in the capsule and the adjacent connective tissue, where their number had notably increased by the third day of treatment, and also the cortex contained many such cells after a treatment of 6 or 8 days.

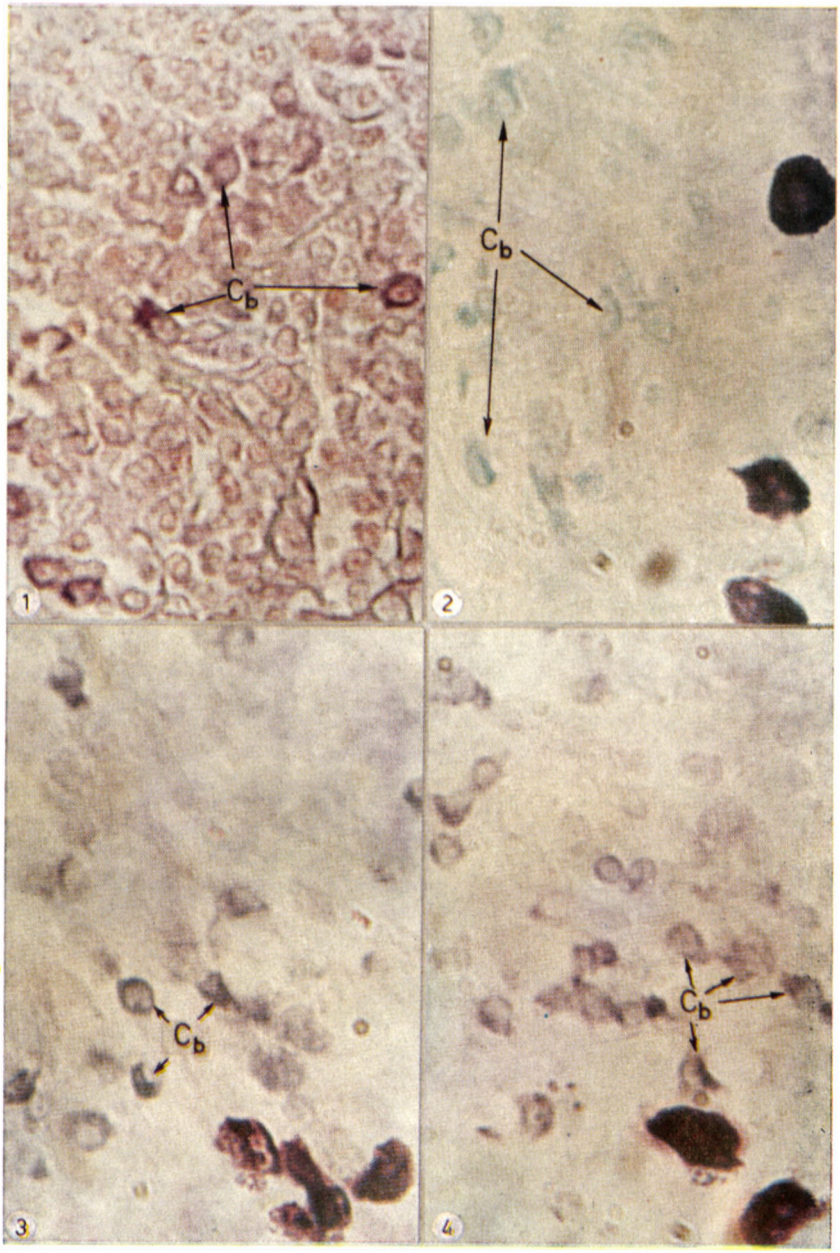


Fig. 1. Cortex of thymus after a six-day treatment with cortisone. Gomori-positive small, C_b cells (→). Paraldehyde-fuchsin after oxidation, ×300

Fig. 2. Subcapsular area in the thymic cortex after a six-day treatment with cortisone. Note metachromic mast cells and non-metachromic small, C_b cells (→). Toluidine blue, ×300

Fig. 3. Subcapsular area in the thymic cortex after a six-day treatment with cortisone. Note mast cells and slightly likewise metachromic small, C_b cells (→). Toluidine blue (after oxidation), ×300

Fig. 4. Subcapsular area in the thymic cortex after a six-day treatment with cortisone. Note mast cells and small, C_b cells showing less pronounced metachromasia (→). Azure A (after oxidation), ×300

Table 3

Amount of mature mast cells after treatment with prednisolone or cortisone

Group	Capsule	Cortex
30 min.	+	—
60 min.	+	—
6 hrs	+	—
3 days	++++	—
6 days	++++	+
8 days	++++	+++
Controls	+	—

Haematoxylin and May-Grünwald-Giemsa stainings were performed in order to detect the possible existence of eosinophilic granulocytes. No such cells were encountered.

Electron-microscopic studies

Chronology

Many lymphocytes, reticular cells, a few plasma cells, further ripe and unripe mast cells were seen in the cortex of the thymus of control animals. Granules appeared in some reticular cells ten minutes after the administration of prednisolone. The pattern showed no change after 30 and 60 minutes and the picture was like that in the controls 6 hours after the administration of Adreson.

Hardly any lymphocytes were encountered after three days of Adreson treatment, and none after six days. We saw numerous reticulocytes containing characteristic granules that appeared to be identical with those contained in immature mast cells. In addition to these cells, we found a still greater number of large reticulocytes filled with lipid granules. At last a few epithelial reticulocytes with characteristic tonofibrils were observed.

Since both light- and electron-microscopy had revealed the predominance of two cell types, it was easy to identify the lipid-filled cells as the type C_a and the other type as cells C_b described above. The method of identification will be dealt with in the Discussion.

Cell types

Grouped in clusters, cells C_b appeared only in the cortex of the thymus (Figs 5, 6). We saw C_a cells around these clusters; they contained numerous lipid granules and much phagocytized matter in the animals that had repeat-

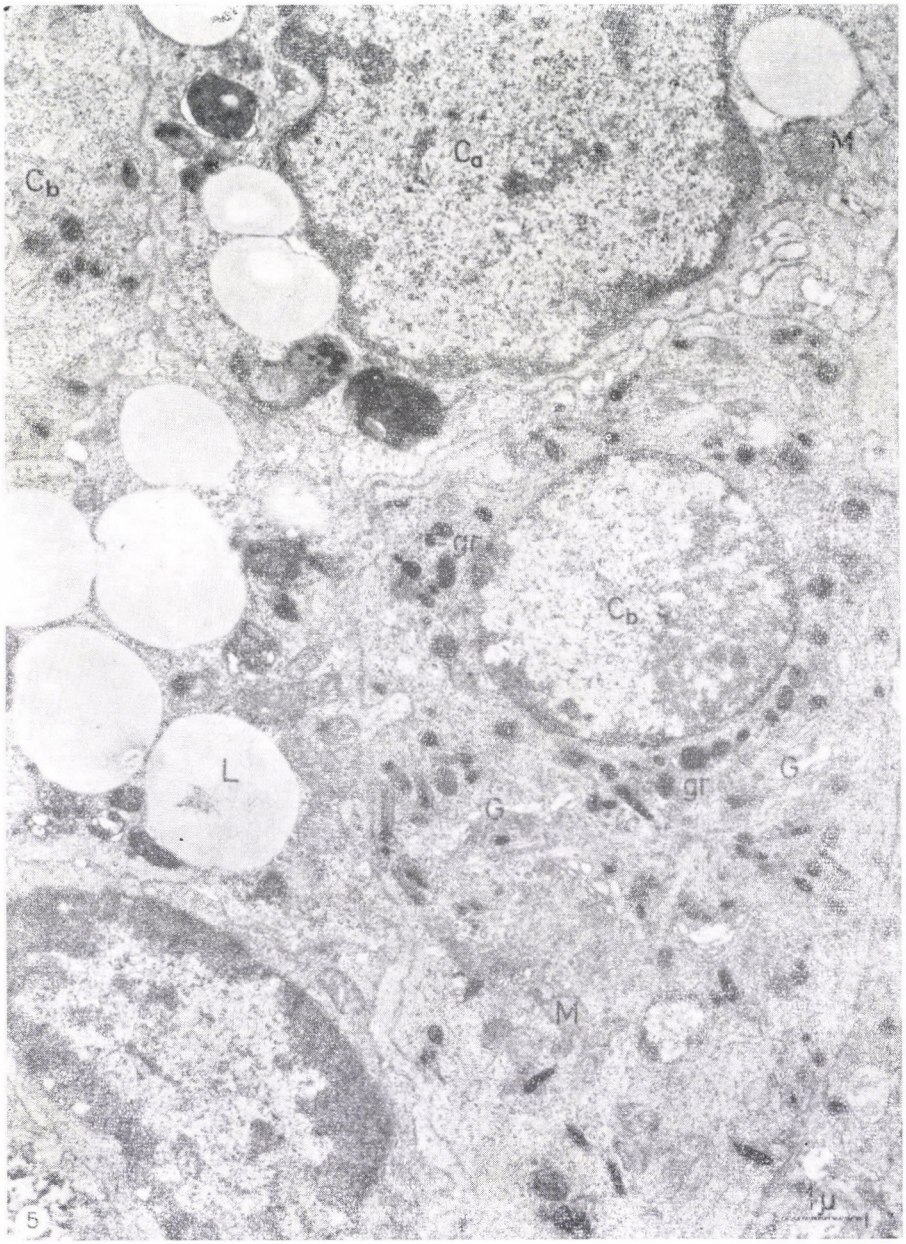


Fig. 5. C_a and C_b cells in the cortex of the thymus after a six-day treatment with cortisone. C_a cells contain numerous lipid droplets (L) and phagocytized matter. The cytoplasm of C_b cells is characterized by the presence of dark granules (gr). Note the well-developed Golgi apparatus (G). M = mitochondrium

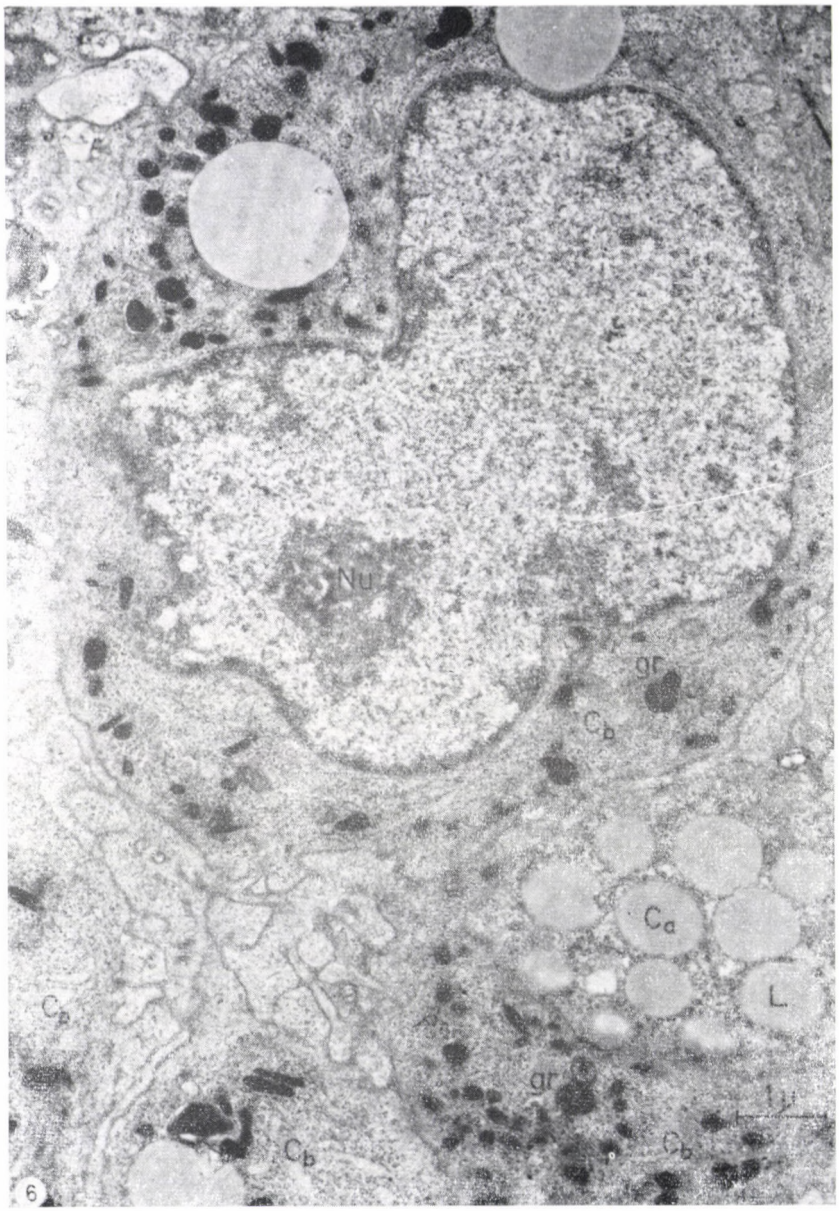


Fig. 6. Picture showing positions of C_b-cell groups. The large polymorphous nuclei of the youngest C_b cells have a loose chromatin structure, and some of them contain nucleoli (Nu). Numerous small, darkly staining granules (gr) in the cytoplasm. Note (on the right) the C_a cell filled with lipid droplets (L). (Rat treated with cortisone for 6 days)

edly received cortisone. As has already been said, the C_a cells are not dealt with in this paper.

Most of the grouped C_β cells were at the same stage of development, although ripe mast cells, too, occurred among them, suggesting a close topographical connection between C_β cells and mature mast cells (Fig. 7). The crystalline structure — which, at first sight, was suggestive of that commonly found in eosinophile granules and is to be described below — was still recognizable in some granules contained in the young immature mast cells of the thymus (Fig. 8).

The usually multilobate nuclei of the youngest C_b cells (Figs 5, 6) were large and polymorphous, had a loose chromatin structure, and contained nucleoli (Fig. 6). Their cytoplasmic granules were small and electron-dense; only few granules revealed a crystalline inner structure.

As regards shape and structure, the nuclei of C_b cells became gradually more and more similar to those of the adjacent mature mast cells (Fig. 7).

The cytoplasm of C_β cells was remarkably rich in ribosomes (Fig. 9) which were either free or attached to the tubules of the endoplasmic reticulum. Coarse-surfaced cisternae of the endoplasmic reticulum were scattered all over the cytoplasm: they contained a fine nebulous substance (Figs 5, 6, 9). The Golgi apparatus of the C_b cells was frequently found in the concavity of the nucleus, its cisternae were distended at some points; numerous, mostly still structureless, vesicles — containing a dark substance — were seen in their vicinity (Fig. 11). Crystalline inner structure was generally most conspicuous in the granules around the Golgi apparatus. Presumably crystallization takes place inside of already preformed granules.

The mitochondria, mostly oval in shape, are scattered over the entire cytoplasm among the rough surfaced cisternae of the ergastoplasm (Fig. 8). A few lipid droplets may appear also in C_b cells after three- or six-day of cortisone treatment. (Figs 6, 9, 12).

The granules with crystalline structure (Fig. 10) are spindle-shaped, 0.25 to 0.5 μ in length and 0.10 to 0.25 μ in diameter. The rod-shaped crystalline structure of high density is situated along their longitudinal axis and has a diameter of about 0.25 μ. Limited by a membrane, the granules assume an oval form and lose their crystalline structure in the course of ripening; they increase in size, accumulate at the periphery of the cytoplasm, and a bright zone develops along the inner surface of the membrans. Also rarified granules of low electron density, similar to those present in mature degranulating mast cells, were observed among the peripherally accumulating granules in the cytoplasm of the C_b cells of animals that had received cortisone for 6 days (Fig. 12).

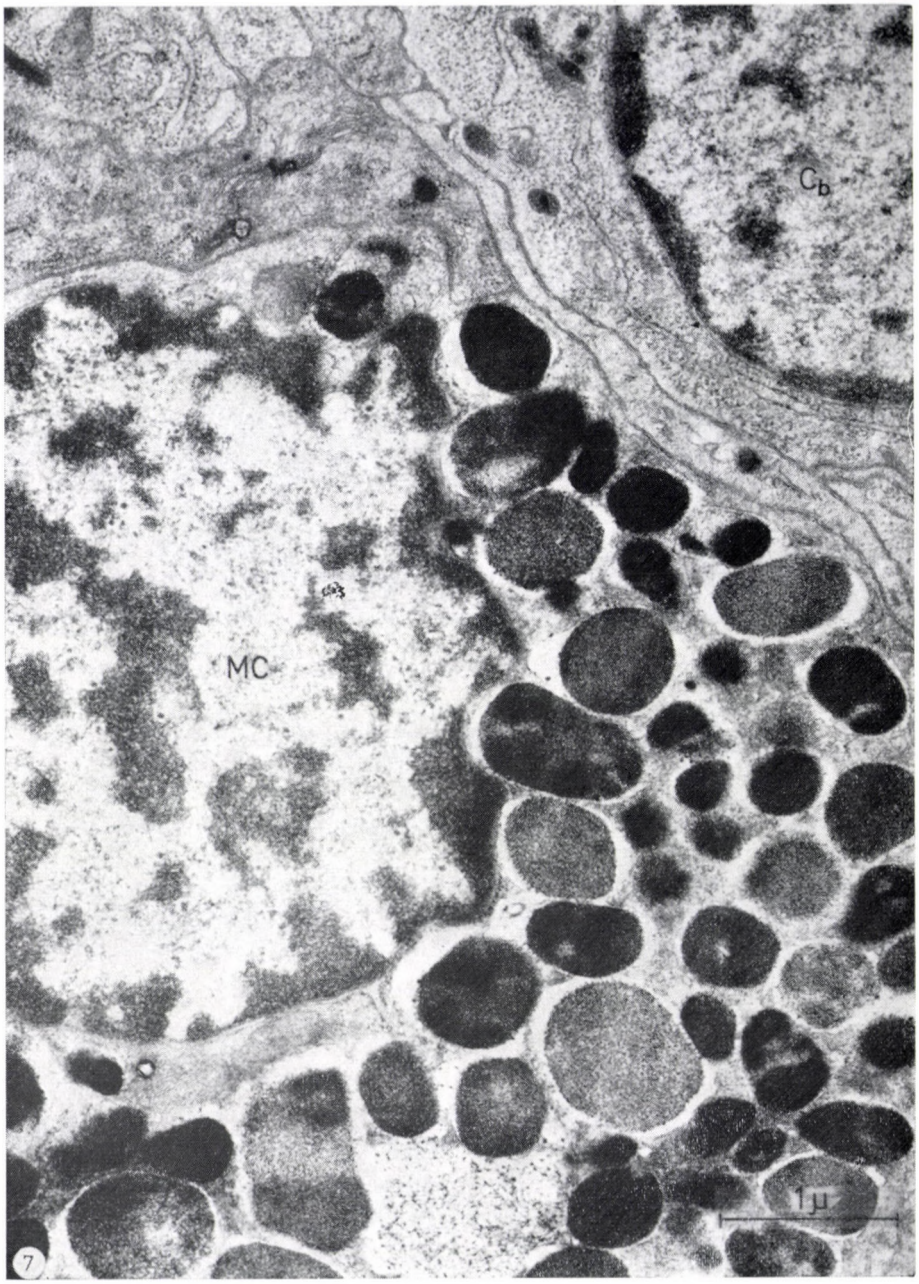


Fig. 7. Mature mast cell (MC) in the vicinity of C_b cells. At this stage of development, the nuclear structure of C_b cells is already similar to that of mature mast cells. (Rat treated with cortisone for 3 days)

Discussion

Following short-term prednisolone or long-term cortisone treatment, two characteristic kinds of cells, termed C_a and C_b appear in great numbers in the rat thymus.

Both types occur in the thymus of healthy untreated rats as well, but in very small number. C_a cells are present in the thymic medulla of control ani-

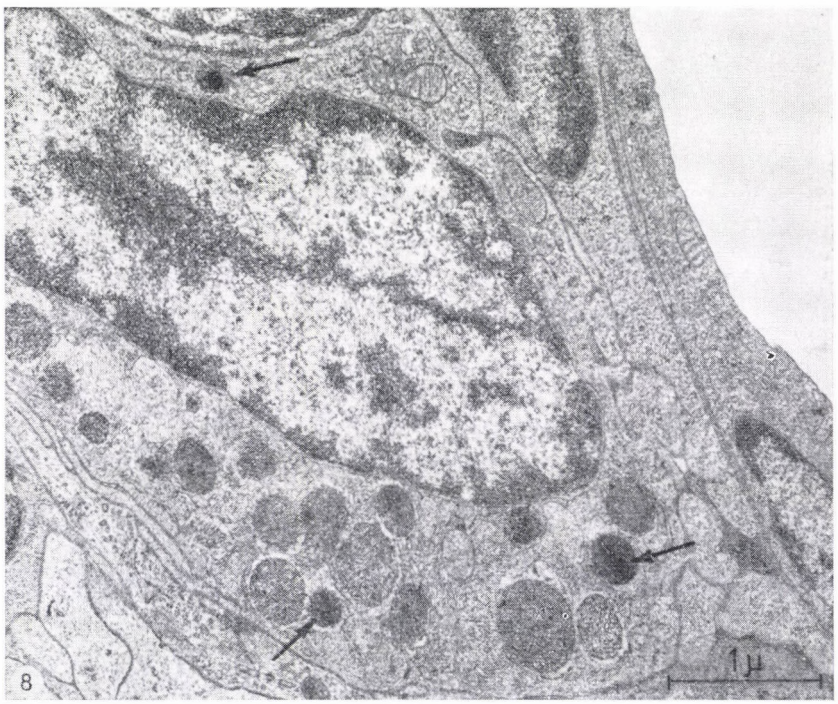


Fig. 8. Young pericapillary mast cell. There are still some granules with crystalline inner structure (→) among the typical mast-cell granules. (Control animal)

mals, but not in the cortex, where they appear some time after prednisolone treatment. This cell will be the subject of a later study. It will suffice here to note that cells of this type contain considerable amounts of lipids and mildly acid glycoprotein. They multiply intensively in the cortex on long-term treatment with cortisone.

Neither the medulla of treated, nor that of untreated animals contains C_b cells. These occurring in small numbers in the thymic cortex of controls showed no histochemically detectable multiplication after a single dose of prednisolone. They became, however, well observable after long-term cortisone treatment: we saw them scattered over the wide subcapsular zone of the cor-

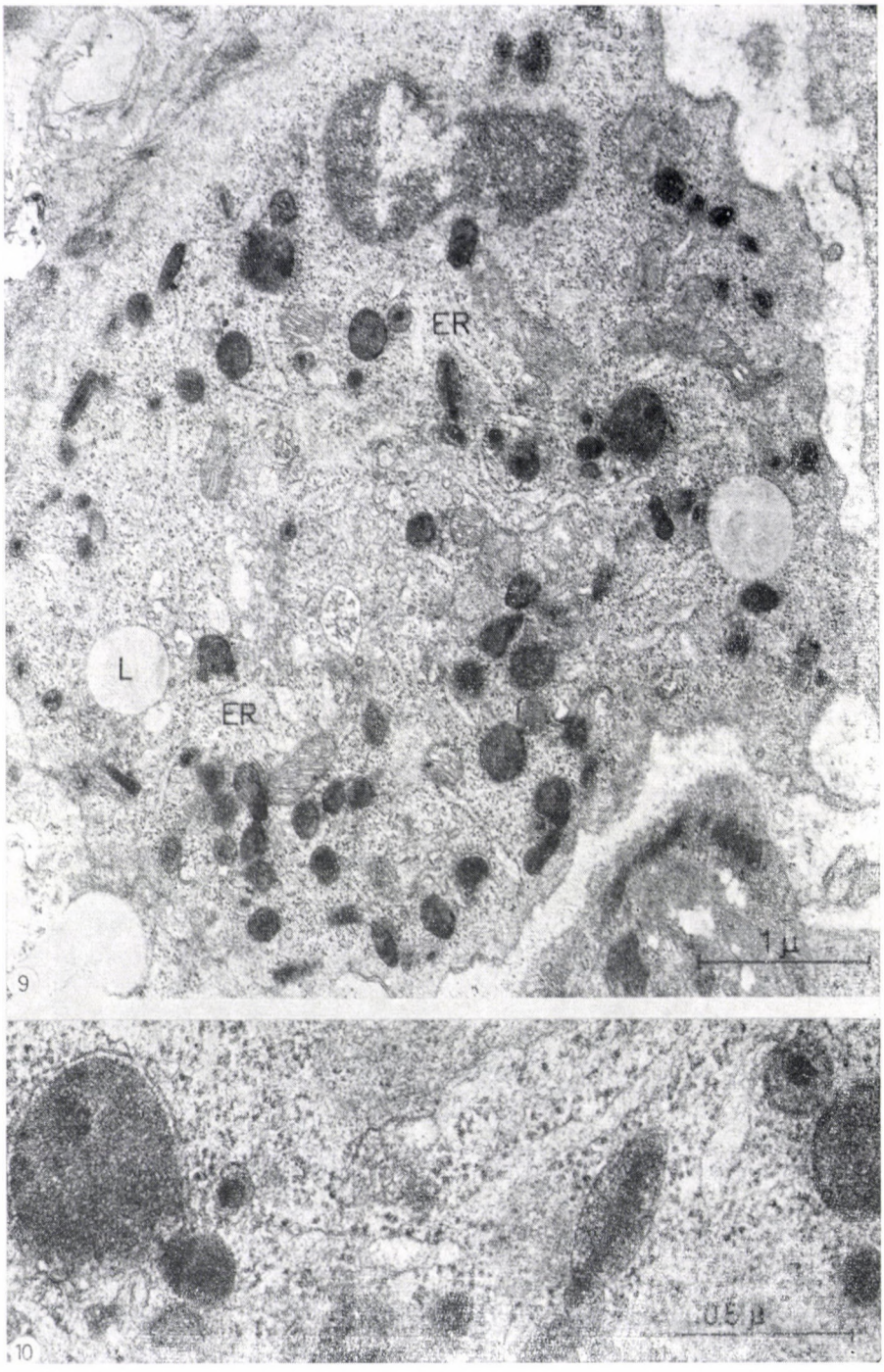
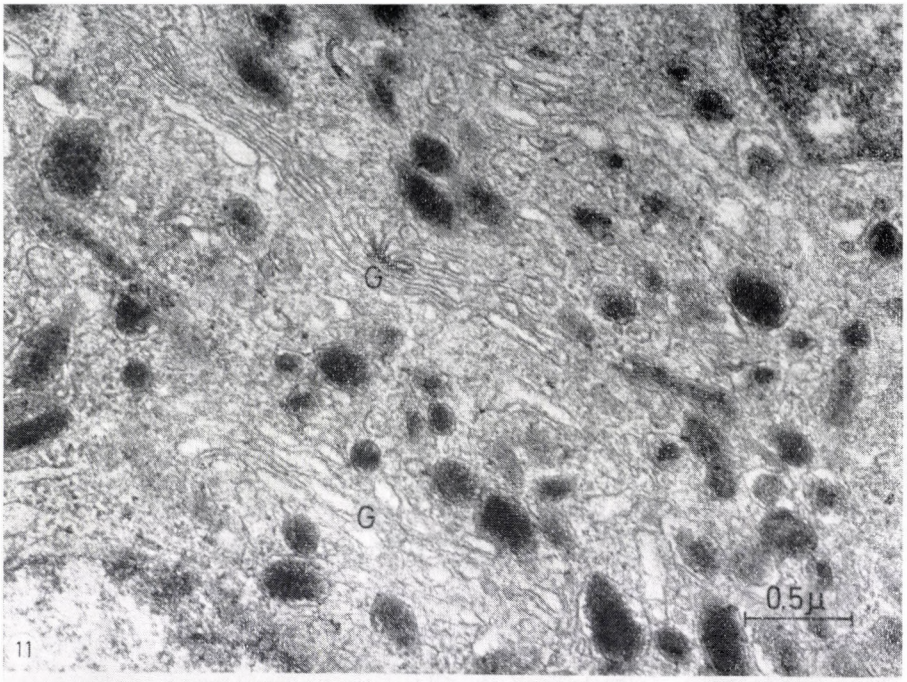


Fig. 9. C_b cells contain a great number of free ribosomes and — scattered over the cytoplasm — well-developed coarse-surfaced endoplasmic reticulum (Er). The granules are mainly accumulated at the periphery of the cells. Droplets of lipid may appear under the effect of cortisone. (Rat treated with cortisone for 6 days)

Fig. 10. Detail from Fig. 9. Highly-magnified granules with inner crystalline structure and granules without inner structure. Both kinds of granules are limited by unit membrane. The cross section of the crystalline granule clearly shows that the inner electrondense figure is cylindrical. (Rat treated with cortisone for 6 days)



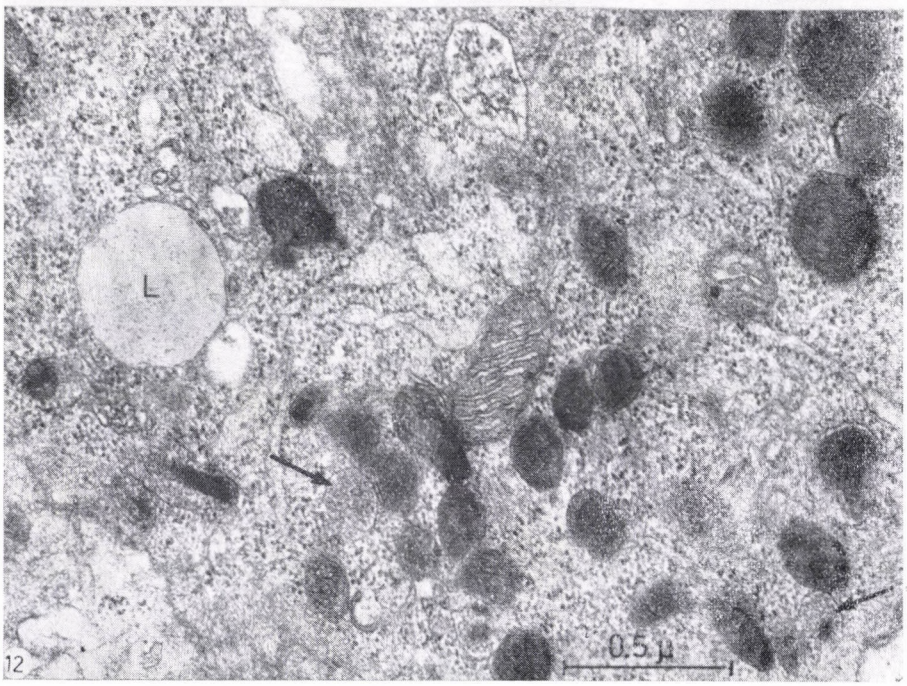


Fig. 11. Numerous amorphous granules in the wide Golgi zone (G) of a C_b cell. (Control animal)
Fig. 12. Detail from Fig. 9. The inner crystalline structure has disappeared from the enlarged and rounded granules; the granular pattern is similar to that observed in mature mast cells. The cell also contains granules that are presumably at the stage of granulolysis (→). Lipid droplets (L). (Rat treated with cortisone for 6 days)

tex, and in groups immediately beneath the capsule. Oxidized with potassium permanganate, they turned slightly metachromic with toluidine blue and azure A, suggesting that they might represent precursors of mast cells.

The major part of our experiments had the purpose to identify the C_b cells by electron microscope. The detection of C_a cells presented no difficulties since the electron microscope visualized reliably the lipid vacuoles that had already been observed under the light microscope. Their very size prevented them from being mistaken for other cells. Being small, the C_b cells might have been lymphoid cells. However, the electron micrographs obtained after long-term treatment with cortisone, failed to show the presence of lymphoid cells: it was dominated by lipid-containing cells of the C_a type and by another type of cells whose granules were suggestive of immature mast cells. As — apart from epithelial cells containing tonofibrils — no other kind of cells was encountered, it was safe to suppose that these small, granulated cells were identical with the C_b cells. Their structure, and their relationship with the mast cells require, however, further discussion.

Ripe mast cells, encountered in both the treated and control animals, were somewhat different from immature ones. The granules contained in mature mast cells range between 0.3 and 1.0 μ in diameter. They are highly electron-dense, strongly granular, and the picture is dominated by the granules at the expense of organelles. All these phenomena suggest that mast cells are functionally differentiated cells destined to store some end product. Young mast cells, on the other hand, have lobate nuclei, their granules are more variable in size, they contain mitochondria and abound in ergastoplasm. These findings are in agreement with literary data [35]. It may have been due to our method of fixation by glutaraldehyde perfusion that we succeeded in observing a hitherto unknown structure in certain granules of control mast cells. This crystalline figure, described in detail in the foregoing, is situated in the centre of the granule and, being absent in mature cells, constitutes a characteristic feature of immature mast cells. We frequently encountered granules of this nature in many cortical reticulocytes as soon as ten minutes after a single dose of prednisolone. Groups of such cells appear in especially large numbers after prolonged cortisone administration in the zone in which the cells histochemically defined as C_b cells are situated.

Both histochemical and electron-microscopic examinations make it, therefore, probable that the cells in question are identical with the C_b cells and represent growing mast cells. That these cells have so far not been described may be due to that the majority of investigators was concerned with the ultrastructure of mature mast cells [37], with those of the loose connective tissue in particular. Further, the method of fixation was not perfusion with glutaraldehyde as in the present experiments, so that the ultrafine structures may have remained hidden. That mast cells have a granular [1, 35], lamellar

[35] and fingerprint-like [39] structure has long been known; FEDORKO and HIRSCH [17] observed even crystal-like formations in them. It appears that the crystal in the centre has hitherto escaped notice. Our attention was directed to the crystalline structure by the great number of C_b cells encountered in the thymus of animals that had received prolonged cortisone treatment. This prompted us to look for them in the controls, in which both the young mast cells and the granules characteristic of C_b cells are considerably less numerous.

The crystalline structure of the granules made us first think that we were dealing with eosinophile cells. That mast cells and eosinophils may occur concurrently has been proved by ORFANOS and STÜTTGEN [31], who described their co-existence in mastocytomas. However, a closer scrutiny of the granules at issue made it clear that they were different from those of eosinophile granulocytes. Sections stained with May-Grünwald-Giemsa and with haematoxylin-eosin settled the problem definitively: not even an occasional eosinophile granulocyte was found in the thymus of the examined animals.

The granules — especially the smaller ones — are surrounded by a membrane, a phenomenon observed also by FUJITA [19]. It has been claimed by FERNANDO and MOVAT [18] as well as by KLUG [25] that the granules of certain mast cells are provided with membranes.

We are not in a position to take a definite attitude concerning the origin of mast cell granules. The substance which is not yet granular but has already granular density seems at some points to be connected with Golgi apparatus. This would be consistent with FUJITA's results [19] suggesting that mast-cell granules are produced in the Golgi apparatus.

We are in the dark as regards the chemistry of the observed granules. The fact that they react positively with Gomori's, Hale's stains and PAS points to the presence of neutral mucopolysaccharides or glycoproteins. That the cytoplasm of these cells shows homogenous staining under the light microscope does not argue against the existence of the granules, for both the size of the granules and the thickness of the sections are below the resolving power of the light microscope.

Presumably other types of cells with granular ultrastructure may also occur in the thymus. OLÁH and co-workers [30] described an interesting type of granulated cells they had found in the thymic medulla of healthy animals. These cells were not identical with the young forms of mast cells. They were provided with sharply distinct ectoplasm and endoplasm; the granules, structurally different from mast-cell granules, were contained in the latter; they had a marked cytocentrum, occurred only in the medulla and presumably represented reticulocytes. Mast cells and their various developing forms, as observed by us, are invariably in the cortex.

In the thymus mastocytopoiesis has repeatedly been observed. Our observations in this respect [4 to 12, 14] have been confirmed by KLEIN [24]

and GINSBURG [21]. The combined results of the present histochemical and electron-microscopical observations seem to show that we have succeeded in detecting the precursors of mast cells. Since mast cells divide but rarely, they are presumably evolved from another cell-type, probably from reticulocytes present in the cortex of the thymus. It is, of course, still possible that mast cells may originate from other cell-types and in other organs as well. The site of cell formation in the thymus seems to be the subcapsular area [20], and it may be assumed that mast cells too are produced there, a phenomenon that would explain why thymic mast cells are mostly found in and beneath the capsule.

SMITH [36] has advanced the hypothesis that the mast cells produced in the thymus are really heparinphagocytizing reticulum cells. The present electron-microscopic investigations have revealed no sign of phagocytosis, and the micrographs pointed to normal cell development.

The present experiments warrant the conclusion that mastocytopoiesis takes place in the thymus. The effect of short-term prednisolone or long-term cortisone treatment on this organ is, first of all, quantitative and not qualitative. Those two types of cells which multiply as a result of the treatment are present in the thymus of normal animals as well. Qualitative changes occur inasmuch as the cells appear also in sites where they do not occur under normal conditions; besides, the accelerating action of cortisone causes the processes of granulopoiesis and granulolysis to occur simultaneously so that the cells are unable to attain maturity. It should, of course, be borne in mind that cortisone may affect the thymus in healthy animals as well, and it is therefore not impossible that this hormone is responsible for the existence of the above-discussed cell types in normal animals, too.

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DIE WIRKUNG VON GLUKOKORTIKOIDEN AUF DIE THYMUSDRÜSE

I. Histochemische und elektronenmikroskopische Untersuchung der Mastzellenbildung

In den Thymusdrüsen von erwachsenen Ratten wurde die durch akute und chronische Kortisonbehandlung provozierte Mastzellenbildung untersucht. Ein Vergleich der histochemischen und elektronenmikroskopischen Untersuchungsergebnisse ließ in der Thymusdrüse eine Mastzellenbildung nachweisen. Die licht- und elektronenmikroskopische Morphologie ihrer Entwicklungsformen wird beschrieben. In den Körnchen der sich entwickelnden Mastzellen wird über eine neue, kristalline Struktur berichtet.

ДЕЙСТВИЕ ГЛИКОКОРТИКОИДОВ НА ЗОБНУЮ ЖЕЛЕЗУ

I Гистохимические и электронномикроскопические исследования образования тучных клеток

Авторы исследовали образование тучных клеток, вызванное в зобной железе взрослых крыс острой и длительной дачей кортизона. На основании сравнения результатов гистохимических и электронномикроскопических исследований можно доказать, что в зобной железе на самом деле происходит образование тучных клеток. Дается описание свето- и электронномикроскопической морфологии их отдельных форм развития, а также новой кристаллоидной структуры, наблюдаемой в зернышках развивающихся тучных клеток.

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RECENSIONES

E. N. PAVLOVSKY: **Natural Nidality of Transmissible Diseases**
Ed. by N. D. LEVINE, translated by F. K. PLOUS, jr., University of Illinois Press, Urbana and London (1966), pp. 261, ill. 126, $ 8.

The importance of infectious diseases transmitted by animals to man, the zoo-anthroponoses, has come into prominence in our days when it was found that further diseases are spread by animals in addition to the "classical" rabies, pestilence, tuberculosis and others. By now, there are more than a hundred of them. The tendency initiated by the Soviet academician PAVLOVSKY, head of the Parasitological Department of the Leningrad Zoological Institute, has represented a further progress in this branch of science. At the time of the economical exploration of Siberia, in the 'thirties, he studied encephalitis-type diseases spreading epidemically among the scientists and workers engaged in that country and has found that these diseases occur in what he termed "natural nidus". PAVLOVSKY demonstrated that the pathogens of certain diseases can be found in animals living in a state of nature and are transmitted by vectors to man if he penetrates these areas. "Natural nidality" means a new outlook and research trend, requiring the co-operation of the physician and the epidemiologist and of a number of other specialists, such as, the veterinarian, the zoologist, the botanist, the philologist and the climatologist, in order to explore, to prevent and to heal the infectious diseases of animal origin.

The fact that PAVLOVSKY's book, published in 1964, has already been edited in English translation proves the increased interest manifested towards the subject on the one hand, and on the other, it is an acknowledgement of the excellent epidemiologist, who presents in this book the results and conclusions of several decades of work.

The book is an excellent synthesis of all we know about zooanthroponoses and first of all about infectious diseases of the "natural nidality" type; nobody is more competent for doing this work than Pavlovsky who has lead several expeditions into different regions of the Soviet Union and whose extremely thorough observations have become scientific theses acknowledged all over the world. Due to his results the WHO has asked in 1960 the Soviet Union to organize a course of lectures for foreign experts about the research method of natural nidus. The successful course was followed in 1962 by another one, and all over world institutes and laboratories have been founded in order to study the problem.

The author deals with theoretical as well as practical questions and, by them, familiarizes the reader with the special properties of these diseases, with animal reservoirs and their way of life. He describes the properties of the most different vectors — fleas, mosquitoes, ticks —, their role in the transmission of diseases, the spreading of the diseases, the circulation of pathogens in the natural nidus. In a special chapter he deals with the most important diseases of the natural nidality type as well as with the methods of their exploration. A conclusion of the author is of outstanding importance and is treated in a separate chapter: the different diseases are characteristic of a given geographical area, i.e., they are of regional character.

The book contains more than 200 most useful bibliographic references and is completed with an explanation of special technical terms, which largely contributes to the understanding of the subject. The contents of the book are very well demonstrated by technically excellent original photos and drawings.

Translation and edition were done with great expertise by the Center for Zoonoses Research of the University of Illinois, rendering a very good service to all experts dealing with infectious diseases and their epidemiology by opening up PAVLOVSKY's results and rich experiences to the English-speaking world.

Z. ALFÖLDY (Budapest)

Egg Implantation
Ciba Foundation Study Group No. 23
Ed. by G. E. W. WOLSTENHOLME and M. O'CONNOR. Churchill, London (1966), pp. 112, Figs 25

This meeting was arranged in honour of the 70th birthday of Professor R. COURRIER, who started the egg implantation experiments some decades ago. The number of participants was considerably higher than that of the lectures: altogether 5 papers were read before an audience of 26 members. Although the lectures are very useful, the value of this small book lies chiefly in the discussions, a fact not uncommon in Ciba symposia. This is reflected also in the proportions of the text: 46 pages are taken by the lectures, 57 pages by the publication of the discussions.

The main problem of the meeting was to find out the factors playing a role in egg implantation. In this regard the importance of hormonal factors and their timing (PSYCHOYOS), the luteolytic activity of the uterus (BLAND and DONOVAN), the progestational activity of undomesticated badger (CANIVENC) as well as the mechanical and metabolic factors involved in the placentation in the rabbit (BÖVING, GOTHIÉ) were discussed.

Despite the low number of lectures, the book furnishes us with a comprehensive picture on the problems of implantation prevailing also today, and this is, in the first line, the merit of the lively discussion. Besides the debate connected directly with the lectures, the principal topic was whether there are predisposed loci for implantation in the uterus, or simply, the decidua starts to develop at the site of implantation. On the other hand, the role of the zona pellucida in implantation was discussed. Finally the importance of certain biogenic amines in implantation was dealt with.

The book was issued by the publisher with his well-known carefulness and in excellent presentation.

G. CSABA (Budapest)

D. G. SENN: **Über das optische System im Gehirn squamater Reptilien (On the optic system in the brain of squamate reptiles)**
Acta Anatomica, **65**, Suppl. 52. Ed. by D. G. SENN, S. Karger, Basel—New York (1966), pp. 87, sFr/DM 23.

The monography is concerned with the comparative, structural and topographical examination of the optic system of squamate reptiles. The results were obtained by the application of 2 methods (Bodian's impregnation and cresylviolet Nissl-staining). A special chapter deals with the progressive and regressive development of the fibres of the optic tract, of the optic chiasm, the N. geniculatus lateralis, tectum opticum and of the nuclei of the oculomotorius nerves occurring in the phylogenesis and with the ensuing structural differentiation and de-differentiation.

The monography is completed by 29 illustrations, mainly photomicrographs.

With its comparative aspect, this monography can be used not only for specialists of this field, but also for biologists with an interest in general comparative biology.

J. HÁMORI (Budapest)

The State and Movement of Water in Living Organisms
Symposia of the Society for Experimental Biology, No. 19.
Ed. by G. E. FOGG, Cambridge Univ. Press (1965), pp. 432, 75s.

The last of the interesting volumes in the symposium series issued by the Company of Biologists elucidates — in conformity with the significance of the water — comprehensively numerous qualities of this very common but highly important and unique compound. The most recent knowledge on water is dealt with by 26 famous experts in 22 lectures, divided into 3 topics.

The first topic was concerned with the physical background.

P. G. KOHN treated various physical and chemical characteristics of the water, inclusive of heavy water, presenting a number of data and tables on different subjects including molecular weight, ionization constants, thermodynamical indices, diffusion coefficients of water-solved electrolytes, etc., each being important to physicists and biologists.

For the latter the chapter by J. D. BERNAL, entitled "The structure of water and its biological implications" deserves perhaps the greatest interest. The author points to the fact that the water structure known at the molecular level gains various aspects in biological systems in the presence of ions, neutral molecules, giant molecules and macro-molecular surfaces. All these are biologically different, and a great number of intracellular processes can be understood only on the basis of this manifold appearance of water structure.

The further chapters of the first topic are as follows: "Kinetic theory and transport in ice and water" by G. WYLLIE; "Recent research into the properties of water in thin films and in microcapillaries" by B. V. DERJAUGIN; again the lectures "Water flow in the presence of active transport" by O. KEDEM and "Osmotic flow" by J. DAINTY.

Under the second topic entitled "Water in the plant" 7 lectures were delivered. E. G. YOUNGS dealt with the movement of water in the soil, approaching the problem from the general aspects of the isothermic flow of liquid water in porous media. R. O. SLAYER and W. R. GARDNER elucidated comprehensively the water movement in the soil and in the plants, establishing accurate relations between the rate of transpiration and the water content of the soil as well as between the transpiration rate and size of stomata. R. BROUWER spoke about the water movement taking place through the roots to the xylem vessels and underlined that this is considerably influenced, besides by physico-chemical factors, by the living protoplasm. M. H. ZIMMERMANN in his lecture entitled "Water movement in stems of tall plants" drew the conclusion that he does not know proofs against the cohesion theory, though it is involved with many open questions. P. E. WEATHERLEY investigated the condition and movement of the water in leaves and concluded that the whole plant and its environment (including soil) constitute a homogeneous system. H. MEIDNER discussed the stomatic control of water losses by transpiration and J. L. MONTEITH explained the connections of transpiration and environment.

The title of the third topic was "Water in the animal". The first reader, J. B. ROBINSON stressed in his paper entitled "Water regulation in mammalian cells" that our knowledge on the water of animal cells and organisms and its regulation are often speculative, and that several seemingly negligible factors, e.g., the water of cells beside extracellular water, the osmolarity of cells, etc., would deserve more attention. The bearings upon cytoplasmic contraction and water distribution in *Amoeba* were attractively analysed by R. D. ALLEN and D. W. FRANCIS whereas J. W. L. BEAMENT expounded the facts, models and mechanism of active water transportation in Arthropoda, chiefly in insects. R. P. DURBIN and F. G. MOODY discussed the movement of water through the transportation membrane, the gastric mucous membrane, and pointed out that it is still impossible to calculate — on the basis of the current osmotic gradient — the water flow for multi-membrane systems corresponding to transportation membranes. The explication given by D. H. SMYTH on the water movement through the mammalian intestine is very interesting. In his opinion this water movement is governed by active cell function, but we have no experimental system in which these biophysical strengths could be demonstrated. Besides living material there are no examples to maintain such a phenomenon. In his lecture "The mechanism of isotonic water absorption and secretion" J. M. DIAMOND treated the problem how a dissolved molecule transports hundreds of water molecules; this fact cannot be explained either by the classic osmosis or by electro-osmosis, pinocytosis, co-diffusion, etc. According to the author near the cell membrane local osmosis occurs: the active cell function creates a solution of high concentration and the water passes this local osmotic gradient. All these, however, can only be assumed in case of a special structure. H. DAXSON and M. BRADBURY discussed the liquor exchange of the central nervous system. In the brain the situation is more complicated because of the existence of two types of extracellular water, namely, the pericellular water and the cerebrospinal fluid. A. J. ZOTIN, in his lecture entitled "The uptake and movement of water in embryos" described the particular water economy displayed by the developing embryo during its early morphogenesis. "The uptake and distribution of water in the developing amphibian embryo", paper by P. TUFT revealed that the binding and distribution of water are governed not only by the pressure gradient and cell permeability but also by certain oriented cell layers similar in polarity to the egg surface from which they originated. The water flow is due to the asymmetric water regulation sites in the cell membrane.

The interesting volume discloses many new aspects of the discussed problems and clearly points — especially in the third part — to unknown factors connected with the condition and movement of water and manifesting themselves in biological systems. No doubt that particularly the processes taking place in animal cells and organisms of more complex systems are far less known than those in the plants.

At the end of each chapter a review of recent literature is presented.

This book may be of great interest not only for biophysicists, but also for physiologists interested in botany and zoology, furthermore embryologists and even pathologists will certainly find many useful advices in the book. Besides being very instructive it gives rise to numerous thoughts and attracts attention to the trends of future research.

G. KISZELY (Szeged)

N. I. LAZAREV: **Dyshormonal tumors. The theory of prophilaxis and treatment** Consultants Bureau, New York (1966), pp. 136, Figs 39

In his monograph about the prophylaxis and therapy of hormone-dependent tumours and the hormonal causes of their formation, Lazarev reviews the data of technical literature and sums up the results of his own research work in four parts and eighteen chapters. The first part reviews the hormone-dependent tumours, using instead of the term "hormone-dependent" the less known expression "dyshormonal". In the first experiment he examines the development of the mammary tissue on sucking rats and proves that oestrogen dosage cannot cause hyperplasia of the mammary tissue unless the hypophysis is intact. FSH plays the most important role among hypophysis hormones. He separates the increasing effect exerted on mammary tissues by progesterone from that due to oestrogen. Oestrogens cause hyperplasia of excretory ducts of the mammary glands progesterone that of lobuli, both in the presence of FSH. Progesterone intensifies the secretion of the hypophysis FSH hormone, while prolactin secretion needs simultaneous administration of oestrogens and progesterone.

In the general chapter the author points out that the latency time required for tumour formation is equal to the time of sexual maturity in case of the mouse, rabbit, dog, monkey and man. E.g. the puberty time of man is 10—15 years, while the latency time of tumour formation is 12—15 years.

The second part deals with the carcinogenetic effect of oestrogens. The author demonstrates with experiments, that ovarian cystae produce an abnormal hormonal milieu that may result in tumour formation in other organs, particularly in the mammary glands. With the removal of cystic ovaries, the abnormal endocrine situation is eliminated and the mammary glands do not become tumorous.

The third part is one of the most valuable chapters of the monograph. As a prophylaxis to the formation of tumours the author suggest curing of the precancerous state. On the ground of literary data and the author's own observations mastopathy should be considered as a precancerous symptom. He produced experimental cystae (by X-ray radiation, etc.) in the ovary of rats and observed that, after the ovarian cystae had developed, the mammary glands first became mastopathic and then developed tumours. Veterinaries treated the ovarian cystae of cows and the resulting sterility with small doses of iodine. The author's collaborator, E. ISMIRNOVA, has found that iodine intensifies the LH production of the hypophysis through the thyroid gland and that this results in elimination of cystae. Relying upon the animal experiments, she treats her mastopathic patients with small doses of iodine. Mastopathic women are given continuously, for 6—8 months, daily a spoonful of 0.25% solution of potassium iodide, resulting in the cessation of ovarian cystae and in the healing of mastopathy at a rate of 60—70 %. Among 33 mastopathic patients 15 were symptomless, 8 improved and 10 remained unchanged after 3 months of this treatment.

The fourth part deals with the hormone therapy of mammillary cancer. The chemical and chirurgical hormonal treatments are reviewed and their mechanism of action discussed. Hormonal treatments of the uterus, the ovarian thyroid-gland and prostate cancer are also reviewed. Among cytostatic agents, Sarcolysin intensifies the secretion of FSH in the hypophysis of male rats, and Thio-Tepa in that of female rats. In case of seminoma, the intensified FSH secretion causes cellular proliferation and consequently, manifests the cytostatic effect of Sarcolysin. In case of mammillary cancer, on the other hand, intensified FSH secretion and the consequent cellular proliferation can be evoked by Thio-Tepa, which thus promotes the cytostatic effect.

The monograph contains 233 bibliographic references. Special experiments are described which might be of particular interest to research workers dealing with similar subjects. The clinical experiences can be widely used, and the principles laid down at the discussion of precarcinoma are most valuable.

F. MASSZI (Budapest)

D. J. DEWHURST: **Physical instrumentation in medicine and biology**
Pergamon Press (1966), pp. 205, 60 s.

The author is assistant professor at the Melbourne University. His book reported here deals comprehensively with the principles, construction, and application of electric devices and equipments used in the experimental biology.

Experiments especially those of biological grounding, engaged in biological research, have often to cope with difficulties presenting themselves in the installation and correct use of a suitable equipment, one of the prerequisites of performing biological research with an adequate accuracy.

This book was written to facilitate the use of instruments by beginners. It offers little to experienced investigators. A great deal of items are treated, consequently all devices are only outlined, pointing merely to the most important details.

The volume is divided into 28 chapters. The *first* of them expounds some technical particulars of instrument fabrication. In *Chapters 2 to 7* the main data of electric current as well as the lay-out and utilization principles of electrical basic devices are discussed. *Chapter 8* deals with the data, construction and the characteristic of the transistor amplifier, whereas *Chapter 9* with the vacuum-tube amplifiers, treating the characteristics, types, parameters and also utilization possibilities of vacuum-tubes. *Chapter 12* is devoted to the cathode-ray tube, *Chapters 13 to 15* are engaged in the construction and details of oscilloscope. In *Chapters 16 to 20* the types and application of mono- and bistabile multivibrators are described. *Chapter 21* explains how to register the electric phenomena of biological tissues, in *Chapter 22* and *23* the methods of tissue stimulation and — separately — the detection of β- and γ-radiation as well as the characteristic data and function principles of the Geiger—Müller tube are presented. *Chapter 24* deals with the lay-out of scintillation counters, touching upon their types and the statistical valuation of the counting. *Chapter 25* presents some examples of electric regulation. In *Chapter 26* the function and types of transducers are described, whereas in *Chapter 27* a review on the transfer and storage of data, on some applications of information theory and on memory equipments is given. *Chapter 28*, finally, treats some analytical methods of biology, so the use of flame photometers for Na-, K- and Ca-determination, the gas-analysis of O_2, CO_2 and N in respiration samples and the measuring of pH.

The practicability of the book is facilitated by a short subject index. At the end of each chapter suggestions in a brief literature review and practical directives are found. Many simple circuits, diagrams, graphs, tables and figures promote the understanding of the material and the use of this work, which has been issued in excellent typographic presentation.

A. NIEDECZKY (Pécs)

Proceedings of the International Symposium on the Cardiovascular and Respiratory Effects of Hypoxia

Symposium in Kingston, Ontario, June 1965

Ed. by J. D. HATCHER and D. B. JENNINGS. S. Karger, Basel—New York (1966), pp. 406, Fr 96.

The subject of Symposium is divided into six chapters, the present proceedings contain 25 lectures, the pertaining discussions and at the end of the chapters, the round-table conferences. The first chapter deals with the oxygen tension in cells. Diffusion is examined from two aspects: as the determining factor of oxygen transport through the lungs and as that of oxygen transport from the blood to the tissues. The driving force of oxygen diffusion in the tissues is the PO_2 gradient. Several direct and indirect methods are suggested for determining that gradient.

The second chapter deals with the correlation between low oxygen tension and metabolism. Correlations are sought between enzymatic process concerning glycolysis and respiration. In case of hypoxia, the functional changes are said to depend primarily on the correlation between energy production and consumption.

The lectures of the third chapter deal with reflex responses to hypoxia. First of all, the attention is called to the peripheral receptors, playing a major role in the reaction to hypoxia. Comparative tests are described about the values of blood and CSF, pH and bicarbonate. At last the mathematical formulation of the correlation between ventilation, hypoxia and H^+ can be found in this chapter.

The fourth chapter deals with the mechanism of the cardiovascular responses to hypoxia and anaemia. A cardiotonic agent, with a component from the adrenals and the kidneys, was detected in the blood of dogs suffering from hypoxia. In grave cases of anaemia, lower vascular resistance can always be discovered, with reduced blood viscosity as the main factor.

The fifth chapter deals with hypoxia and pulmonary circulation. The effect of hypoxia on the cardiorespiratory system and on energy management was examined primarily in infants. According to the experiments, this correlation is fundamentally modified by temperature drop. Another lecturer dealt with the question of hypothermia and infants suffering from anoxia.

The sixth chapter deals with the correlation between hypoxia and physical performance. Physically supportable load depends on the correlation between oxygen uptake and consumption. The last lecture reviews the transport line of oxygen within the organism. This line consists of two convective forwarding systems: ventilation and blood circulation, and of two diffusion systems: (1) capillaries of the pulmonal alveoles and (2) the capillary cell system of the tissues. The energy requirement in the various phases of oxygen transport is unequal. Among the statements of the round-table conference it is worth mentioning that the inclination to coronary diseases and staying at great altitudes are in correlation to each other.

The question of hypoxia is examined in detail from many aspects. The present proceedings are of utmost importance for any medical research worker or biologist whose interest is focused on hypoxia. Excellent illustrations, tables and a large number of references contribute to easy understanding and to usefulness of the volume.

ÉVA ORBÁN (Budapest)

KLAUS AUS DER MÜHLEN: **The Hypothalamus of the Guinea Pig**
S. Karger, Basel—New York (1966), pp. 97, Figs 22

Completed with fine microphotos, the topography of the guinea pig's hypothalamus is presented in three languages — English, German and Spanish. The growing interest in the morphology of the hypothalamus and the common use of the guinea pig in such studies makes this book valuable assistance to research workers interested in this field. Luckily, the author is using not only the conventional frontal plane, but presents certain regions of the hypothalamus in sections prepared in both the sagittal and frontal planes. The advantage of succinct description often involves the draw-back of essential parts being omitted. The nomenclature of KRIEG and RIOCH is being used, but we miss some actually current terms to be mentioned occasionally as synonymes. The hypothalamus is discussed according to the standard classification of LE GROS-CLARK, which is correct in regard to systematization, but results in the omission of area hypothalamica lateralis and area retrochiasmatica; furthermore the anterior hypothalamic nuclear group, recently recognized as a nucleus, is considered as area hypothalamica anterior, whereas nucleus periventricularis is treated as a homogeneous nucleus. A subtle analysis of different nuclear groups is equally missing, and the author failed to avail himself of the quantitative methods. The volume of the nuclei in various nuclear groups were determined, but the karyometric curves were, unfortunately not analyzed, and so the possibility of fine differentiation cell types has remained unutilized.

The author is the first in technical literature to give a description and topographical definition of nucleus magnocellularis praefascicularis mammillaris.

The documentation and, particularly, the photos of low magnification are very fine indeed, above all Fig. 1, presenting the neurosecretory tractus supraoptico-hypophyseos. Unfortunately, most section planes are not precise, enclosing an angle with the desired plane, and so the two sides of the hypothalamus are often dissimilar. The delimitation of certain nuclei is rather subjective, in some cases even erroneous. In Fig. III/b. the area indicated as nucl. ventromedialis anterior is actually the frontal lateral part of nucleus hypothalamicus anterior, the nucl. paraventricularis shown in Fig. IV/b corresponds to nucl. periventricularis ant., in Fig. VIII nucl. dorsomedialis is sited above, and to the side of the marked area, and the marking of nucl. ventromedialis includes only part of the nucleus. Unfortunately, the number of demonstrable sagittal and horizontal sections is not enough to give us a threedimensional impression of every area. Since the areas were not marked in a coordinate system, some marks are not in accordance in different pictures; major disagreements may be found particularly in the case of area hypothalamica anterior and nucl. dorsomedialis.

In spite of all this, the book offers a substantial help to all those who deal with the hypothalamus of the guinea pig, and considerably facilitates their orientation in this field

M. PALKOVITS (Budapest)

Modern Trends in Neuromorphology
Symp. Biol. Hung. Vol. 5. Ed. J. SZENTÁGOTHAI. Akadémiai Kiadó, Budapest (1965) pp. 316

The present volume includes the proceedings of the neuro-morphological symposium held in Budapest, July 5—6, 1963, in honour of the centenary of the birth of MIHÁLY LENHOSSÉK. In compliance with the traditions of the great Hungarian anatomist and neurohistologist, the subject-matter of the symposium was composed with the intention to underline the functional and biological aspects in the neuro-morphological studies; the nervous structures are presented according to the latest techniques.

By way of introduction I. TÖRŐ commemorates the career and the human assets of MIHÁLY LENHOSSÉK, while I. KÖRNYEY deals with his scientific results, emphasizing his merits in supporting the neuron theory and in having it adopted. The scientific contents of the volume are divided into the following main parts: receptors, vegetative nervous system, neuromuscular correlations, central nervous system and neurosecretion. In the chapter dealing with the receptors the most thorough electron-microscopic work of H. ENGSTRÖM and co-workers on the types and innervation of vestibular sensory epithelium, as well as MILOCHIN's work made with impregnation technique, discussing the sensorial innervation of the ganglion cells of the autonomous nervous system should be emphasized. The chapter on the vegetative nervous system includes the following neuro-histological works: A. ÁBRAHÁM deals with the intracardial ganglion cells and the neuromuscular correlations of the heart, V. P. BABMINDRA with the morphology of synapses to be found on vegetative neurons, A. STAMMER reports on the histological and histochemical analysis of the ciliary ganglion and G. LASSMANN publishes data on the nervous supply of the veins of the iris. The fine structure of the vegetative synapses of blood vessels is analyzed by an important electron-microscopic work (H. BETTSCHNEIDER).

Three lectures deal with neuromuscular correlation: quantitative changes of acetylcholine esterase are treated by B. HORÁNYI and G. BOZSIK, postjunctional calcium release caused by acetylcholine by G. SÁVAY and B. CSILLIK, and the fine structure of the myoneural junctions of insects by J. HÁMORI.

The chapter on the central nervous system contains valuable lectures, such as, that of W. J. KRIEG on the junctions of the cortex of the monkey, that of A. BRODAL on the experimental-anatomical examination of cortico-spinal and cortico-rubro-spinal junctions, that of P. ANDERSEN and L. C. ECCLES on the localization and identification of postsynaptic inhibitive synapses, that of F. WALBERG on the light- and electron-microscopic examination of bouton degeneration of oliva inferior and that of J. SZENTÁGOTHAI on the fine structure of synapses of short local neurons.

The last chapter includes works in the field of neurosecretion (B. ÁROS and co-workers: Structure of the neurosecretory system of the earthworm, I. Zs. NAGY and K. BENKŐ: Electron-microscopic examination of the cerebrovisceral connectivum of the shell-fish), ontogenesis (L. GONTCHARENKO: Development of the trigeminus nuclei), neuro-cytology (M. MÜLLER: Nucleolus extrusion in ganglion nuclei) and neuro-histochemistry (D. PICARD: Advantages and drawbacks of perfusion fixation in neuro-histochemistry).

P. RÖHLICH (Budapest)

A. WISEMAN: **Organization For Protein Biosynthesis**
Blackwell Scientific Publications, Oxford (1965), pp. 217, 45s.

The book contains some 190 pages, and — relying on the most important recent results obtained in biochemistry, microbiology and cytobiology — the author presents a clear survey of present knowledge regarding the mechanism and regulation of protein synthesis. The concise and, consequently, comparatively short monograph cannot fail to command the interest of university students and teachers engaged in biology or medical science in general.

The work contains an introduction, and four parts divided into 12 chapters. The introduction consists of a short treatise concerning the structure of proteins and nucleic acids. The four parts have the following titles: Biochemical Mechanism in the 'Classical Scheme' of Protein Biosynthesis; Specificity and Controlling Mechanisms of Enzyme Biosynthesis (classical scheme); Regulation of Enzyme Biosynthesis; Other Pathways of Protein Biosynthesis.

The *first part* of the work deals with ATP synthesis; the activation of amino acids; the activating enzymes; the structure, various forms, properties, role and analysis of transfer-RNA and, finally, with the structure and function of ribosomes.

The *second part* comprises three chapters. The structure of DNA, the methods of its study, the mechanism of replication, the gene-conception and the synthesis of messenger-RNA are discussed in the first chapter. The second chapter deals with the messenger-RNA, especially with those of its properties that are known from bacteriological studies. The next chapter discusses the codons determined by nucleotide triplets, the coding of amino-acid sequences, synthetic polynucleotides and the experiments concerned with the amino-acid incorporation of cell-free systems.

The *third part* of the book treats on the regulation of enzyme biosynthesis partly in micro-organisms and partly in animal tissues or cell-free systems. The passages dealing with the operon conception, induced enzyme synthesis and the differentiation of animal cells (gene repression) are particularly noteworthy.

The *fourth part* of the work describes those forms of protein synthesis which are in no connection with the cytoplasmic ribosomes but take place in the nucleus (e.g., histones), in the mitochondria (e.g., certain structural proteins) or in bacterial plasma membranes. This part of the book deals further with activated peptides and their function as also with the activity of the enzyme system which regulates the production of activated peptides.

Each chapter concludes with a brief summary. Besides, the most recent data and future trends regarding the given subject are presented at the end of the chapters.

To sum up, besides being concise, clear and highly instructive, the great value of the work consists in treating all data with severe and expert criticism so that a careful study of the book helps the reader in forming a coherent picture of the subjects discussed. Being a biochemist, the author analyses the problems from a biochemical viewpoint, but pays due regard to their morphological aspects as well.

The bibliography at the end of the volume lists 361 literary sources referred to the text.

I. Törő Jr. (Budapest)

ANNOUNCEMENT

On May 9, 1967 at the University of Washington, Seattle, an Organizing Committee (selected by the votes of 229 invertebrate pathologists responding affirmatively to the questionnaire) initiated a new association of invertebrate pathologists, to be known as the *Society for Invertebrate Pathology*. Officers chosen by the committee were EDWARD A. STEINHAUS, president; ALBERT K. SPARKS, vice-president; and ARTHUR M. HEIMPEL, secretary-treasurer. Others on the Committee that took action to establish the Society were T. A. ANGUS, M. E. MARTIGNONI, C. J. SINDERMANN, and V. SPRAGUE.

Beginning January 1, 1968 the official publishing organ of the Society will be the *Journal of Invertebrate Pathology*. All persons interested in the promotion of invertebrate pathology (i.e., the diseases — infectious and noninfectious — of invertebrate animals) are invited to apply for membership by communicating with the Secretary-Treasurer, Arthur M. Heimpel, at the address given below. The regular dues of the Society are $14 for those desiring to receive the *Journal*, or $4 for those who wish to become members but no receive the *Journal*. Although the Society is already an active organization, general membership will begin January 1, 1968. All who submit $14 prior to that date will be considered as "Charter Members"; those who pay a single initial year's fee of $25 will be considered as "Founding Members", and will be so indicated in the roster of members.

Although the name of the Society does not include the word "International" it is to be emphasized that membership is open to invertebrate pathologists anywhere in the world. Announcement of the Society's first general meeting, to be held sometime during 1968, will be made at a later date. In the meantime, all who wish to apply for membership need only contact the Secretary-Treasurer. Ratification of a constitution, now being drawn, will be accomplished by mail ballot. The possibility of periodically distributing a mimeographed newsletter is being considered.

ARTHUR M. HEIMPEL

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Beltsville, Maryland 20705

GEBURTSHILFLICHE STRAHLEN- UND RÖNTGENDIAGNOSTIK

Grundlagen, Methodik und praktische Anwendung

Von Professor Dr. med. habil. WILFRIED MÖBIUS

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DIFFERENTIATION OF THE MYELON AND THE LIFE CYCLE OF GRANULOCYTES IN RATS

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(Received December 28, 1966)

Synopsis

Phases and quantitative relationships of granulocyte differentiation in albino rats were studied. Comparing the relative values of myelo- and haemograms, six morphologically distinguishable forms (GC 1—GC 6)* of the neutrophils are determined, instead of the two known formerly. Development of the eosinophils starts in the rat bone marrow during the PMC phase, too, and comes to an end in the course of GC 3 stage in peripheral blood. Concerning the origin of the ring-shaped nuclei, characteristic in rodent granuloid cells, it was shown that *a*) 18.79 per cent in PMC I, 71.50 and 80.18 in MC and MMC respectively, include perforated nuclei *b*) the value Q_{open}/Q_{closed} increases during the MC → GC development from 0.12 to 1.31 and *c*) among nucleus-variants transitional forms between "band" to ring-shaped nuclei are found in a very low proportion. Consequently the morphogenesis of rodent myeloid elements passes through the perforation of the compact nucleus of the precursor cells; the "band" forms, well-known in the human haematology have no differential-diagnostic significance in rat. In the sample examined, the granuloid mitotic index mounts up to 18.36 per thousand. Based on microscopical observations, it was rendered probable that elements more differentiated than MC's are capable sometimes of mitosis, too.

Introduction

The differentiation of intermitotic vegetative cells and their development into postmitotic, non-dividing cells in rat granulocytes were examined. First of all the mode of morphogenesis, the phases of maturation, the quantitative distribution of developmental forms in interphase and mitosis were studied.

Morphogenesis and nomenclature of the human granuloid cells [1, 11, 25] show only partial identity with those of the rat. The initial and final stage: MB and GC, resp., is similar, but in the case of intermediate forms (PMC, MC, MMC and "band" granulocyte) significant deviations occur. The cause is due to the appearance of *ring-shaped* granuloid nuclei in the course of rodent morphogenesis [14, 22].

In judgement of the maturational degree of perforated cells there exist great differences: the majority of authors divides them in series from GC to PMC, Vogel [28] classifies them exclusively into the transitional phase be-

* The abbreviations of the developmental stages are: MB = myeloblast, PMC = promyelocyte, MC = myelocyte, MMC = metamyelocyte, GC = granulocyte, MI = mitotic index.

tween MC—MMC. In connection with the extent of differentiation the *mode and date of ring-shaped nucleus formation* is contradictorily evaluated. According to KÖHLER [14], recalling former opinions [13], the closed nucleus type originates — as in human blood — through the "Jugend" → "Stab"

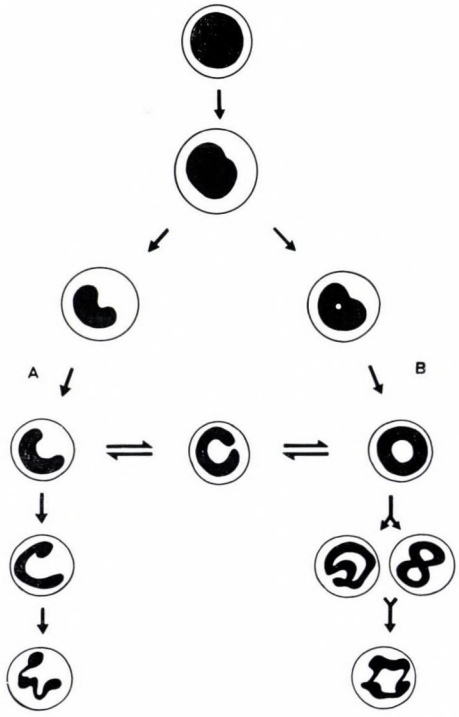

Fig. 1. Possible ways of the morphogenesis in rat neutrophil granulocytes

(= band) transformation and the fusion of the latter on their two apices (Fig. 1, A). Others assume the perforation of an originally compact nucleus (Fig. 1, B), but on different degree of cell life-cycle; as the time of perforation the MMC [23], transitional stage between MMC and MC [28], MC [19] and PMC [3, 6, 12] are mentioned.

We aimed to make clear whether the great *variation in nuclear forms* ("polymorphonuclear leukocytes") existing in granulocytes correlates with the differentiational degree, or not; namely the variations in nuclear morphology could be used as criteria of maturity and age. This was required for the judgement of the parallelly performed cytophotometric DNA and RNA measurements. For the same purpose it became necessary to classify the MMC's and GC's according to their *size*. Namely, in distribution diagrams of GARCIA [8] MMC's 8—10 μ in diameter are considered diploid, while "megametamyelocytes" of 13—15 μ in a high degree polyploid. Larger α- and smaller β-proleu-

kocytes (= MMC) and GC's are also distinguished [18] in the bone marrow, and one scale, small granulocytes in peripheral blood. Contrary to this, other authors did not apply a dimensional distinction either for granulocytes, or for metamyelocytes.

The data on the differentiation of the *eosinophils* are incomplete, too. In their myelograms only a few authors distinguish them according developmental stages [27, 21]; they are generally classified into one common group [4, 12, 14, 24]. In this manner it is known only from observations of human electron microscopic material [16] that eosinophil granules appear as soon as in the PMC stage, and the nuclear differentiation comes to an end in an earlier degree than in the case of neutrophils. However for rat eosinophils quantitative data are lacking.

Finally, beyond the granuloid cells in interphase, we registered the *mitoses*. Until the last years, as final limit of the differentiating myeloid series the *myelocytes* were specified. However, after irradiation in rats [15] and following haemorrhage of guinea pigs [10] it was proved — by means of autoradiography and cytophotometry — that MMC's are also capable of DNA synthesis. So it became more important to know the distribution of mitotic numbers for GC precursors more precisely, all the more, because recently, the MI is calculated not for the total, but only for these granuloid cells, which are capable of division [1].

To resolve the above problems, using albino rats, we prepared myelo- and haemograms. It was necessary to obtain own data because those of the haematological literature are deficient, and deviate relating to nomenclature, judgement and separation of developmental forms, even within the scope of the same species.

Material and method

Ten healthy Wistar rats, both males and females, 80—100 g in weight were killed in ether narcosis; then we obtained with a heparinized syringe 2—4 ml blood by punction of the left ventricle. The sample was left to sediment for 30 minutes at room temperature, after being mixed with 6 per cent dextran solution (dissolved in Tyrode medium) in a ratio 1 : 2. Taking from the lower layer of the supernatant 0.5 ml leukocyte concentrate cautiously, it was settled for 3 minutes in the GAILLERD—SCHABERG [7] sedimentation chamber. By cutting the femoral diaphyses and removing the plug of the same animal we obtained the bone marrow sample, which was, likewise after a mild shaking, settled in the sedimentation chamber. The air-dried preparations were stained mainly according to PAPPENHEIM. To improve the differential diagnosis peroxidase reaction according to SATO and GRAHAM—KNOLL resp., and the Lepehne-reaction were made; for identification of alkaline phosphatase diazonium-staining, for demonstration of non-specific esterase the naphthol-AS-acetate method were applied. For preparing the granulograms we examined 1,000 myeloid cells in each bone marrow and, in addition, the granuloblast mitoses found among them were registered (magnification: ×1,250). From each peripheral blood sample 200 neutrophil granulocytes and the occurring eosinophil and basophil cells were classified. Thus in the Tables and Figures 12,297 cells in interphase and divisions are included.

Since the literary data, concerning the judgement and nomenclature of the maturity of rat haemopoietic elements are rather heterogenic, we enumerate the characteristics which

were used as bases of the differential diagnosis: (a) *cell volume* (MB < PMC > MC > MMC > GC) (b) *nucleo-cytoplasmic ratio:* from MB to GC it decreases (roughly 1.1 → 0.2), (c) *basophilia* and *azurophil granulation*, (d) cytochemical characteristics: first of all differences in *peroxidase*, *alkaline phosphatase* and *nonspecific esterase* activity, (e) volume and density of the *nucleoli* (they decrease from MB to GC [9]). Finally (f) the *distribution* of nuclear *chromatin* varies characteristically, too. The differential diagnosis of human granuloid cells is based on the same criteria.

Results

Correlating the karyotypes with the characteristics, we obtained the following groups in the bone marrow:

MB: uniform groups, mostly including a round nucleus. PMC I. and PMC II: three categories: (a) compact nucleus with round stretched kidney-shape or irregularly indented shape, (b) "band" form (horseshoe- and crescent-shaped) and (c) perforated or ring-shaped nucleus. We emphasize that, contrary to the human haematology, the kidney-shaped nucleus in rodents has no significance concerning morphogenesis in the "band" direction, since in the overwhelming majority of the promyelocytes an indentation arises, localized to the cytocentral region, including the perforated and ring-shaped nuclei. MC: three groups: (a) compact nucleus (round, ovoid, or irregular shape), (b) "band"-form, (c) perforated and ring-shaped, resp. MMC: (a) closed forms (ring- and 8-shaped) (b) open forms (band-like elongated and horseshoe nucleus) (c) intermediate ("false" ring=a transition between the horseshoe-shaped open and ring-shaped closed forms). GC: (a) closed nuclear variations (including rings with smooth contour, 8-shape, invaginated or wound ring, 1-, 2-, 3-fold segmented, furthermore hypersegmented nuclei), (b) open forms (band-, horseshoe-, nuclei wound to different degree and the same with nuclear bridges resulting segments of variable number), (c) intermediate karyotypes, with broken pieces of nuclei.

For the sake of comparability relating to the blood the same categories were formed, in similar distribution.

In the course of differential diagnosis there occurred in about 8—10 per cent transitional forms. E. g., cell volume has a delimiting significance only in the case of extreme values. A cell 15 μ in diameter could be identical with GC, MMC, but with MC, PMC or MB, too. We always took into consideration the *collective* of the criteria; e. g., if a cell is 17 μ in diameter, exhibits vigorous azurophil- and specific granulation, 0.7 in nucleo-cytoplasmic ratio, exhibits one well-contoured nucleolus, we define it as MC, even if its cytoplasm is markedly basophil.

Following determination of the main categories of myelo- and haemograms it was examined whether the division of GC and MMC's into two-dimensional groups would be reasonable. In fact, between granulocytes there have been found extremely small (7 μ ∅) and large ones (15 μ ∅), that is, calculated

from $\frac{4\pi}{3} \cdot r^3$, cells of 180.0 and 1771.8 μ^3; this indicates a tenfold difference in volume. Whether these are the characteristic values of *two* different types or the extreme values of cells belonging to *one* dimensional "set" is shown by the slope of the frequency diagram.

Summarizing the GC-sizes in a distribution function, equally in bone marrow (Fig. 2) and in peripheral blood (Fig. 3) we obtained curves with one peak, which approximate the ideal gaussian distribution. Similarly, the distri-

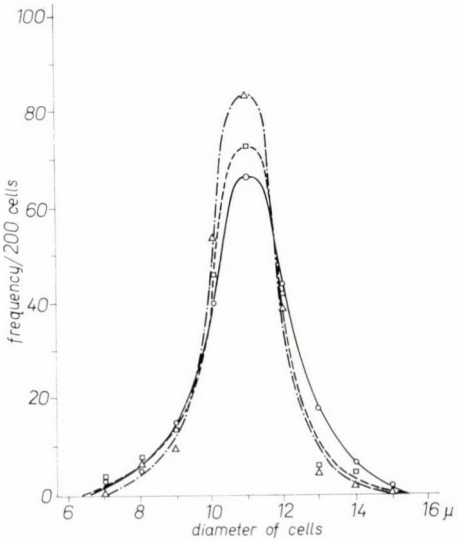

Fig. 2. Dimensional frequency diagram of neutrophil granulocytes in the bone marrow

bution diagram of MMS's on bone marrow (Fig. 4) does not exhibit a second maximum at the higher values corresponding to the "megametamyelocytes". Consequently, we did not distinguish either α- and β-granulocytes, or megametamyelocytes. Among the distributional maxima bone marrow MMC-s ($\bar{x} = 12.02\ \mu$) are right-shifted, compared to the GC-s ($\bar{x}_{\text{bone marrow}} = 10.90$, $\bar{x}_{\text{blood}} = 11.04$). The latter difference shows no significance: $P > 0.5$.

Having determined the categories and the problems of differential diagnosis, we summarized the data of the *bone marrow* and obtained the myelogram as follows:

The distribution of granulocytes in the *peripheral blood* is shown in Table 2.

Beyond fixation of the quantitative data of the main categories we examined the course of morphogenesis. To make clear, on which maturational stage of the precursors the GC type with *ring-shaped nucleus*, characteristic of the rodents, appears for the first time, we registered separately the cells with compact and perforated nuclei *inside* the main categories (Fig. 5). The greater part

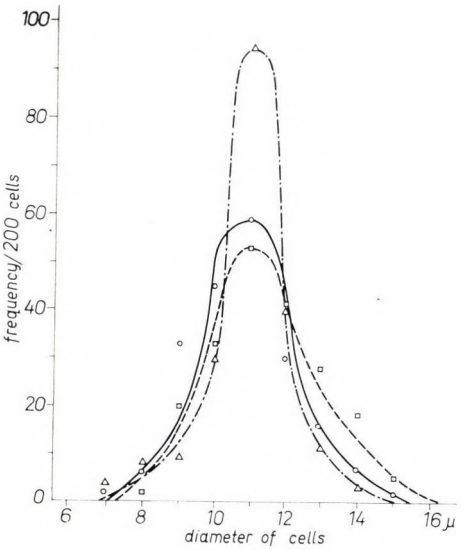

Fig. 3. Dimensional frequency diagram for the peripheral blood

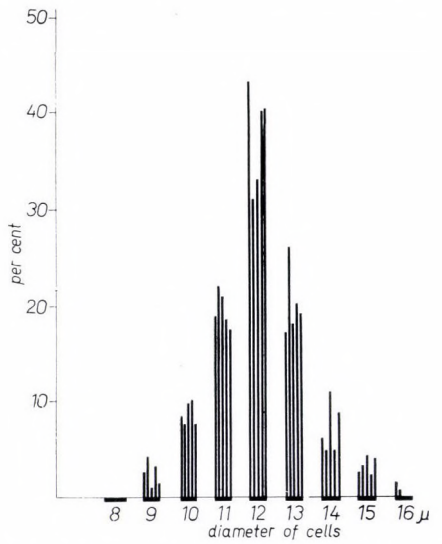

Fig. 4. Distribution of metamyelocytes according to size in the bone marrow

(2,239 in number) of the 3,780 neutrophil precursors counted exhibited ring-shaped nucleus. In Fig. 5 it is clearly seen that perforation occurs not only in the MMC or MC phase, as it was supported by the majority of authors, but it already occurs in PMC I; a half of the cells (49.84 per cent) which maturated to PMC II contained no compact nuclei. The highest value occurred in the MMC-phase, where 80.18 per cent of the cells had ring-shaped nuclei.

Table 1

Composition of the myelon in the bone marrow of Wistar rats, based on differential diagnosis of 10,187 cells

Cell-type	$\bar{x}$		s	
	neutr.	eos.	neutr.	eos.
Myeloblast	24.4		1.65	
Promyelocyte I	69.7	5.6	4.35	2.63
Promyelocyte II	86.9	12.1	23.05	5.38
Myelocyte	116.5	19.1	9.47	6.74
Metamyelocyte	104.9	22.3	15.99	6.39
Granulocyte	487.9	40.7	54.97	12.13
Basophil	9.9		3.84	
Myeloid mitosis	18.7		5.08	

Remark: $\bar{x}$ values are concerned on 1,000 cells of each animal.

Table 2

Distribution of granulocytes in the peripheral blood

Cell-type	neutrophil			eosinophil			basophil		
	$\bar{x}$	s	per cent	$\bar{x}$	s	per cent	x	s	per cent
Segmented	102.7	14.50	48.67	—	—	0	—	—	0
Non-segmented	97.3	14.50	46.11	6.1	2.69	2.89	4.9	601	2.32

Remark: $\bar{x}$ values are concerned on 210 cells of each animal.

At the same time, this high, with maturation parallelly growing ratio of perforated cells gave an explanation for the possible mechanism of *ring-formation*. If the "Jugend" → "Stab" transformation were the most important way of differentiation and segmentation in rodents, too (Fig. 1 A), then, obligatorily, the ratio of open to closed nucleus forms would decrease. Data in Table 3 prove the contrary of this.

The value of this quotient increases for peripheral blood granulocytes to such a high degree that it is reasonable to presume: the open nuclei with horse-shoe- and band-shape come into being even following the slendering and separation of ring-shaped nuclei. The number of perforated nuclei overwhelm that of the open forms (833 : 100) already in the MC phase. Therefore, if we presume that the ring-shaped nucleus originates by streching, slendering, leaning and closing of the compact one, these processes must take place in the

Table 3

Distribution of open and closed nuclear forms in the neutrophil granulocytes and their intermediate precursors

Karyotype	bone marrow			peripheral blood
	MC	MMC	GC	GC
Open ("Jugend", "Stab")	100	97	1,002	1,052
Closed (perforated)	833	841	3,858	803
$Q_{open:\ closed}$	0.120	0.115	0.260	1.310

differentiation process before MC. In fact, the Q value decreases in this period: $Q_{PMC\ I} = 0.661$, $Q_{PMC\ II} = 0.270$. However, the absolute number of horseshoe and crescent-formed nuclei was rather low (from 1566 neutrophil PMC only 95) and among them the violently leaning forms, which precede the fusion, are

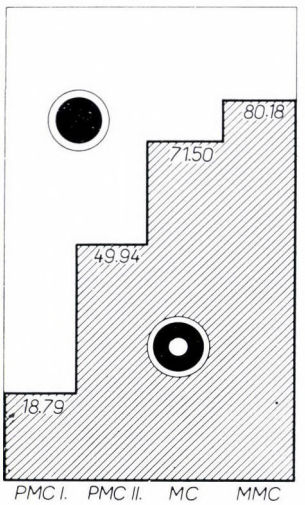

Fig. 5. Ratio of granulocyte precursors with perforated nucleus at the myelon

rare. All these facts show that in the rat (and presumably in other rodents) segmentation is preceded by *perforation* of the *compact* nucleus of the myeloid elements (Fig. 1, B).

It was examined furthermore whether the GC-differentiation could be divided into morphologically well-definable phases. Therefore the frequency of single GC nuclear forms in bone marrow and those in the blood were compared. The values obtained are shown in Table 4.

Karyotypes with similar relative frequency were regarded as identical in maturity. Thus, we established six developmental stages of the GS's, on

Table 4

Differentiation degree of granulocytes, based on the comparison of bone marrow and blood neutrophils

Degree of maturation	Karyotype	bone marrow		periph. blood		Q
		cell number	per cent	cell number	per cent	
1	Smooth-contoured, invaginated ring-, and 8-shaped	1,822	37.34	139	6.95	5.38
2	Wound ring-, horseshoe- and band form	2,107	43.18	444	22.20	1.96
3	1x segm. ring-, wound band- and wound horseshoe-shape	751	15.39	581	29.05	0.54
4	1x segm. band and 2—3x segm. ring	155	3.18	391	19.55	0.16
5	Hypersegm. ring and 2—3x segm. band	33	0.63	215	10.75	0.06
6	Hypersegm. band and broken nucleus	11	0.23	230	11.50	0.02

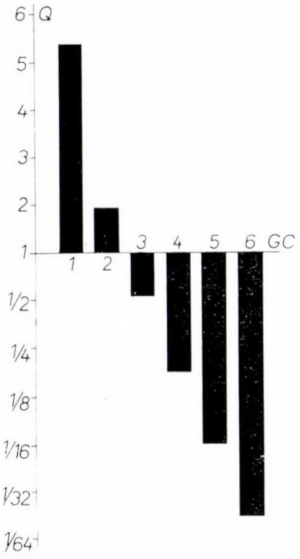

Fig. 6. Alteration of the value $\frac{Q_{\text{bone marrow}}}{\text{blood}}$ by GC karyotypes representing the single stages of maturation

the basis of a decreasing $Q_{\frac{\text{bone marrow}}{\text{blood}}}$ value from GC 1 (Q = 5.38) to GC 6 (Q = 0.02) (Fig. 6). Nuclei with smooth-contoured, infolded ring and 8-shape are the youngest (GC 1) and their ratio, together with the next group, is significantly higher in bone marrow than in the blood (Fig. 7). Cells in stage GC 4—GC 6 (Fig. 8) occur more frequently in the peripheral blood. The highest degrees of differentiation were exhibited by the granulocytes with hypersegmented,

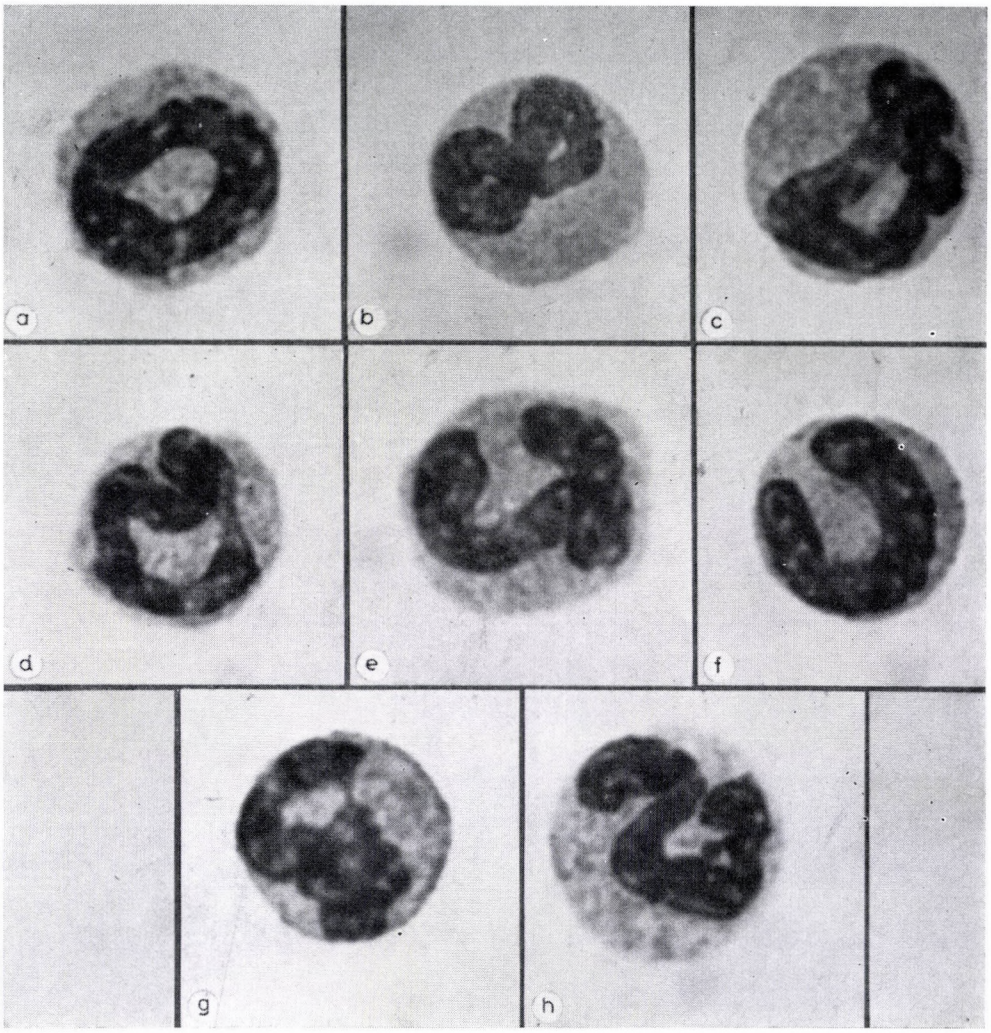

Fig. 7. Maturational forms of neutrophils in the bone marrow
a—c: $Q_{GC\,1} = 5.38$; d—f: $Q_{GC\,2} = 1.96$; g—h: $Q_{GC\,3} = 0.54$. Pappenheim, ×2,700

bandformed and of broken nuclei. The connection between the single categories and some transitional form is demonstrated in Fig. 9.

Data in Table 4 have proved, furthermore, our previous hypothesis suggesting that a part of closed, ring-shaped nuclei became open in the course of time. Namely, the open forms with a homologous structure usually appear by one category later than the corresponding closed ones. E. g., the smooth-contoured ring belongs to GC 1, the smooth-contoured band and horseshoe to GC 2; the onefold segmented ring was classified into GC 3, the onefold segmented bandform owing to its lower Q value, into GC 4.

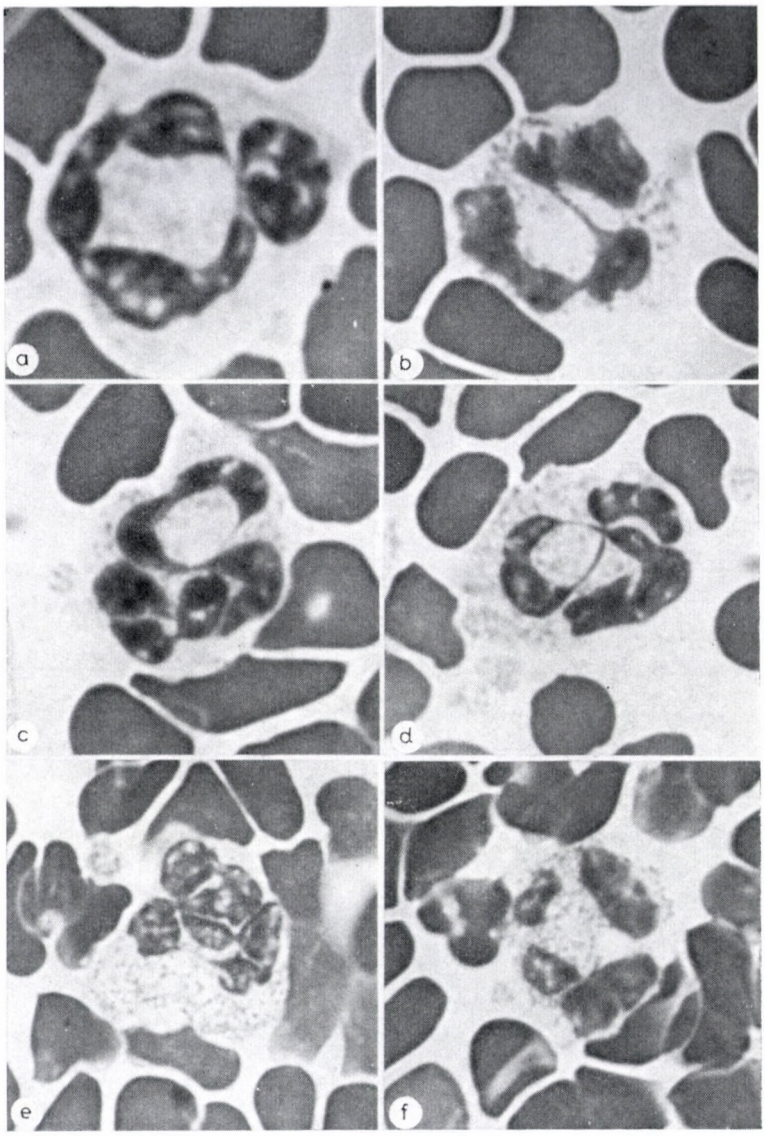

Fig. 8. Maturational forms of neutrophils in the peripheral blood
a—b: $Q_{GC\ 4} = 0.16$; c—d: $Q_{GC\ 5} = 0.06$; e—f: $Q_{GC\ 6} = 0.02$. Pappenheim, $\times 2{,}700$

Concerning the starting and final points of *eosinophil* granulocyte differentiation we concluded the following: in many cases there are discernible cells (Fig. 10) characterized by eosinophil granulation, 15—22 μ in diameter, basophilia in some sites of their cytoplasm, and a relatively large nucleus. Consequently, eosinophil granules occur in an early developmental phase (similarly to the specific neutrophil ones already following the mitoses of MB's),

in PMC I stage. Their numerical distribution was shown in Table 1; it is visible that among 10,000 granuloid cells in interphase 176 (= 17.6 per cent of the eosinophils) were eosinophil PMC I or PMC II.

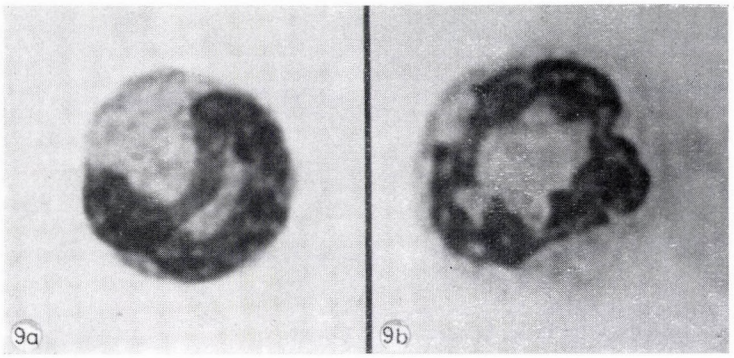

Fig. 9. Transitional granulocyte forms: a) mechanism of bending and winding; b) segmentation of the ring-shaped nucleus. Pappenheim, × 2,700

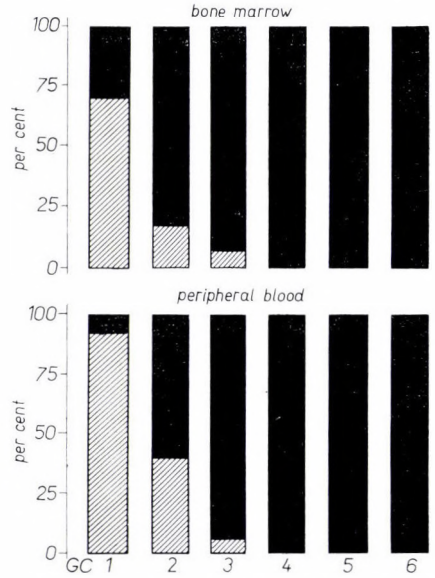

Fig. 12. Ratio of young and mature cells by neutrophil and eosinophil granulocytes in the bone marrow and peripheral blood

The *final phases* of eosinophil differentiation are shown by the diagram of Fig. 12. The majority of GC's contain ring- or 8-shaped nuclei (89.43 per cent in the bone marrow and 83.61 in the blood). Their differentiation is less complicated, compared to the neutrophils, and does not develop beyond phase GC 3 (Fig. 11). We failed to find eosinophil granulocytes, exhibiting 2—3 times

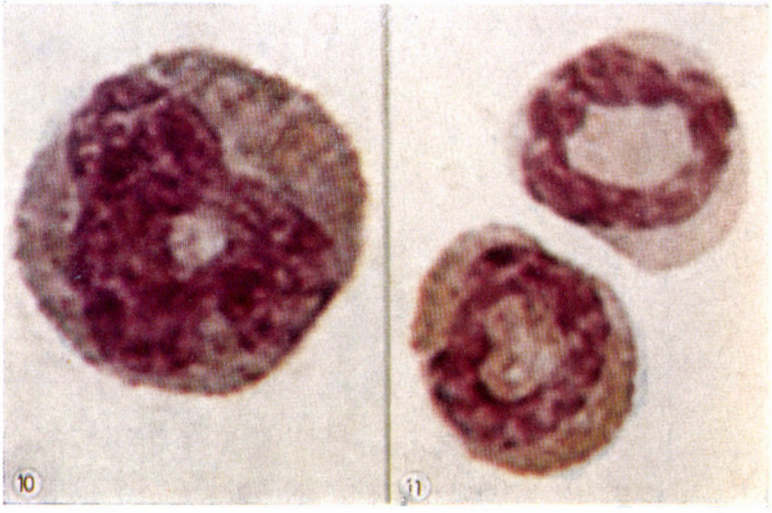

Fig. 10. Eosinophil granulation occurs during morphogenesis at the PMC stage. Pappenheim, × 2,700

Fig. 11. Neutrophil and eosinophil granulocytes from the bone marrow. Among equally smooth-contoured, ring-shaped nucleus the eosinophils pass through two, the neutrophils through five further maturational degrees. Pappenheim, × 1,250

segmented- or hypersegmented nuclei among more than 12,000 myeloid elements.

Among *mitoses* only those were registered which originated presumably from MB, or cells containing azurophil granulation (the diagnosis of the former, due to their similarity with proerythroblasts, is rather difficult). The 187 mitoses, occurred to the total number of granuloid elements, indicate an MI = 18.36 per thousand. The majority of the mitoses was represented by meta- and anaphases of PMC and MC's. It should be noted that we found dividing cells which, based on the criteria defined on page 374 as (a), (c) and (d) can be regarded as *metamyelocytes*. The diameter of the latter reached 12—14 μ, and their pink cytoplasm, free of basophilia, contained rare azurophil granulation.

Discussion

The comparison of the myelograms published by different authors is rather difficult, owing to their different nomenclature and categorization. Calculated on a common, percentual basis the great deviations between the quantitative values of the cells become visible.

Table 5

Comparative data of rat myelograms calculated on percentual values

Author	MB	neutrophil				eosinophil				basophil granulocytes
		PMC	MC	MMC	GC	PMC	MC	MMC	GC	
Stasney and Higgins [24]	8.66	7.23	12.27	7.76	52.89	11.19				
Ramsell and Yoffey [20]	6.82	9.84		78.86		3.80				0.67
Sachetti and Bianchini [21]	0.59	14.75	5.66	16.03	43.12	1.12	4.96	7.28	6.48	
Burke and Harris [2]	1.46	24.22		74.32						
Töppner [27]	1.77	6.19	31.86	7.08	35.40	2.65	8.85	3.54	1.77	0.88
Higgins and Machella [cit.: 14]	0.71	4.10	5.00	37.50	30.18	20.89				
Dumas [4]	3.01	4.82	27.71	6.02	44.58	13.86				
Köhler [14]	0.77	5.03	10.46	28.88	49.81	3.88				
Vogel [28]	5.81	1.45	19.03		59.27	—	0.40	1.53	12.34	0.16
Hulse [12] (ser.II.)	5.47	3.36	14.90	5.97	57.99	11.21				1.10
Balázs and Rappay, 1967	2.44	15.66	11.65	10.49	48.79	1.77	1.91	2.23	4.07	0.99

The *cause of differences* can be explained by alterations in age [2, 5], sex, strain, nutrition, etc. Into the MB category, caused by the uncertainty of differentiation on the basis of the nucleolar shape [25] some authors include the proerythroblasts and lymphoblasts, too [20, 24]. Others united some groups among both neutrophils and eosinophils: many authors do not mention basophil granulocytes at all. The latter error may originate not only from the low number of basophils, but — especially in the peripheral blood — from the high individual standard deviation, too (in our measurement $\bar{x} = 2.32$ per cent, but in 3 animals no basophil was found). In previous myelograms the greatest source of error was due, as already mentioned, to a wrong ranging of the cells with ring-shaped nuclei, which represent 59.23 per cent of the neutrophil precursors. The high ratio of precursor cells in the myelograms is striking, too; probably this causes the diminished sensibility of the rat against X-ray irradiation, compared to the man or dog [1, 12].

Inside the single categories the majority of authors have distinguished two groups: cells with closed ring-shaped and those with "complicated" nuclei [18], or "staff-cells" and mature granulocytes [28], etc. The six developmental stages, characterized by us quantitatively, too, offers the possibility of a more exact evaluation of the granulocyte dynamics and aging.

In the *morphogenesis* of ring-shaped nuclei, as main mechanism the perforation in PMC and MC phases can be specified. If closing follows the "Jugend" and "Stab" stages, would be performed, in agreement with SCHERMER [22], this is only the minor case; in contrary, the opening of the ring during segmentation occurs more frequently. It remains an unresolved question, whether it takes place in the interphase, or, as presumed by TERAO and GROPP [26], in the course of mitosis.

For her excellent, enthusiastic technical assistance the authors are indebted to Mrs. ERZSÉBET FAZEKAS-HOLLÓSI.

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DIE DIFFERENZIERUNG DES MYELONS UND DER LEBENSZYKLUS DER RATTENGRANULOCYTEN

Es wurden die Phasen und quantitativen Verhältnisse der Differenzierung von Granulocyten an Albino-Ratten untersucht. Durch die Abmessung der Myelo- und Hämogramme wurden sechs Reifungsformen (GC 1—GC 6) der Neutrophilen den bisher bekannten zwei Formen gegenüber morphologisch bestimmt. Die Getrenntheit der Entwicklung der eosinophilen Granulocyten beginnt auch in den Ratten im Promyelocytenstadium und im peripheren Blut geht die Differenzierung dieser Zellen nur bis zur Reifungsform GC 3 vor. Hinsichtlich der Bildung der ringförmigen Kerne der myeolopoetischen Zellen wurde festgestellt, daß (a) 18.79% der Promyelocyten, 71.50% der Myelocyten und 80.18% der Metamyelocyten ringförmige Kerne besitzen, (b) der Quotient der nicht perforierten/ringförmigen Kerne in den Entwicklungsstufen Myelocyt—Granulocyt von 0.12 auf 1.31 zunimmt, ferner Übergangsformen unter den stabförmigen und ringförmigen Kernvarianten nur in einem geringen Prozentsatz vorkommen. Daraus folgt, daß die Morphogenese der Kerne in der Myelopoese von Ratten durch die Lochung der Vollkerne der Prekursorzellen vorgeht; so haben die aus der menschlichen Hämatologie bekannten stabförmigen Zellkerne keine differentialdiagnostische Bedeutung bei den Ratten. Der Mitose-Index der myelopoetischen Zellen des Knochenmarkes betrug 18.36‰; es wurde mit Mikroaufnahmen demonstriert, daß auch die reiferen Zellformen als die Myelocyten mitosefähig sind.

ДИФФЕРЕНЦИАЦИЯ МИЭЛОНА И ЖИЗНЕННЫЙ ЦИКЛ ГРАНУЛОЦИТОВ У КРЫС

Стадии дифференцирования и квантитативные соотношения гранулоцитов были изучены на крысах-албиносах. На основании сопостовления приготовленных миэло- и гемограмм, вместо до сих пор принятых двух морфологических форм созревающих нейтрофильных гранулоцитов было выявлено шесть (ГЦ 1—ГЦ 6). Отделение развития эозинофилов и у крыс происходит в стадии промиэлоцитов и заканчивается в стадии 3 гранулоцитов в периферической крови. В отношении возникновения гранулоидных клеток с характерным для грызунов кольцевидным ядром было установлено, что (а) кольцевидное ядро имеется в стадии ПМЦ 1 18.79%, а в стадии МЦ и ММЦ 71.50% и 80.18%, (б) соотношение открытое/закрытое от МЦ до ГЦ с 0.12 увеличивается на 1.31, (в) и среди разнообразных ядер только в небольшом количестве имеются переходные формы от «палочкоядерных» — в кольцевидные. Из этого следует, что морфогенез миэлоидных элементов у грызунов происходит путем перфорации плотного ядра прекурзоров, а «палочкоядерные» формы, известные с гематологии человека, у крыс не имеют дифференциально-диагностического значения. В нашем материале митотический индекс было 18.36‰ (тысячных). Имеются данные, что в костном мозгу более дифференцированные формы, чем МЦ, тоже способны к делению.

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THE UTILIZATION OF L-SORBOSE BY AGARICUS MACROSPORUS (MOELL. ET J. SCHAEFF.) PILÁT

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Synopsis

Being an activity based on enzyme induction, the utilization of sorbose by the fungus *Agaricus macrosporus* starts with a considerable delay in media inoculated with mycelia consisting of "resting" cells. In the presence of small quantities of d-glucose, the delay is shorter; first glucose and subsequently sorbose will be utilized. The length of the phase of induction shows a well-defined correlation with the concentrations of both sorbose and glucose. If the inoculum is a growing mycelium colony, the utilization of sorbose begins practically without delay and its degree seems to be in correlation with the activity level of other enzyme systems and enzyme-forming mechanism of the cells. Above a certain — not high — sorbose concentration some delay in sorbose utilization occurs, even in case of a growing mycelium colony. Supposedly the relative quantity or the activity of the induced enzyme is more or less "counter-balanced" by the concentration of the specific substrate. From the experimental data it may be concluded that in non-growing cells the enzyme systems of generally low activity providing the utilization of the carbon source are inhibited even by low concentrations of sorbose, until the beginning of induction. No inhibition is caused in enzyme systems of higher activity below a certain sorbose concentration. If the concentration of sorbose is raised, it inhibits successively the enzyme systems in the order of their growing activity. As to sorbose utilization the nine examined species of the Agaricus genus represent different degrees. In *A. macrosporus* most likely a temporary enzyme system, and not a genetic change, is induced by sorbose.

Introduction

In the course of experiments performed with *A. macrosporus* inductive utilization of sorbose was noted. The present paper deals with some circumstances of the sorbose-caused induction.

Investigations conducted by Lilly and Barnett [5] on many fungus species have revealed that l-sorbose exerts a wide range of species-dependent effects: from good utilization to toxicity. According to these authors "it is somewhat surprising to find that a sugar causes such diverse effects".

Material and method

The *A. macrosporus* culture (marked as P. c. 14.) was taken from the collection of this Department. It excelled with a constant growth rate for the whole period of cultivation (1954—1966). — To culture the strain, 50 ml nutrient solution was placed in Erlenmeyer flasks of 100 ml. The flasks were cleansed twice in boiling distilled water before use. — Composition of the basal nutrient solution: 0.2 g KCl, 0.2 g $CaCl_2$, 0.2 g $MgSO_4 \cdot 7\,H_2O$, 0.2 g KH_2PO_4, 0.72 g

$Na_2HPO_4 \cdot 7H_2O$, 1 g peptone, 1 ml 1 per cent solution of $FeCl_3$, 50 μg aneurin, 0.1 g malt extract (for completion with growth substances); supplemented to 1,000 ml with distilled water made in glass apparatus. Except peptone all compounds were of "pro analysi" or "purissimum" quality. To the 1000 ml nutrient solution 2 g of agar-agar were added to keep the mycelium in the supernatant. (Agar-agar is no carbon source for *A. macrosporus.)* The medium of inoculum was the same as the basal nutrient solution, but 1 per cent d-glucose served as carbon source and agar-agar for solidification. The inoculum was not older than six weeks. Method of autoclaving: as soon as the pressure came up to 2 atmospheres the heating was discontinued. To avoid excessive caramelization the flasks were removed from the autoclave few minutes after the steam pressure was down. — After autoclaving the pH remained 6.5. — The dry matter weight was established as follows: the mycelium was collected on filter, washed with water and predried (mostly in a refrigerator) and subsequently kept in an exsiccator at about 80°C for 24 hours till complete exsiccation. The dry material was immediately weighed on a torsion balance. (Later, at room temperature the weight increased by 10 to 15 per cent.) The average weight of the inoculum (1.7 mg) was subtracted from the dry matter weight. Number of parallel test: generally four. — Practically no microbial contamination occurred.

Results and discussion

(a) If inoculation was carried out with a mycelium consisting of "resting" cells, the utilization of l-sorbose took a relatively long time (even if the Seitz-filtered sorbose was added in cold state). Accordingly, the sorbose-induced enzyme synthesis or increase in enzyme activity requires a considerable period of time to reach the level necessary for the growth. (Recent investigations — e. g. those of LYR and LUTHARDT [6] — have proved that the enzyme appearing due to induction results from de novo protein synthesis; Tables 1 and 2.)

Table 1

Temporal process of sorbose adaptation at 23.2 to 23.8°C
l-sorbose: 0.2 per cent

Incubation period: days	7	14	21	28	35	42
Mycelial dry matter: mg	0	0	2	6	13	21

In the presence of a small quantity of d-glucose the induction period was shorter. This can be attributed to the fact that — like in other microbes — first glucose permits a growth, sufficient to the synthesis of the inductive enzyme needed for the utilization of sorbose (Tables 2, 3 and 4).

With 0.2 per cent glucose and 0.8 per cent sorbose (Table 4) the utilization of sorbose begins in 15 to 18 days and after 30 days the dry matter weight of the mycelium is double that with 0.2 per cent glucose in the absence of sorbose. At the same time in the cultures containing sorbose but no glucose the induction still proceeds, and there is no growth. DOBROGOSZ and DEMOSS [3] examined the xylose induction and supposed that the function of a required extraneous substrate could be to provide the carbon and energy necessary to overcome the stalemate produced by the permeability barrier; further, that

Table 2

The influence of the concentration of sorbose and glucose on growth at 23.0 to 24.5°C

d-glucose	l-sorbose	Maximum colony diameter mm	Mycelial dry matter mg	Maximum colony diameter mm	Maximum colony diameter mm	Mycelial dry matter mg
per cent medium		10	20	30	40	60
		days after inoculation				
0.02	0.02	25	33			
	0.05	25	31			
	0.1	23	31			
	0.2	23	24			
	0.4	12	16			
	0.6	11	16			
	0.8	6	8			
	1.0	0	7			
0	0.02	17	23			
	0.05	14	16			
	0.1	3	3			
	0.2	0	0	3	12	43
	0.4	0	0	2	9	43
	0.6	0	0	1	6	43
	0.8	0	0	0	2	30
	1.0	0	0	0	2	

Table 3

The influence of the concentration of sorbose and glucose on growth at 23.2 to 23.8°C

l-sorbose	d-glucose	Mycelial dry matter in mg after	
per cent medium		20	40
		days	
0	1.0	104	228
	0.2	62	51*
0.2	0.2	73	101
	0.05	41	63
	0.01	14	43
1.0	0.2	64	155
	0.05	18	86
	0.01	3	20

* The carbon source was utilized earlier, decomposition processes increased

Table 4

Connection of the sorbose-glucose effect at 23.2 to 23.8°C

Time of incubation in days	Mycelial dry matter in mg if the carbon source is		
	0.8% l-sorbose 0.2% d-glucose	0.2% d-glucose	1.0% d-glucose
		as control	
3	0		
6	5		
9	13		
12	37		
15	55		
18	81		
20*		62	104
21	88		
24	112		
27	110		
30	127		

* Taken from another experiment

the extraneous substrate itself, or a derivate thereof, may be the specific carbon component involved in an initiating induction to xylose utilization. The same may be assumed for the sorbose induction dealt with here, and seems to be confirmed by the findings outlined under (b).

There are apparent correlations between the concentrations of sorbose and glucose on the one hand and the length of the induction period, on the other (Tables 3, 4 and 16).

We know however that glucose and some other compounds may have even opposite effects. In recent publications MANDELSTAM [9], MCCLATCHY and ROSENBLUM [8], NAKADA and MAGASANIK [11] and CITTI and co-workers [2] have stated that the synthesis of the inductive enzymes in *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus lactis* is influenced by the repressive effects of glucose and other utilizable carbohydrates. The synthesis of inductive β-galactosidase is even prevented by glucose and considerably inhibited by mannitol and glycerol. NAKADA and MAGASANIK [11] have proved that in the repression of the β-galactosidase synthesis rather the metabolite of glucose than glucose itself is the important factor. On the other hand, CITTI and co-workers [2] established clearly the operation of catabolite repression as a means of control of induced enzyme synthesis. For the induction in question this may supposedly be formulated so that certain carbon sources — while permit-

ting the development of conditions necessary for the inductive synthesis — simultaneously inhibit the induction until their utilization.

It should be mentioned here that on the surface of a medium or nutrient solution containing only l-sorbose as carbon source the inoculum becomes villose with a delay, i. e., at first a pronounced inhibition is apparent, even if the concentration of sorbose is relatively low and later this compound becomes utilized as carbon source at a suitable rate. It is hard to find an explanation for the reason of this phenomenon. One may refer to the investigations by LYR and LUTHARDT [6], according to which "a strong tyrosinase induction could be obtained by the addition of some metabolic inhibitors to the culture". Searching for the causes they established that "it is difficult to find a common basis for an explanation of this effect. Most of the (inhibitor) inducers are able to inhibit the oxidative phosphorylation of respiratory processes in fungi. It would therefore seem that a disturbance of the oxidative metabolism leads to an enzyme induction. At present it is difficult to say whether this depends on the liberation of an internal inducer or whether the induction is bound to the abolition of metabolic repression. By this it may mean that the synthesis of the enzyme commences when a number of factors in the general metabolism have been satisfied and this may be varied from species to species".

(b) If the inoculation is carried out with a growing mycelium colony, sorbose utilization begins in a short time (Table 5), and its degree appears to be connected with the activity level of other enzyme systems and enzyme-forming-mechanisms of cells, furthermore with the fact that the cells are provid-

Table 5

Sorbose utilization at 23.2 to 23.8°C after inoculation with growing mycelium

Time of incubation in days	Sorbose per cent medium		
	0.2		0.1
	Inoculum:		
	mycelium consisting of resting cells	mycelium colony developed for 7 days on a nutrient solution containing 0.4% d-glucose as carbon source	
	Mycelial dry matter in mg*		
7	0	10	15
14	0	15	15
21	2	36	26
28	6	48	27
35	13	55	32
42	21	46	63

* Weight of inoculum subtracted

Table 6/a

Interrelation between sorbose utilization and the "activity level" of inoculum at 23.0 to 23.8°C, using sorbose as carbon source

Carbon source	Inoculum: mycelium consisting of growing cells cultured for the indicated number of days in the presence of		Mycelial dry matter after 20 days in mg (Weight of inoculum subtracted)
	0.4% d-glucose 0.1% peptone	0.5% peptone	
	as C- and N-sources		
0.2% l-sorbose	3		16
	4		23
	5		25
	6		31
	7		37
	8		45
		8*	11
		11	23
		12*	23
		16*	23
	Inoculum: mycelium consisting of resting cells		5

* Average from 8 experiments performed at 23.0 to 23.8°C

Table 6/b

Interrelation between sorbose utilization and the "activity level" of inoculum at 23.0 to 23.8°C, using glucose as carbon source

Carbon source	Inoculum: mycelium consisting of growing cells cultured for the indicated number of days in the presence of		Mycelial dry matter after 20 days in mg (Weight of inoculum subtracted)
	0.4% d-glucose 0.1% peptone	0.5% peptone	
	as C- and N-sources		
0.2% d-glucose (control)	3		66
	4		64
	5		62
	6		56
	7		59
	8		57
		11	56
	Inoculum: mycelium consisting of resting cells		48

ed already to a certain extent with the energy needed for enzyme synthesis (Tables 6/a, 6/b, 7 and 8).

Naturally, like the utilization of other carbon sources, the degree of adaptive utilization is influenced by external factors (Table 9).

Table 7

*Interrelation between the growth rate of growing inoculum and the utilization degree of sorbose**

Carbon source in the nutrient solution of the inoculum	Growth rate (increase of dry matter weight) mg/day	l-sorbose per cent medium	Mycelial dry matter in mg after 20 days (Weight of inoculum subtracted)
0.4% d-glucose	1.3	0.2	16–25
0.1% peptone	1.8–2.6		31–45
0.5% peptone	0.8–1.2		14–23

* Averages from 8 experiments conducted at 23.0 to 23.8°C

Table 8

Sorbose utilization after inoculation with growing mycelium, using asparagine as N-source, at 23.2 to 23.8°C

l-sorbose per cent medium	Nitrogen source	Inoculum	Mycelial dry matter in mg after 20 days (Weight of inoculum subtracted)
0	0.1% asparagine	Growing mycelium colony developed for 7 days on a nutrient solution containing 0.4% d-glucose and 0.1% peptone as C- and N-source	12
0.2	0.1% asparagine		39
	0.1% peptone		31
	0.1% asparagine	Mycelium consisting of resting cells	4
	0.1% peptone		1

In case of inoculation with a growing mycelium colony the carefulness exerted in transferring the colonies seemed to be of importance. Injured colonies grew somewhat slower. The slight deviation observable after washing the inoculum in sterile distilled water prior to inoculation may probably be traced back to this reason. When an inoculum developed in a glucose-containing nutrient solution had been used, it might have been supposed that the unwashed inoculum had transferred a small amount of glucose; but this deviation mani-

Table 9

Interrelation between the pH of nutrient solution and sorbose utilization at 23.2 to 23.8°C
(Inoculum: growing mycelium colony developed for 7 days on a nutrient solution containing 0.4% d-glucose as carbon source)

l-sorbose per cent medium	pH = 6.5		pH = 6.0*		pH = 5.0*	
	After 20 days					
	Final pH value	Mycelial dry matter: mg**	Final pH value	Mycelial dry matter: mg**	Final pH value	Mycelial dry matter mg**
0	7.1 7.2	18	6.5	28	6.3 6.4	26
0.1	6.8 7.1	16	6.2 6.3	38	6.0 6.1	28
0.66	6.5 6.7	8	6.1 6.2	27	5.7	14
1.0	6.5 6.8	15	6.2 6.2	14	5.8 6.0	18

* Adjusted with phosphate buffer
** Weight of inoculum subtracted

fested itself even then, if for the inoculum a glucose-free nutrient solution and as carbon source the same peptone was applied on which served as nitrogen source in the experimental nutrient solutions (Table 10). (Due to this reason the washing of mycelium colonies in sterile distilled water was omitted later.)

If the inoculation is performed with mycelia consisting of resting cells, the results are to a certain degree influenced by the age and state of mycelia. To study the source of error appearing — due to inoculation with growing

Table 10

Effect of washing the growing mycelium colonies in sterile distilled water on growth at 23.2 to 23.8°C

l-sorbose per cent medium	Treatment of the inoculum	Inoculum	
		developed for 7 days on a nutrient solution containing 0.4% d-glucose as carbon source	developed for 14 days on a nutrient solution containing 0.5% peptone as carbon source
		Mycelial dry matter in mg after 20 days (Weight of inoculum subtracted)	
0.2	Washed in distilled water	29	13
	Not washed	36	18

mycelium colony — in the effect connected with the size of the colony and the higher number of cells, inoculations were also made with larger inoculum fragments consisting of resting cells. It was concluded that the effect exerted by the size of the mycelium colony must also be taken into consideration (Table 11).

Table 11

Interrelation between the weight of the inoculum and of the mycelial dry matter
l-sorbose: 0.2 per cent

Weight of the inoculum: mg	6	2
Mycelial dry matter after 20 days: mg (Weight of inoculum subtracted	8	2

(c) Above a certain — not high — concentration some delay in the utilization of sorbose occurs even in case of inoculation with a growing mycelium colony. Based on this fact we suppose that in the cell — in the present instance — the relative quantity or activity of induced enzyme must more or less be "counter-balanced" by the concentration of the specific substrate (Table 12). Alternately the delay may be caused by a mechanism reducing the self-inhibition, in conformity with the theory of LANDMAN [4]. (According to LANDMAN inducers are self-inhibiting above a certain concentration).

Table 12

Sorbose concentration, activity level of the inoculum and sorbose utilization at 23.2 to 23.8°C

l-sorbose	d-glucose	Inoculum			
		Mycelium of resting cells	Growing mycelium colony, 7 days old	Mycelium of resting cells	Growing mycelium colony, 7 days old
per cent medium		Mycelial dry matter in mg (Weight of inoculum subtracted)			
		20		30	
		days after inoculation			
0.1		4	23	15	50
0.2		2	31		61
0.5		0	10	5	57
1.0		0		0	37
	0.1	28			
	0.2	48			
	0.5	71			
	1.0	75			

(d) It may be assumed that in non-growing cells sorbose as an inducer inhibits — even in a low concentration — the enzyme systems of generally low "activity" ensuring the utilization of the carbon source. The enzyme system which ensures — in the absence of other carbon sources — the utilization of peptone as carbon source is also of low activity. In the presence of sorbose, when present above a low concentration limit, peptone is not utilized until sorbose induction had took place (Table 13). A similar phenomenon can be observed with asparagine as carbon source (Table 14). After inhibition had ceased, peptone or asparagine serves as carbon source until, depending on the sorbose concentration, the 20th to 40th day after inoculation. (At this time the weight of mycelia developed in sorbose-containing and sorbose-free nutrient solution are still equal.) Sorbose is utilized only thereafter.

Table 13

The utilization of 0.1% peptone serving as carbon source and its inhibition by sorbose at 23.2 to 23.8°C

1-sorbose	d-glucose (control)	Mycelial dry matter in mg after 20 days
per cent medium		
0		14
0.01		19
0.05		14
0.1		7
0.2		2
	0.01	17
	0.05	27
	0.1	43
	0.2	69

Table 14

The utilization of asparagine serving as carbon source and its inhibition by sorbose at 23 to 24°C

1-sorbose per cent medium	Mycelial dry matter in mg after	
	20	40
	days	
0	10	25
0.01	10	23
0.05	10	21
0.1	8	23
0.2	1	21

The acceptance of this mechanism leads to the conclusion that below a certain concentration limit sorbose, as a specific substrate, does not inhibit or slightly inhibits the enzyme systems of higher activity, e. g., those ensuring the utilization of d-glucose, d-fructose, d-cellobiose (i. e., the normal metabolism of cells is not or hardly disturbed) (Tables 3, 15 and 16).

Table 15

Connection between sorbose induction and enzyme systems of different activity at 23 to 24.5°C

Concentration of		Mycelial dry matter in mg after 30 days
l-sorbose	other sugars	
0.2%	0.1% d-glucose	75
	0.1% d-fructose	78
	0.1% cellobiose	60
	0.1% galactose	28
	0.1% maltose	17
0% (control)	0.1% d-glucose	42
	0.1% d-fructose	44
	0.1% cellobiose	47
	0.1% galactose	24
	0.1% maltose	22

Table 16

Connection between sorbose induction and enzyme systems of different activity at 23.2 to 23.8°C

Percentual concentration			Mycelial dry matter in mg after 20 days
l-sorbose	other sugars		
0	d-glucose (control	2.5	138
		1.0	104
0	d-glucose	0.5	89
0.5			87
1.0			78
2.0			66*
0	galactose	0.5	27
0.5			32
1.0			17
2.0			0
0	maltose	0.5	33
0.5			19
1.0			11
2.0			0

* Inhibition manifests itself chiefly in delayed or protracted growth

If the concentration of sorbose is higher, it "cuts" or inhibits one by one the enzyme systems of higher activity. This is the explanation for the observations made by TATUM and co-workers [12] as well as by LILLY and BARNETT [5]. TATUM and co-workers [12] have noticed that the linear growth of *Neurospora crassa* decreased when at constant sucrose level the concentration of sorbose was increased. According to LILLY and BARNETT [5]: "the inhibition due to sorbose tends to increase as the relative concentration of sorbose to maltose or glucose is increased. This is more evident with sensitive fungi and during early growth. When the amount of maltose was kept constant at 20 g per liter and the amount of sorbose was increased (0, 10, 20, 40 g), the mycelial weights of *Sordaria fimicola* after 5 days were as follows: 215, 87, 20, 11 mg. The weights of *Endoconidiophora fimbriata* after 5 days with glucose constant

Table 17

Utilization of l-sorbose by different Agaricus species at 23 to 24°C

Species	Percentual concentration of		Mycelial dry matter in mg after		Remarks on the commencement of growth
	l-sorbose	d-glucose	20 days	40 days	
campestris L. ex FR.	0.2		12	37	No delay
		0.2	48	81	
bitorquis (QUÉL.) SACC.	0.2		8	41	Delay of several days
		0.2	34	90	
semotus FR.	0.2		5	24	
		0.2	40	54	
maškae PILÁT	0.2		4	24	After 15 days weak growth
		0.2	57	46	
augustus FR.	0.2		4	7	
		0.2	11	19	
macrosporus (MOELL. et J. SCHAEFF.) PILÁT	0.2		2	20	
		0.2	62	51	
bernardii (QUÉL. apud CKE. et QUÉL.) SACC.	0.2		1	10	
		0.2	28	47	
xanthodermus GENEV.	0.2		1	2	
		0.2	33	70	
bitorquis (QUÉL.) SACC. var. *nánayi* BOHUS	0.2		0	2	
		0.2	51	56	
bisporus (LANGE) TRESCHOW	0.2		0	1	
		0.2	58	38	

at 20 g per liter and the amount of sorbose increased (0, 1, 5, 10, 20 g) were as follows: 122, 113, 94, 32 and 10 mg."

(e) The components of the enzyme or enzyme system ensuring the utilization of l-sorbose do not appear outside the cell in the nutrient solution. This is supported by experiments in which adding of a filtrate of cultures developed on sorbose-containing nutrient solution remained ineffective.

(f) It could be established that as to the utilization of l-sorbose some species of the *Agaricus* genus — as controls — represent different degrees and are grouped accordingly (inclusive of *A. macrosporus)* in Table 17. *A. campestris* heads the Table, its growth commences without delay (l-sorbose is, naturally, also for this species a weaker carbon source than d-glucose), whereas *A. bisporus* stands at the end of the Table, showing no growth for 20 days and only a minimum one even by the 40th day.

It seemed justified to examine the utilization of sorbose in both latter species more thoroughly (Table 18).

Table 18

Utilization of sorbose at 23.4 to 24°C

Species	Percentual concentration of sorbose	Diameter of mycelium colony in mm			Mycelial dry matter mg	
		10	30	50	20	60
		days after inoculation				
Agaricus bitorquis (QUÉL.) SACC. var. *nánayi* BOHUS	0	21	35	42	21	28
	0.01	18	39	43	32	33
	0.05	6	29	41	3	39
	0.2	0	0	12	0	14
Agaricus bisporus (LANGE) TRESCHOW	0	39	50	50	14	20
	0.01	33	46	50	17	22
	0.05	21	45	49	16	21
	0.2	0	9	13	0	5

(g) In the Agaricus genus among the intraspecific taxonomic categories relatively fairly remote from one another (subspecies, varieties) there are naturally differences also in this field. *A. bitorquis* utilizes sorbose quite well, while *A. bitorquis* var. *nánayi* only with a long delay, by means of induction. As to the utilization of sorbose among the taxonomic categories standing close to one another (forms, mutants) there are or may also be differences. From the three examined *A. macrosporus* mutants one is able to utilize sorbose by induction, but both the others cannot at all.

Concerning bacteria the problem of enzyme induction and repression has been more thoroughly investigated from the aspect of genetics [7]. Among others MCCLATCHY and ROSENBLUM [8] have stated, that "mutants lacking

β-galactosidase or galactosidepermease, as well as a constitutive mutant for lactose utilization, may be isolated. Like that in *Escherichia coli*, the genetic system of *Staphylococcus aureus* seems to consist of two structural genes for the synthesis of the two enzymes and at least one regulatory gene simultaneously controlling the expression of the structural genes".

There is also an opinion [1], suggesting that "none of the data . . . show that response to the substrate is due to the formation of temporary enzyme systems. Rather, the probability of selection of variants is good, since in all of instances the cells were proliferating and no attempts to obtain immediate reversion to the original condition were made".

With fungi the situation is quite different, as pointed out by CHRISTENSEN and DALY: "Genetic analysis of multicellular fungus colonies poses problems not encountered in bacterial population studies, problems which are augmented if the fungus is dicaryotic or multinucleate. Genetic variation in asexually propagated cultures is, then, a consequence of nuclear dissociations or reassociations (heterokaryosis) in addition to classical chromosomal aberrations and point mutations" [1]. In case of the examined *A. macrosporus* strain, however, under the influence of l-sorbose in all probability a "temporary enzyme system" develops. This supposition is confirmed by the observation that l-sorbose is utilized by an inoculum consisting of resting cells grown on a sorbose-containing medium with the same delay as by an inoculum obtained from a sorbose-free nutrient solution.

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DIE NUTZUNG VON L-SORBOSE DURCH *AGARICUS MACROSPORUS* (MOELL. ET J. SCHAEFF.) PILÁT

Diese Pilzart nützt die l-Sorbose durch Enzym-Induktion aus. Deshalb erfolgt die Verwertung bei einer Impfung mit aus »ruhenden« Zellen bestehendem Myzel nur nach einer längeren Zeit. Bei Vorhandensein einer kleinen Menge an Glykose verkürzt sich die Phase der Induktion: zuerst wird die Glykose und nachher die Sorbose verwertet. Hinsichtlich der Induktionszeit besteht zwischen den Konzentrationen von Sorbose und Glykose ein deutlich wahrnehmbarer Zusammenhang. Wird die Impfung mit im Wachstum begriffenen Myzelkolonien vorgenommen, so beginnt die Nutzung der Sorbose — praktisch — alsbald, und ihr Ausmaß scheint mit dem Aktivitätsniveau der Enzymsysteme, enzymbildenden Mechanismen in den Zellen verknüpft zu sein. Auch bei Verwendung von im Wuchs befindlichen Myzelkolonien läßt sich in der Sorbosenutzung oberhalb einer gewissen — nicht hohen — Konzentrationsgrenze eine größere oder kleinere Verspätung feststellen. Man kann also annehmen, daß die relative Menge oder Aktivität des induzierten Enzyms mit der Konzentration des spezifischen Substrats mehr oder minder im »Gleichgewicht« sein muß. Die Versuchsangaben lassen vermuten, daß in den nicht-wachsenden Zellen für eine Zeit — bis zu Beginn der Induktion — die Sorbose bereits in niedrigerer Konzentration jene, die Nutzung der Kohlenquelle sichernden Enzymsysteme hemmt, deren »Aktivität« i. allg. gering ist. In Enzymsystemen höherer Aktivität tritt unter einer gewissen Konzentrationsgrenze keine Hemmung ein. Mit steigender Konzentration werden durch die Sorbose die Enzymsysteme fortschreitend höherer Aktivität der Reihe nach gehemmt. In der Sorbosenutzung stellen die neun untersuchten Arten der *Agaricus*-Gattung verschiedene Stufen dar. Es ist sehr wahrscheinlich, daß sich bei *A. macrosporus* unter dem Einfluss der Sorboseinduktion ein zeitweiliges Enzymsystem entwickelt und nicht eine genetische Änderung stattfindet.

УСВОЕНИЕ L-СОРБОЗЫ ГРИБАМИ *AGARICUS MACROSPORUS* (MOELL. ET J. SCHAEFF.) PILÁT

Исследуемый вид грибов усваивает сорбозу посредством индукции. Следовательно, в случае прививки мицелия, состоящего из клеток находящихся в «состоянии покоя», усвоение сорбозы начинается лишь после длительного времени. В присутствии значительного количества d-глюкозы, время индукции короче. Вначале усваивается глюкоза, а потом сорбоза. Относительно времени индукции между концентрацией сорбозы и концентрацией глюкозы существует хорошо выявляемая корреляция. В случае прививки мицелия, находящегося в стадии роста, усвоение сорбозы начинается быстро, и его степень зависит, повидимому, от активности прочих энзиматических систем, от механизмов, образующих энзимы, имеющихся в клетках. Даже в случае применения мицелия, находящегося в стадии роста, выше определенной — незначительной — концентрации наблюдается более или менее большое запаздывание усвоения сорбозы. Следует предполагать, что относительное количество или активность индуцированного энзима должна более или менее находится в «равновесии» с концентрацией специфического субстрата. Из экспериментальных результатов можно заключить, что сорбоза в клетках находящихся в состоянии покоя, до определенного времени — начала индукции — задерживает и при более низкой концентрации энзиматические системы, обеспечивающие использование источников углерода, «активность» которых в общем незначительна. В энзиматических системах с большей активностью сорбоза — ниже определенной концентрации — не вызывает торможения. При повышении концентрации сорбоза задерживает также энзиматические системы со все повышающейся активностью. Девять исследовавшихся видов рода *Agaricus* представляют собой с точки зрения усвоения сорбозы различные ступени. В случае гриба *A. macrosporus* при действии сорбозной индукции возникает, по всей вероятности, временная энзиматическая система, а не генетическое изменение.

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PROTEIN AND FREE AMINO ACID CHANGES IN DIFFERENT HETEROZYGOTES OF DROSOPHILA MELANOGASTER MEIGEN IN THE COURSE OF ONTOGENESIS

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Synopsis

Free and protein amino acids of the *Oregon-R wild*, *white*, *Bar* and *white-Bar* parent stocks of *Drosophila melanogaster* MEIGEN, as well as of their heterozygote combinations have been examined at various stages of ontogenesis. The analyses have led to the following conclusions: (1) Among all the stages of ontogenesis it is the larval stage where the differences between genotypes are the most obvious. (2) The trend of changes in the concentration of protein amino acid is characteristic of the respective genotypes. In case of both the pure genetic lines and their various heterozygote combinations, these changes show roughly the same tendency, apart from differences in the order of magnitude. All of the examined genotypes differ from the *Oregon-R wild* stock. (3) It is only against the white genotype the *Oregon-R wild* stock is able to enforce its dominance. The pupal and adult stages of the heterozygote genotype w^+/w are dominated by *Oregon-R*, while the white genotype is dominating in the larval stage of the same heterozygote. (4) The phenomenon of heterosis holds true of every heterozygote.

Introduction

In the earliest studies the genotypic determinatedness of the protein metabolism of *Drosophila melanogaster* MEIGEN was examined by analyzing the free amino acids in the larval haemolymph of mutants possessing lethal factors, in comparison to the *Sevelen wild* stock (*lme* — CHEN and HADORN [6], SCHMID [14]; *ltr* — CHEN [3]; *1-8* and *1-52* — GLOOR [8]; *Sevelen* — CHEN [3]). Differentiated on ontogenetical and, in the adult stage also sexual, basis the analyses of the free and protein amino acids of the *Oregon-R wild* stock and of the *white*, *Bar* and *white-Bar* eye-mutant stock have been conducted by the authors [16, 17] with the distant aim of comparing, again on an ontogenetical basis, these stocks with their heterozygotes. We also tried to get information of the relationship between the conditions of dominance known for the eye-phenotype of these mutants, and those presumed for protein metabolism.

Material and method

Material examined: The following heterozygote genotypes of *Drosophila melanogaster* MEIGEN were analyzed: $w^+ + {}^B/w^+B$, $w^+ + {}^B/w+{}^B$, $w^+ + {}^B/wB$, $w + {}^B/w^+B$, $w + {}^B/wB$, and w^+B/wB. The data obtained earlier for *Oregon-R wild* stock and the mutant stocks *white*, *Bar* and *white-Bar* were used for comparison.

Culturing: The material was grown at 25°C in a culture medium of the following composition: 1,000 ml water, 145 g yeast, 57 g molasses, 50 g maize meal, 50 g maize grit, 13 g salt-free agar-agar. Fresh yeast pulp was dropped on the surface of the ready nutrient.

Ontogenetical stages examined: 120 hours old larva (before entering the pupal stage), colourless (young) pupa and five days old virgo female imago.

Procedures: Larvae and pupae were washed with distilled water and then dried with filter paper. The imagoes were killed by ether before homogenized. The material was ground with quartz sand and extracted with a solution composed of 96% methanol + conc. NH_4OH + + H_2O at a volumetric ratio of 75 : 1 : 24. After homogenization and extraction enzymes were denatured by boiling to prevent decomposition of peptides, and then the material was centrifuged three times. The supernatants containing free amino acids were decanted and collected. Hydrolysis was made with $Ba(OH)_2$ in combustion tube in nitrogen atmosphere for 48 hours at 150°C. The hydrolysate was washed three times with 70% ethanol, the residual $Ba(OH)_2$ was precipitated by bubbling CO_2 through the cooled fluid and the resulting $BaCO_3$ was removed by centrifuging. Excess CO_2 was eliminated by boiling. After adjusting pH to 7, the thus purified hydrolysate was concentrated in an incubator at 80°C and, finally, in an exsiccator at room temperature. The dried hydrolysate and the dried collected supernatant were redissolved in 50% ethanol, and an aliquot of solution, corresponding to 20 specimens was dropped from a micropipet on the start line of the radial chromatogram 16 cm in diameter (Schleicher-Schüll 2043/b). The solvent used was a 16 : 5 : 2 : 2 mixture of n-butanol + glacial acetic acid + phenol + H_2O. In order to achieve complete separation of the amino acid strips, the chromatogram was developed twice. Between the two developments the chromatogram was dried at room temperature. The different amino acids were determined by the method of FISCHER and DÖRFEL [7]. The amino acids detected by ninhydrine and transferred in copper complex were eluted with 96% methanol and examined at 510 mμ in a UVIFOT photometer. The extinction values are expressed in α-alanine and the data obtained are presented. Taking into consideration the data of LINSKENS [13] the measured extinctions have been multiplied by the following numbers:

1.08 α-alanine	1.75 arginine
1.33 leucine	2.50 isoleucine
1.50 threonine	3.16 β-phenylalanine
1.58 glutamic acid	3.42 tyrosine
1.58 valine	3.42 aspartic acid
1.67 lysine · 2HCl	

The extinction values measured were thus referred to 20 specimens, this being the optimal quantity for quantitative chromatography. The material of 600 individuals was pooled each time, and that of 100 individuals was actually chromatographed. In the larval and pupal stages the material was a 1 : 1 mixture of male and female individuals. In the imago stage, however, the sexes were separated and only the heterozygote females were examined. The extinction data for the imago stage calculated to the sex ratio of 1 : 1 are available from our previous experiments [16, 17]. Since our experiments were aimed at comparing the heterozygote females not only with the homozygote females of the parent stocks, but also with their hemizygote males, every extinction value of the adult animals are referred to the unit weight of the males. Therefore the extinction values of the female imagoes were multiplied by a constant of male/female = = 0.65, obtained from our own data [17].

Results

We have detected the following protein amino acids in the three examined ontogenetical stages of the heterozygote individuals $w^+ + {}^B/w^+B$, $w^+ + {}^B/w+{}^B$, $w^+ + {}^B/wB$, $w + {}^B/w^+B$, $w + {}^B/wB$ and w^+B/wB of *Drosophila melanogaster* MEIGEN: arginine, histidine, serine, glycine, asparagine, aspartic

acid, glutamine, glutamic acid, α-alanine, threonine, proline, tyrosine, valine, β-phenylalanine, leucine and isoleucine. In the free amino acid fraction β-phenylalanine was absent in the 120 hours old larval and in the pupal stage (Figs 1, 2 and 3).

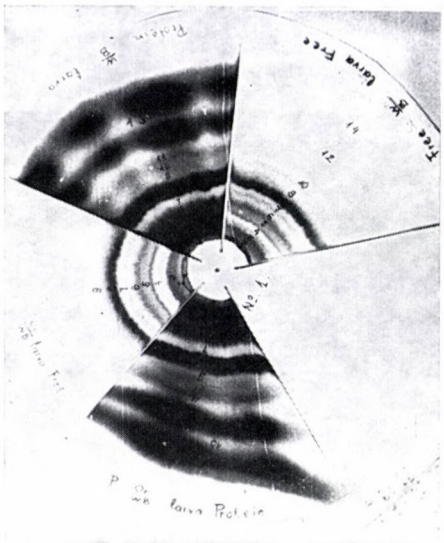

Fig. 1. 120 hours old larvae

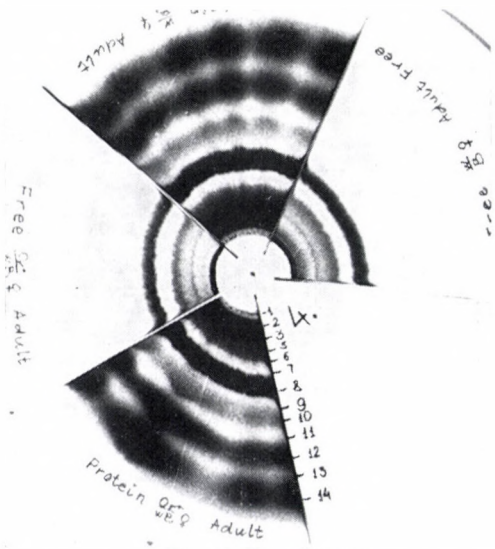

Fig. 2. Pupal stage

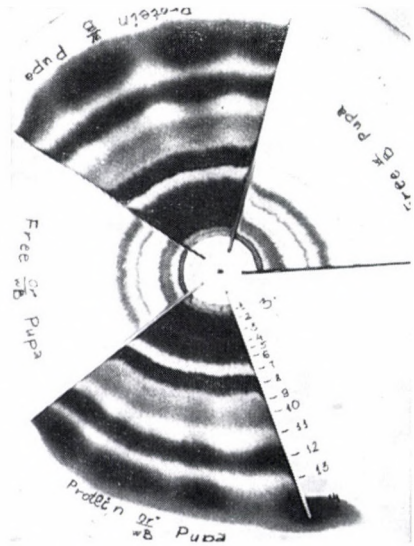

Fig. 3. Adult stage

Figs 1—3. Protein and free amino acids of the di-heterozygotes $w\ {}^{+B}/w^{+}B$ and $w^{+}\ {}^{+B}/wB$ on the radial chromatogram: 1 — arginine + histidine, 2 — asparagine, 3 — serine + glycine, 4 — aspartic acid, 5 — glutamine, 6 — glutamic acid, 7 — threonine, 8 — α-alanine, 9 — tyrosine, 10 — proline, 11 — tryptophane, 12 — valine, 13 — β-phenylalanine, 14 — isoleucine + leucine

The analysis of *protein amino acids* shows that in the ontogenetical stages examined the concentration of *arginine + histidine* is the lowest and there is hardly any difference in this respect between homozygotes and heterozygotes.

The *serine + glycine* values for the parents, though increasing with the progress of ontogenesis, are inferior to those for the heterozygotes in all of the three ontogenetical stages.

A comparatively high concentration of *aspartic acid* was found in the homozygote individuals, except for the *Bar* stock exhibiting a very low concentration in the larval stage. In the heterozygotes of the genotypes w^+/w and $+^B/B$ the high concentration characteristic of the larval and pupal stages decreased by the time the adult age is reached. The aspartic acid contents of the four other genotypes hardly change during the three ontogenetical stages.

For *glutamic acid* the parental values are surpassed by those of heterozygotes in the larval and pupal stages, but in the adult stage the concentration is higher in the homozygote parents.

The concentration of *threonine* being variable in different genotypes is levelled up in the adult stage.

The concentration of *α-alanine* increases during the ontogenesis of homozygotes, but remains by far inferior to that observed in heterozygotes, with the exception of the adult stage.

Tyrosine is at a particulary high level in the larval stage of homozygotes, as opposed to heterozygotes.

Valine concentration in the larval stage is almost twice as high in heterozygotes as in homozygotes; this difference decreases somewhat in the course of ontogenesis.

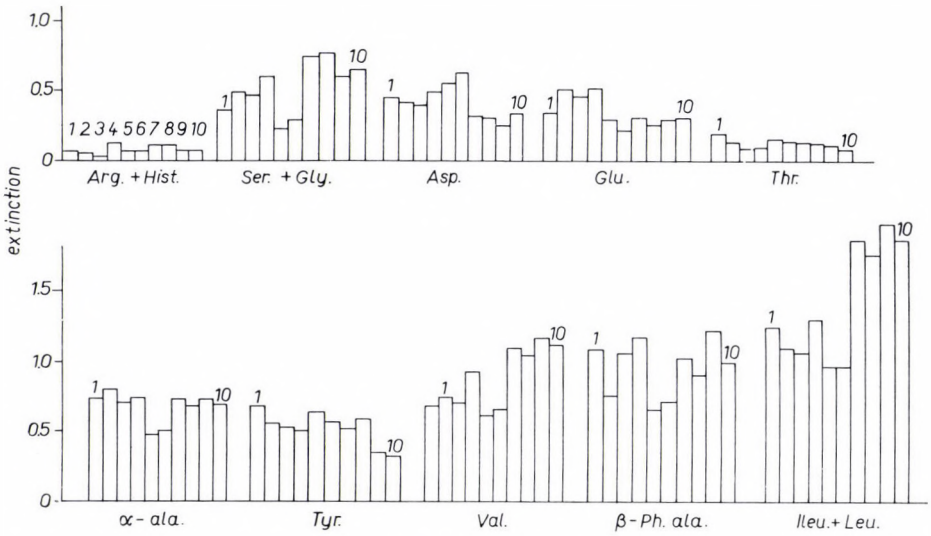

Fig. 4. Adult stage

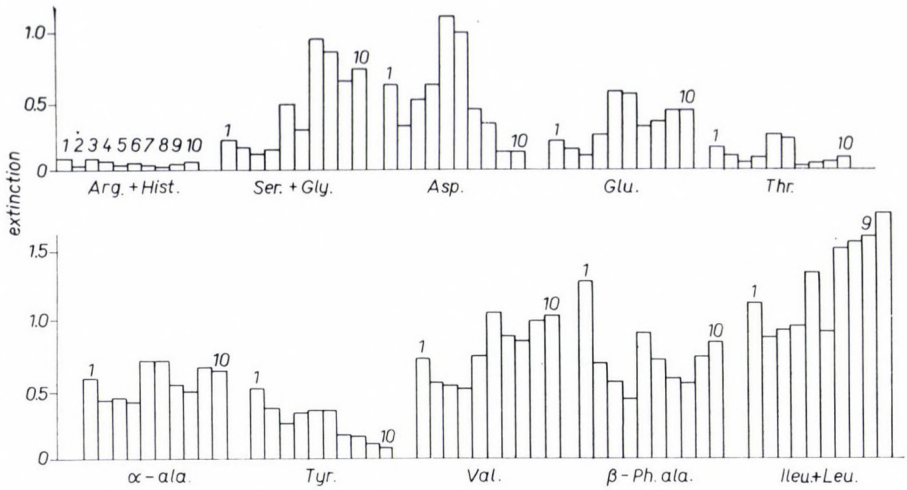

Fig. 5. Pupal stage

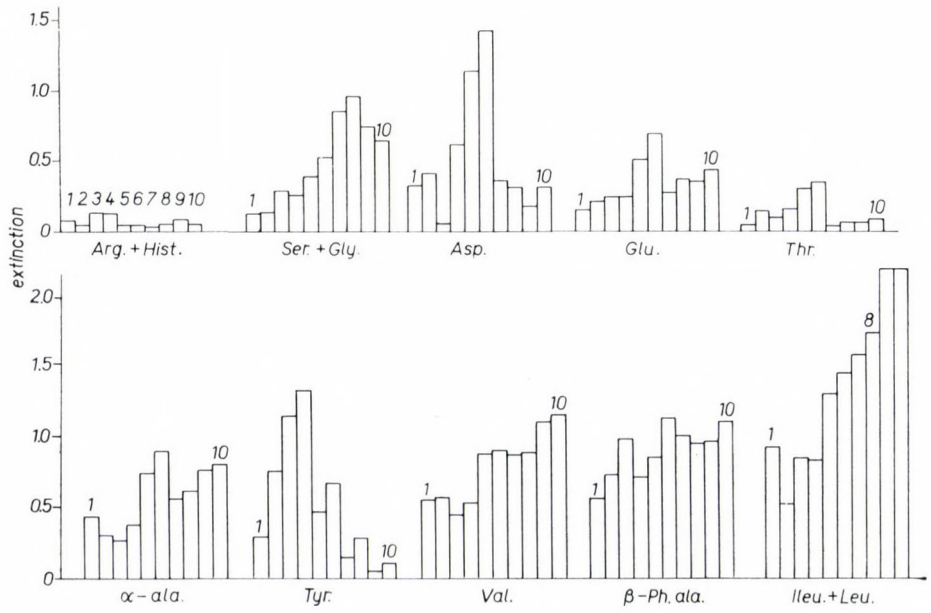

Fig. 6. 120 hours old larvae

Figs 4—6. The quantity of the different protein amino acids of following genotypes of *Drosophila melanogaster:* 1 — *Oregon*-R, 2 — *w*, 3 — *B*, 4 — *wB*, 5 — w^+/w, 6 — $+^B/B$, 7 — $w +^B/w^+B$, 8 — $w^+ +^B/wB$, 9 — $w +^B/wB$, 10 — w^+B/wB

In all of the three stages of development, the concentration of both *β-phenylalanine* and *isoleucine + leucine* is much higher than that of any of the other amino acids, both in homozygotes and heterozygotes. β-phenylalanine is at a particulary high level in the *Oregon-R wild* stock in the pupal stage. In the larval and adult stages the leucine + isoleucine contents of heterozygotes are by far superior to those of homozygotes (Figs 4, 5 and 6, Table 1).

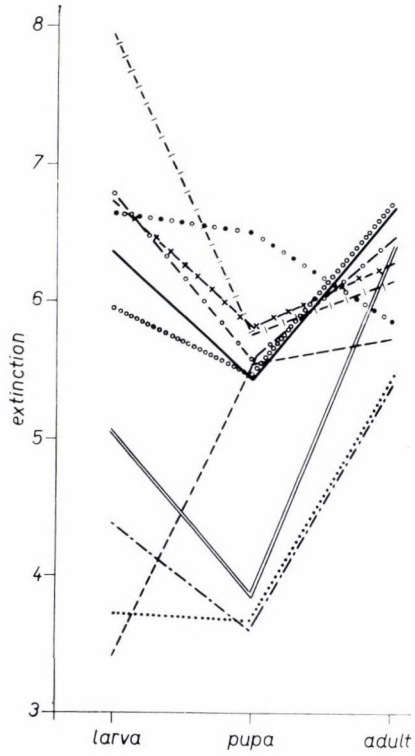

Fig. 7. Change in total quantity of protein amino acids in the course of ontogenesis. Total extinctions. — — — — = *Oregon-R* . · · · = w, — · — · — · = B, ═══ = wB, ○ ● ○ ● = w^+/w, — | — | = $+^B/B$, ○ ○ ○ = $w + {}^B/w^+B$, — ○ — ○ = $w^+ + {}^B/wB$, — = $w + {}^B/wB$, — × — × = w^+B/wB

It is thus *a general phenomenon* that the protein amino acid concentration is higher in heterozygotes than in homozygotes in all of the three ontogenetical stages, with the most distinct difference in the larval and pupal stages.

Though the numerical values are different, the *total extinction values* of protein amino acids present, in general, the same tendency in homozygote mutant stocks and in heterozygotes. In the *Oregon-R wild* stock, on the other hand, the lowest values were shown by 120 hours old larvae, whereas the maximum

Table 1

Extinctions of cupric complex of protein amino acids of heterozygotes at S_{510}, expressed in α-alanine

Mark of genotype	w^+/w	$+^B/B$	$w+^B/w^+B$	w^++^B/wB	$w+^B/wB$	w^+B/wB
larval stage						
Arg. + His.	0.03	0.03	0.02	0.04	0.08	0.05
Ser. + Gly.	0.38	0.52	0.84	0.93	0.73	0.63
Asp.	1.14	1.39	0.36	0.31	0.17	0.32
Glu.	0.50	0.68	0.26	0.33	0.33	0.42
Thr.	0.30	0.35	0.03	0.05	0.05	0.08
α-ala.	0.73	0.79	0.55	0.60	0.75	0.78
Tyr.	0.46	0.65	0.14	0.27	0.03	0.09
Val.	0.98	0.99	0.97	0.98	1.07	1.11
β-Ph. ala.	0.84	1.12	1.01	0.96	0.98	1.11
Leu. + Ileu.	1.27	1.42	1.55	1.70	2.15	2.15
Total ext.	6.63	7.94	5.73	6.17	6.34	6.74
pupal stage						
Arg. + His.	0.03	0.04	0.03	0.05	0.04	0.05
Ser. + Gly.	0.48	0.29	0.94	0.86	0.65	0.73
Asp.	1.11	0.89	0.46	0.34	0.14	0.14
Glu.	0.56	0.55	0.32	0.35	0.44	0.44
Thr.	0.26	0.23	0.03	0.03	0.06	0.09
α-ala.	0.71	0.71	0.53	0.50	0.67	0.63
Tyr.	0.37	0.37	0.20	0.19	0.13	0.10
Val.	0.75	1.05	0.89	0.85	0.99	1.02
β-Ph. ala.	0.90	0.71	0.51	0.54	0.73	0.83
Leu. + Ileu.	1.32	0.90	1.49	1.54	1.58	1.75
Total ext.	6.49	5.74	5.40	5.25	5.43	5.78
$\frac{♀+♂}{2}$ adult stage						
Arg. + His.	0.06	0.06	0.10	0.10	0.05	0.06
Ser. + Gly.	0.23	0.29	0.76	0.77	0.61	0.66
Asp.	0.55	0.63	0.32	0.31	0.26	0.35
Glu.	0.30	0.24	0.32	0.27	0.31	0.30
Thr.	0.17	0.15	0.15	0.14	0.13	0.10
α-ala.	0.46	0.48	0.70	0.66	0.71	0.67
Tyr.	0.60	0.55	0.50	0.57	0.33	0.30
Val.	0.52	0.62	1.05	1.00	1.12	1.07
β-Ph. ala.	0.62	0.68	0.97	0.85	1.15	0.94
Leu. + Ileu.	0.93	0.93	1.80	1.70	1.92	1.81
Total ext.	4.44	4.63	6.67	6.37	6.59	6.25

Remark: Values of homozygote stocks in reference No 17

Table 2

Extinction values of free and protein amino acids of

Free amino acids					
Mark of genotype	Hemizygote ♂	Mark of genotype	Heterozygote ♀	♀ × 0.65	$\frac{♀ + ♂}{2}$
white	2.31	$w +^B/w^+B$	1.15	0.75	1.73
Oregon-R	2.24	$w^+ +^B/wB$	1.31	0.85	1.78
white	2.31	$w +^B/wB$	1.66	1.08	1.99
Bar	1.94	w^+B/wB	1.71	1.11	1.83

value of imagoes were almost achieved in the pupal stage. From among the heterozygotes w^+/w, $+^B/B$ and $w^+ +^B/wB$ containing the *Oregon-R* maternal genotype, the heterozygote w^+/w is the only one where the influence of *Oregon-R* can be revealed, and only in the pupal stage, though giving here extremely high value.

The *change in total concentration* of protein amino acids in the ontogenetical stages examined *is characteristic* of the respective genotypes (Fig. 7). In the *120 hours old larval stage* the total concentration in the *Oregon-R wild* stock represents the minimum, and that of the heterozygote $+^B/B$ the maximum value.

In the *pupal stage* the total concentration of protein amino acids substantially decreases in every genotype, except for *Oregon-R* and w^+/w. The sharpest decrease is in the heterozygote $+^B/B$.

From the pupal to the adult stage the homozygote stocks present a higher increase in concentration. The differences between heterozygotes are less marked than in 120 hours old larvae. The homozygote genotypes *Bar* and *white* and the heterozygote genotypes $w+^B/w^+B$ and $w+^B/wB$ largely agree with one another in the trend of change.

The phenomenon of heterosis can be observed in both the total extinction of protein amino acids and the concentration of the single amino acids. In fact, the values of homozygotes are surpassed by the total extinction values to such an extent that even the minimum values for heterozygotes are higher than the maximum values for homozygotes (Fig. 7).

In the *imago stage*, the heterozygote females and hemizygote males of generation F_1 can already be compared. As shown in Table 2, the protein amino acid contents of hemizygotes are always lower by 30% than those of heterozygotes, while the free amino acid values for hemizygotes are by 50—75% superior to those for heterozygotes.

In examining the heterozygotes we followed with attention how the quantity of protein amino acids was affected by the doses of the dominant

hemizygote males and heterozygote females at S_{510}, in adult stage

Protein amino acids					
Mark of genotype	Hemizygote ♂	Mark of genotype	Heterozygote ♀	♀ × 0.65	$\frac{♀ + ♂}{2}$
white	4.43	$w + ^B/w^+B$	8.91	5.79	6.67
Oregon-R	3.94	$w^+ + ^B/wB$	8.90	5.78	6.42
white	4.43	$w + ^B/wB$	8.79	5.71	6.61
Bar	3.54	w^+B/wB	8.95	5.82	6.25

allele. On the basis of the data specified in Fig. 7, the following genotypes are compared with one another, considering the doses of the dominant allele: *white* — $w^+ + ^B/w + ^B$; *Bar* — $w^+ + ^B/w^+B$; *white-Bar* — $w + ^B/wB$ — w^+B/wB.

Table 3

Comparison of homozygotes and heterozygotes considering the doses of the dominant alleles (w^+ and B) on the basis of the total extinctions of protein amino acids (Fig. 7)

Dosis of dominant allele		Result of comparison
Homozygotes	Heterozygotes	
$w + ^B/w + ^B$ nulli-dominant 0 dose	$w^+ + ^B/w + ^B$ mono-dominant 1 dose	In heterozygote: *heterosis*. Homo- and heterozygotes change in the opposite sense
w^+B/w^+B bi-dominant 4 doses	$w^+ + ^B/w^+B$ bi-dominant 3 doses	In heterozygote: *heterosis*. Agreement in trend
wB/wB mono-dominant 2 doses	$w + ^B/wB$ mono-dominant 1 dose	*Heterosis* in both heterozygotes in larval and pupal stage. Agreement in trend
	w^+B/wB bi-dominant 3 doses	

As shown by this analysis the heterosis effect due to heterozygoty surpasses the effect of the dominant allele dosage, as compared with homozygotes. If, in regard to the dose of the dominant allele, the heterozygotes are compared with one another, the following result is obtained:

$w^+ + ^B/w + ^B$, 1 dose, and $w^+ + ^B/w^+B$, 3 doses: the higher value is attained in larva and imago for the 3-dose heterozygote;
$w + ^B/wB$, 1 dose, and w^+B/wB, 3 doses: the higher value is attained in larva and pupa for the 3-dose heterozygote.

There are thus at least two ontogenetical stages where the higher value is always attained by the heterozygote possessing more doses of the dominant allele.

Finally, let us compare the heterozygotes containing the same dosage of dominant allele:
$w^+ +^B/w+^B$ with 1 dose w^+ and $w+^B/wB$ with 1 dose B:

$$w^+ > B \quad \text{in larva and pupa}$$
$$w^+ < B \quad \text{in imago.}$$

$w^+ +^B/w^+B$ with 2 doses w^+ and 1 dose B, and w^+B/wB with 1 dose w^+ and 2 doses B:

$$2w^+ > w^+ \quad \text{in larva}$$
$$B > 2B \quad \text{there is no effect of } B\text{-dose!}$$

The *number of w^+ alleles is thus decisive* and not the number of *Bar* allele doses.

The trend of the concentration of *free amino acids* was found opposite to the trend of protein amino acids, and the concentration of free amino acids is higher in homozygotes than in heterozygotes (Fig. 11).

In the *larval and pupal stages* the difference is particularly remarkable in free arginine + histidine, aspartic acid, α-alanine and tyrosine contents. Further, the high valine concentration in $+^B/B$ larvae, and the large quantity of arginine + histidine and leucine + isoleucine in the pupal stage are conspicuous. In the heterozygotes w^+/w and $+^B/B$ the tyrosine contents remarkably increase as compared to the parents. In these two stages there is no threonine to be detected in the homozygotes, while β-phenylalanine is lacking everywhere (Figs 8, 9).

These major differences between homozygotes and heterozygotes are somewhat levelled up in the *adult stage*. Theronine, tyrosine, valine and leucine + isoleucine are at lower levels in heterozygotes, while the serine + glycine, glutamic acid and α-alanine contents of the heterozygotes $+^B/B$ and w^+/w are remarkably lower than in the other homo- and heterozygote genotypes. It is surprising that only the homozygote females contain β-phenylalanine, which cannot be found in the hemizygote males of these stocks, nor in the heterozygotes (Figs 1, 2, 3, 8, 9, 10 and Table 4).

The ontogenetic development of the *total extinction of free amino acids* shows that, in the homozygotes, the high concentration characteristic of the larval age decreases significantly in the imago stage, whereas the low value in the larval age of heterozygotes increases in the imagoes. The only exception is the heterozygote $+^B/B$, where the decreasing maternal effect exerts itself in the imago stage; in the pupal stage, however, it shows a remarkably high con-

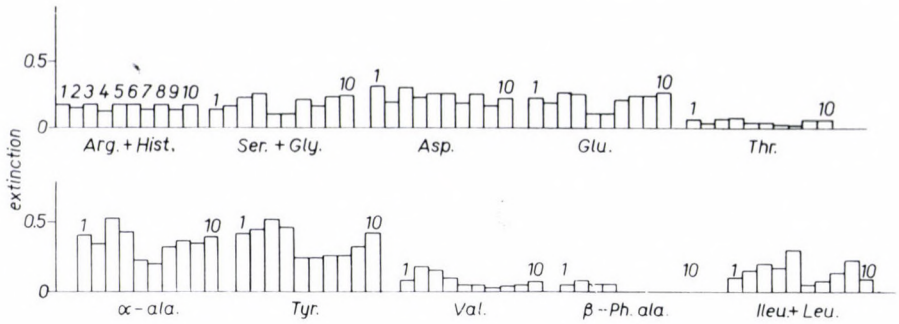

Fig. 8. Adult stage

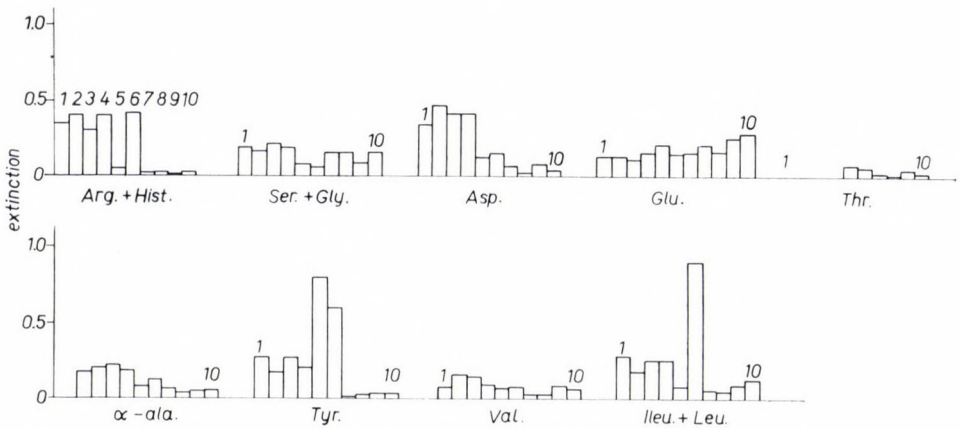

Fig. 9. Pupal stage

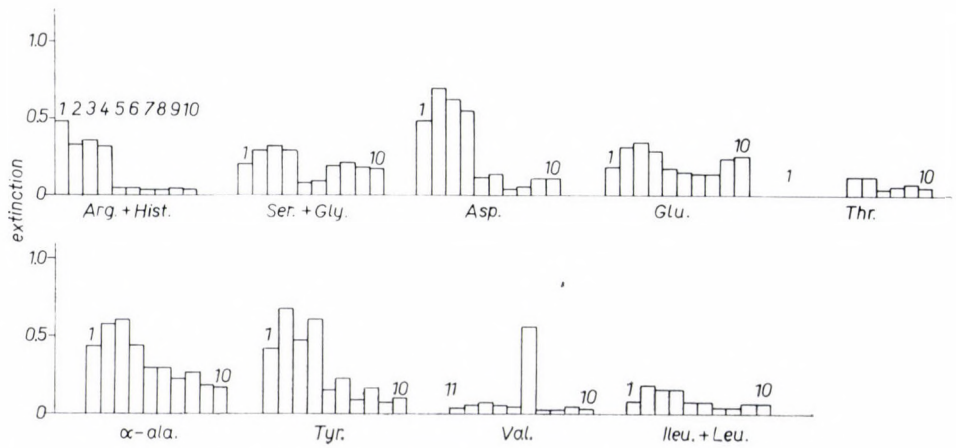

Fig. 10. 120 hours old larvae

Figs. 8—10. The quantity of the different free amino acids of following genotpyes of *Drosophila melanogaster:* 1 — *Oregon-R*, 2 — *w*, 3 — *B*, 4 — *wB*, 5 — w^+/w, 6 — $+^B/B$, 7 — $w+^B/w^+B$, 8 — $w^+ +^B/wB$, 9 — $w+^B/wB$, 10 — w^+B/wB

Table 4

Extinctions of cupric complex of free amino acids of heterozygotes at S_{510} expressed in α-alanine

Mark of genotype	w^+/w	$+^B/B$	$w+^B/w^+B$	$w^+ +^B/wB$	$w+^B/wB$	w^+B/wB
larval stage						
Arg. + His.	0.03	0.03	0.02	0.02	0.04	0.01
Ser. + Gly.	0.08	0.09	0.18	0.20	0.18	0.17
Asp.	0.11	0.13	0.03	0.05	0.10	0.10
Glu.	0.16	0.14	0.13	0.13	0.24	0.25
Thr.	0.11	0.11	0.04	0.05	0.06	0.05
α-ala.	0.28	0.28	0.22	0.26	0.17	0.16
Tyr.	0.15	0.22	0.09	0.16	0.07	0.10
Val.	0.05	0.56	0.02	0.02	0.03	0.03
β-Ph. ala.	—	—	—	—	—	—
Leu. + Ileu.	0.06	0.06	0.03	0.02	0.05	0.05
Total ext.	1.03	1.62	0.76	0.91	0.94	0.92
pupal stage						
Arg. + His.	0.04	0.04	0.02	0.02	0.02	0.02
Ser. + Gly.	0.08	0.07	0.13	0.09	0.19	0.16
Asp.	0.13	0.15	0.07	0.02	0.09	0.05
Glu.	0.20	0.15	0.20	0.16	0.25	0.27
Thr.	0.07	0.06	0.02	0.01	0.05	0.02
α-ala.	0.08	0.12	0.06	0.04	0.10	0.10
Tyr.	0.79	0.59	0.01	0.02	0.03	0.03
Val.	0.06	0.07	0.02	0.02	0.08	0.06
β-Ph. ala.	—	—	—	—	—	—
Leu. + Ileu.	0.07	0.89	0.05	0.05	0.09	0.13
Total ext.	1.52	2.14	0.58	0.43	0.90	0.84
$\frac{♀+♂}{2}$ adult stage						
Arg. + His.	0.15	0.16	0.13	0.16	0.13	0.16
Ser. + Gly.	0.10	0.10	0.20	0.16	0.23	0.24
Asp.	0.25	0.25	0.18	0.24	0.16	0.21
Glu.	0.10	0.10	0.20	0.22	0.22	0.25
Thr.	0.03	0.03	0.02	0.02	0.05	0.05
α-ala.	0.22	0.20	0.31	0.36	0.35	0.39
Tyr.	0.25	0.23	0.26	0.26	0.33	0.43
Val.	0.05	0.05	0.03	0.04	0.05	0.07
β-Ph. ala.	—	—	—	—	—	—
Leu. + Ileu.	0.30	0.05	0.07	0.12	0.22	0.09
Total ext.	1.45	1.17	1.40	1.58	1.74	1.89

Remark: Values of homozygote stocks in reference No 17

centration. In the pupal stage the high concentration in the heterozygote w^+/w is equally remarkable (Fig. 11).

As a general conclusion it may be stated that the free amino acid levels in homozygotes are superior to those in heterozygotes, whereas the opposite is valid for the protein amino acid levels.

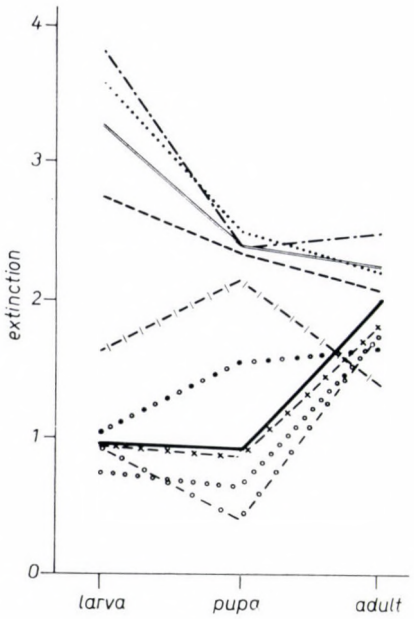

Fig. 11. Change in total quantity of free amino acids in the course of ontogenesis. Total extinctions. — — — — = *Oregon-R*, · · · · · = *w*, — · — · — = *B*, ═══ = *wB*, ○ ● ○ ● ○ = w^+/w, — | -- | — = + $^B/B$, ○ ○ ○ ○ ○ = $w+\ ^B/w^+B$, — ○ — ○ — = $w^+ +\ ^B/wB$, —— = $w\ +\ ^B/wB$, — × — × — × — × = w^+B/wB

Discussion

In many works the physiological and biochemical investigation of the protein metabolism of *Drosophila melanogaster* MEIGEN includes an analysis of the relationship existing between genetical basis and protein synthesis. Accordingly, the free and protein amino acid contents of wild and mutant stocks as well as some of their proteins including enzyme proteins, are examined.

The examinations on *Drosophila* mutants possessing lethal factors furnish new evidence of the relationship between genotype and protein metabolism. In this respect, SCHMID [15] was the first to demonstrate on the mutant lethal-meander *(lme)* that the corresponding mutation manifests itself in a

metabolic disturbance of the protoplasm. In the haemolymph and the extract of larvae of the *lme* homozygote CHEN and HADORN [5] found an abnormally large quantity of glycine, whereas valine, leucine, isoleucine, histidine, arginine and lysine were completely absent and threonine could not be found but in traces. CHEN [3] found a strongly reduced protein synthesis in the larval haemolymph of the *lethal-translucida* mutant. He also demonstrated a very high concentration of free amino acids in the haemolymph, although the development of the larvae had slowed down. From this he concluded on a primary or secondary disturbance of protein metabolism. According to GLOOR [8] *lethal factor No 52* modifies the concentration of free amino acids, while *lethal factor No 8* substantially increases that of free amino acids and peptides, but the various components increases to unequal degrees.

In HCl-hydrolyzed larval haemolymph proteins of the wild stock *Sevelen* CHEN [3] found the following amino acids: arginine, lysine, histidine, serine, glycine, aspartic acid, glutamic acid, threonine, α- and β-alanine, tyrosine, proline, valine, leucine, ornithine and β-phenylalanine. In the mutant stocks we examined previously [16, 17] and in the heterozygotes analyzed actually we failed to detect lysine and ornithine, but found the amines asparagine and glutamine, and the amino acids tryptophane and isoleucine. The differences may be due to the fact that CHEN employed an acid hydrolysis, while we used the alkaline technique. Alkaline hydrolysis has its disadvantages, namely certain amino acids are decomposed or transformed thereby, but it has the advantage of not damaging cyclic amino acids. Accordingly, the low concentration of threonine observed in our tests may be attributed to the alkaline hydrolysis, transforming threonine into glycine and aminobutyric acid.

The relationship between the changes in protein contents and those in nucleic acid contents has been examined under various circumstances. As regards the relationship between DNA synthesis and weight increase in *Drosophila melanogaster*, NIGON and DAILLE [14] have pointed out that, due to the onset of the activity of imaginal discs, the allometric relationship becomes discontinuous by the end of larval stage. Differences in the quantity of DNA were also found between the sexes within stock. LANG and co-workers [11] followed the protein and nucleic acid contents of *Aëdes aegypti* in the course of ontogenesis. They found that its body weight, as well as protein, RNA and DNA contents reach their maximum at the larval age. The peak of both RNA and DNA was reached on the third day of the larval age, while protein and body weight had their peak on its sixth day. The relationship between protein synthesis and the presence or absence of ribosomes was examined by BARTH and co-workers [1] in larvae of *Anthera perny*, of the *Lepidopterae*.

Examining the relationship between protein and age, CHEN [4] demonstrated that the protein contents of the haemolymphs of *Drosophila* and *Culex*

larvae increase with age. In 120 hours old larvae of *Oregon-R wild*, *white*, *Bar* and *white-Bar* mutants of *Drosophila melanogaster* SZABÓ, PUSKÁS and BARTALIS [17] have also found a quantitative increase of protein amino acids, as compared with their concentration in the 84 hours old larvae. Depending on the genotype, a temporary decrease of protein contents can be observed at the beginning of the pupal stage, with the exception of the *Oregon-R* stock presenting at the same time a vigorous increase. The larval age of 120 hours of the *Drosophila melanogaster* heterozygotes under study is characterized by a very high concentration of protein amino acids, which significantly declines in the pupal stage. Histolysis alone cannot account for the decrease of the high protein concentration of the initial pupal stage, since the concentration of free amino acids shows a parallel decline. In this stage considerable quantity of amino acids is probably utilized for respiration.

There are but few experimental data available on the protein metabolism of *Drosophila melanogaster* heterozygotes. Several proteins and enzymes have been analyzed electrophoretically and attempts have been made to localize the genes responsible for enzyme production. The nonspecific enzyme esterase 6 (= Est 6) localized by WRIGHT [18] in chromosome III of *Drosophila melanogaster*, has proved to be polymorphous. Some stocks contain Est 6^F, others Est 6^S and again others both of them. BECKMAN and JOHNSON [2] found isoenzymes in the larval alkaline phosphatase and amino peptidase of *Drosophila*. Examining their hereditary transmission, they have found the genes controlling the enzyme to be localized in chromosome III. Examining the protein mutant of *Drosophila melanogaster* discovered by means of zymograms and genetical analysis, HUBBY [10] found that the electrophoretic mobility of the two dominant multiplex alleles — $Pt_1^{1.01}$ and $Pt_1^{1.13}$ — of the gene localized in chromosome III varies according to the numbers above, and that the heterozygote contains both parental proteins, due to codominance. According to BECKMAN and JOHNSON [2] the codominant pair of alleles localized in chromoseme III controls the synthesis of the iso-enzymes in the leucine-aminopeptidase (LAP) of *Drosophila*.

As regard the conditions of dominance prevailing in the general course of protein metabolism, we have found that, in heterozygotes, the *Oregon-R wild* stock is able to assert its dominance against the *white* gene in the pupal and adult stages, whereas in larval stage the *white* effect is manifest in the heterozygote w^+/w.

We are unable to prove unequivocally the definite effect of parental genotypes in progeny, since an increase of the order of magnitude can be found in the protein amino acid contents of every heterozygote, as compared with the parent stocks. The increase of protein amino acid value can be considered as heterosis, surpassing even the parent stocks of relatively high value. Major heterosis is probably caused by stocks that are not equivalent homozygotes

any more — as compared to one another — in regard to the information of morphologically invisible physiological and biochemical markers. This, however, cannot be decided but by experimental hybridation.

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DIE VERÄNDERUNGEN DER IN EIWEIß ENTHALTENEN UND FREIEN AMINOSÄUREN IM VERLAUF DER ONTOGENESE DER VERSCHIEDENEN HETEROZYGOTEN VON *DROSOPHILA MELANOGASTER* MEIGEN

In den verschiedenen Stadien der Ontogenese der Eltern-Stämme *Oregon-R wild*, *white*, *Bar* und *white-Bar* von *Drosophila melanogaster* MEIGEN sowie ihrer heterozygoten Kombinationen wurden die freien und im Eiweiß vorkommenden Aminosäuren analysiert und festgestellt, daß (1) in den verschiedenen Stadien der Ontogenese die Unterschiede zwischen den einzelnen Genotypen im *Larven*stadium am augenfälligsten sind; (2) für die *einzelnen Genotypen*

ist die Richtung der in den Proteinaminosäuren ablaufenden Konzentrationsänderungen *charakteristisch*. Diese Veränderungen zeigen sowohl in den reinen Linien als auch im Falle ihrer verschiedenen heterozygoten Kombinationen — von Abweichungen in ihrer Größenordnung abgesehen — im großen und ganzen die gleiche Richtung. Vom Stamme *Oregon-R wild* weichen sämtliche untersuchten Genotypen ab. (3). Der Stamm *Oregon-R wild* vermag seine Dominanz nur gegenüber dem Genotyp *white* zur Geltung zu bringen. Im *Puppen-* und *Adulten*-Stadium des heterozygoten Genotyps w^+/w dominiert *Oregon-R*, während im *Larven*stadium des gleichen Heterozygoten der Genotyp *white* vorherrscht. (4) *Die Heterosiswirkung ist für sämtliche Heterozygoten kennzeichnend.*

ИЗМЕНЕНИЯ ПРОТЕИНОВ И СВОБОДНЫХ АМИНОКИСЛОТ В ПРОЦЕССЕ ОНТОГЕНЕЗА РАЗЛИЧНЫХ ГЕТЕРОЗИГОТОВ *DROSOPHILA MELANOGASTER* MEIGEN

При анализе свободных и белковых аминокислот, исследовавшихся в различных стадиях онтогенеза родительских штаммов *Oregon-R wild* (дикий), *white* и *white-Bar Drosophila melanogaster* MEIGEN, а также их гетерозиготных комбинаций было установлено, что (1) в различных стадиях онтогенеза наиболее поразительные отклонения отдельных генотипов наблюдаются в *личиночной* стадии; (2) для отдельных *генотипов характерно* направление изменения белковых аминокислот. Эти изменения показывают, как в чистых линиях, так и в случае различных гетерозиготных комбинаций, в общем и целом одинаковое направление, несмотря на различия порядка величин. Все исследовавшиеся генотипы отклоняются от дикого штамма *Oregon-R*. (3) Господство штамма *Oregon-R* проявляется лишь в отношении генотипа *white*. В *кукольном* и *взрослом* состояниях гетерозиготного генотипа w^+/w господствует генотип *Oregon-R*, в то время как в *личиночной* стадии этого же генотипа господствует генотип *white*. (4) *Явление гетерозиса характерно для всех гетерозиготов.*

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THE EFFECT OF NIGHT TEMPERATURE ON GERMINATION, CARBOHYDRATE AND NITROGEN METABOLISM OF BEAN (VICIA FABA) SEEDLINGS

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Synopsis

The rate of germination of *Vicia faba* increased with the rise in night temperature. The dry weight of the seedlings decreased at a rate which increased with rising night temperature. The total nitrogen contents of the seedlings decreased, due to the leaching of soluble nitrogenous compounds, especially when higher night temperatures were applied. The soluble nitrogen contents of the seedlings increased with the lapse of time at a rate directly proportional with the night temperature. The soluble sugars (reducing sugars and sucrose) increased, at first slowly, then rapidly, and declined on the last three days of the experiment. The polysaccharide contents decreased with the lapse of time at a rate increasing with a rise in night temperature.

Introduction

Numerous investigators have shown that germination of seeds requires in some cases temperature fluctuations in which the time factor does not seem to be critical [6, 17, 28]. It has been observed that night temperature has an effect on the seedling's growth. Thus, the growth of tomato seedlings was retarded by reduction of night temperature to 10°C and this retardation was the most pronounced when the seedlings were young [4]. Loblolly pine seedlings showed the best growth when the difference between day and night temperatures (12—13°C) was the greatest, and poorest growth when nights were as warm as days [23].

The reserve food material of seeds subjected to germination undergoes various temperature-dependent biochemical processes [27]. It was found that during the germination of soybean, one third of the protein and nearly all sugar had been transferred to the seedlings [25]. With the progress of germination of pinus seeds, most of the nitrogen moved from the endosperm to the embryo but its decrease in the entire seed was little during the course of germination [15]. BLACK [2], dealing with subterranean clover, found that the rate of transfer of materials from the cotyledons to the rest of the plant increased with increasing temperature. On the other hand, LAWRENCE and co-workers [24], on studying nitrogen metabolism in germinating seeds, concluded that reserve protein is hydrolyzed and the amino acids so produced are presumably transferred to regions of active synthesis in the seedling, where they are used for the synthesis of new protein.

The transport of carbohydrates from storage organs, such as, the endosperm or cotyledons, to the growing part of the embryo has been shown by some workers [14, 26]. In some cases polysaccharides, in others mono-, di-, or trisaccharides are metabolized first. In *Vigna sesquipedalis*, starch and dextrins as well as soluble sugars appear in the hypocotyl [26]. BOND and GLASS [3], studying sugars of germinated corn seeds, found that glucose and sucrose comprise the largest part of the sugars studied, and that the glucose content remained practically constant for 48 hours then increased rapidly, but fructose remained at a low and approximately constant level.

Methods

For this experiment, uniform-sized seeds of a hybrid (Giza "2") bean *(Vicia faba)* were soaked in tap water in the dark for 24 hours at room temperature, selected for uniformity by choosing those between 1 and 3 mm length of sprouted radicle. These uniform germinated seeds were then planted (15 seeds per pot) at a constant depth of about 3 cm in uniformly washed sand supplied with tap water, the pots were transferred to air-conditioned cabinets adjusted at 5°, 10°, 15° and 20°C as night temperatures, and 20°C day temperature. Light supply inside these chambers is fulfilled by means of fluorescent lamps, each chamber being illuminated by 40 watt day-light lamps. The maximum light intensity measured at the centre of ach chamber was 5,000—6,000 Lux. The length of both dark and light periods was kept constant, being 12 hours each. Watering was carried out regularly every two days, using tap water (100 ml for each pot). Samples were taken at daily intervals till the first leaf was fully expanded. For every sample, three replicates each consisting of 15 seedlings and representing each replicate were taken. After washing the seedlings to free them from sand particles, the seed coat was removed, the length of radicle (from the seed to the root tip), length of plumule (from the seed to the terminal bud), total dry weight determinations, and nitrogen and carbohydrate contents of the whole seedlings were determined.

The methods used for carbohydrate determinations have been published in previous papers [30, 31]. Nitrogen determinations were carried out according to the methods discussed by SAID and EL-SHISHIMY [32].

Confidence intervals (L_1 and L_2) with 95% confidence coefficient were calculated for the true means of radicle and plumule lengths, while for dry weight and chemical analysis of the seedlings, estimates of regression coefficient of each item in the period of the experiment (number of days) were calculated and confidence intervals were constructed for the true regression coefficient (slopes) with 95% confidence coefficient.

Results

Rate of germination: Table 1 shows the number of days required by the seedlings at different night temperatures to reach the first leaf-stage.

Table 1

Days required by the seedlings at different night temperatures to reach the first leaf stage

Night temp.	5°C	10°C	15°C	20°C
No. of days	14	12	10	8

It is clear from Table 1 that night temperature affects the rate of seed germination. The lower the night temperature, the longer the period taken by the seedlings to reach the first-leaf stage. Thus, whereas the germination of seeds at 5°C night temperature took 14 days till the first leaf was fully expanded, germination of the seeds at 20°/20°C day/night temperature took only 8 days.

Table 2

Average values of radicle lengths of Vicia faba seedlings as influenced by the night temperature (measured in cm)

Days	Night temperature							
	5° C				10° C			
	Mean	L_1	L_2	s^2	Mean	L_1	L_2	s^2
1	0.35	0.39	0.31	0.01	0.41	0.45	0.37	0.01
2	1.06	1.11	1.01	0.02	1.15	1.20	1.10	0.03
3	1.49	1.56	1.42	0.05	1.63	1.71	1.56	0.07
4	2.28	2.40	2.16	0.17	2.95	3.09	2.81	0.22
5	3.78	3.94	3.62	0.28	4.64	4.82	4.46	0.37
6	5.51	5.69	5.33	0.36	7.23	7.51	6.95	0.85
7	7.08	7.34	6.82	0.75	8.83	9.12	8.54	0.96
8	8.74	9.04	8.44	0.98	9.90	10.26	9.54	1.42
9	10.92	11.34	10.50	1.95	11.30	11.75	10.85	2.25
10	11.44	11.95	10.93	2.91	12.14	12.67	11.61	3.14
11	12.80	13.36	12.24	3.50	14.98	15.57	14.39	3.85
12	13.05	13.64	12.46	3.87	15.20	15.82	14.58	4.27
13	14.15	14.75	13.55	3.93				
14	14.99	15.60	14.38	4.08				
	15° C				20° C			
1	0.58	0.61	0.55	0.01	0.71	0.75	0.67	0.01
2	1.88	1.95	1.81	0.06	2.05	2.15	1.95	0.10
3	3.67	3.81	3.53	0.20	4.02	4.17	3.87	0.26
4	4.91	5.09	4.73	0.36	7.76	7.99	7.53	0.57
5	7.91	8.19	7.63	0.84	9.98	10.37	9.59	1.71
6	9.26	9.62	8.90	1.42	12.15	12.71	11.59	3.44
7	11.10	11.54	10.66	2.13	14.90	15.56	14.24	4.78
8	13.36	13.88	12.84	3.03	17.03	17.83	16.23	7.04
9	14.60	15.23	13.97	4.41				
10	16.15	16.89	15.41	6.08				

s^2 = Variances between lengths

Radicle and plumule lengths: It is clear from Tables 2 and 3 that the lenghts of radicles and plumules increased gradually during the period of experiment. The rate of increase was directly proportional to the rise in night temperature. Statistical analysis proves that no overlappings in radicle length exist from one day to the other for the same night temperature, indicating that the length of radicles at any two successive days differ significantly. The

Table 3

Average values of plumule lengths of Vicia faba seedlings as influenced by the night temperature (measured in cm)

Days	Night temperature							
	5°C				10°C			
	Mean	L_1	L_2	s^2	Mean	L_1	L_2	s^2
1	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—
5	0.36	0.43	0.29	0.05	0.61	0.68	0.54	0.05
6	1.04	1.13	0.95	0.09	1.77	1.87	1.67	0.12
7	1.09	1.20	0.98	0.14	2.28	2.38	2.18	0.21
8	2.15	2.29	2.01	0.22	2.80	2.99	2.61	0.24
9	2.54	2.70	2.38	0.30	3.43	3.60	3.26	0.31
10	3.62	3.80	3.44	0.36	3.90	4.11	3.69	0.49
11	3.93	4.14	3.72	0.50	4.41	4.65	4.17	0.63
12	4.80	5.05	4.55	0.70	6.49	6.76	6.22	0.82
13	5.11	5.37	4.85	0.74				
14	5.94	6.21	5.67	0.79				
	15°C				20°C			
1	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—
3	0.44	0.50	0.38	0.03	0.60	0.68	0.52	0.08
4	0.95	1.03	0.87	0.07	2.03	2.18	1.88	0.25
5	2.23	2.36	2.10	0.18	3.66	3.89	3.43	0.60
6	2.84	2.99	2.69	0.25	6.42	6.74	6.10	1.15
7	3.97	4.15	3.79	0.35	7.53	7.91	7.15	1.60
8	5.01	5.22	4.80	0.50	8.44	8.39	7.99	2.21
9	5.87	6.12	5.62	0.70				
10	7.01	7.36	6.66	1.37				

— = Plumule not sprouted

values of the variance between the radicle's length increases vertically with days, and horizontally with rise in night temperature. Thus, while the values of variances for the first day of experiment were nearly the same, yet as the experiment proceeded, the behaviour of radicle length proved to be influenced by the night temperature applied.

Although the radicle appeared from the first day of the experiment, yet a certain period of time after planting has to pass before the plumule sprouts from the cotyledons. It is clear from Table 3 that whereas at 5° and 10°C night temperature treatments the plumule sprouted after 4 days, it sprouted as soon as after 2 days at the higher night temperatures 15° and 20°C. At the 1st leaf stage, the plumule reached its maximum length at 20°C night temperature. Compared with other night temperatures, this temperature is the optimum for plumule elongation at this stage of growth.

Statistical analysis of plumule lengths shows some overlappings during the period of experiment at 5°C night temperature only. The first overlapping occurs on the 2nd or 3rd day from the appearance of plumule, the 2nd overlapping occurs between the 10th and 11th day from the day of sowing, and the last overlapping occurs between the 12th and 13th day of germination. All these overlappings mean that no significant difference in the plumule length is found between each two successive days during which the overlapping occurs. When comparing the limits of plumule length at the last day of experiment, we find that they are directly proportional to the rise in night temperature. There is an overlapping between the plumule length at night temperatures of 5° and 10°C only, indicating that the increase in plumule length at the 2nd night temperature is not significant. The variances for plumule length follow the same behaviour of the variances for the radicles but they are comparatively smaller.

Dry weight: There was a continuous decrease in the seedlings' dry weight during the period of experiment, irrespective of the applied night temperature. This decrease was mainly due to the utilization of the reserve food material in the vital processes, especially in the process of respiration.

Statistical analysis of the data and the values of the slopes of the lines illustrated in Table 4 show that both the rate and the loss of dry weight increased with the rise in night temperature, gradually, from 5° to 20°C.

At 5°C night temperature, the period of decrease in the seedlings' dry weight took 13 days. The decrease was somewhat less pronounced during the first four days, then it grew during the next seven days, and declined again during the 12th and 13th days. On the last day of the experiment, an increase in the dry weight of seedlings was observed.

At 10°C night temperature, the loss of the seedlings' dry weight was of low value during the first two days, it became more pronounced during the next seven days, and it decreased further during the 10th and 11th days. At the

Table 4

Average dry weights of Vicia faba seedlings as influenced by the night temperature
(g/100 seedlings)

Days	Night temperature			
	5°C	10°C	15°C	20°C
1	65.65	65.49	64.71	64.35
2	65.30	64.68	63.27	62.14
3	64.76	63.73	62.14	60.11
4	64.07	62.61	60.33	57.47
5	63.19	61.42	58.77	55.92
6	62.25	60.07	57.60	54.50
7	61.14	59.05	56.93	53.26
8	59.85	58.00	56.10	55.02
9	58.51	57.08	55.75	
10	57.67	56.30	56.90	
11	56.56	55.90		
12	56.04	56.70		
13	55.75			
14	56.30			

Night temp.	5°C	10°C	15°C	20°C
Slope	— 0.8742	— 0.9323	— 0.9970	— 1.5249
95% confid. limits	± 0.0961	± 0.1346	± 0.2770	± 0.5398

last day, there was an increase in the dry weight of the whole seedlings, the amount of which was higher than that observed at 5°C night temperature treatment.

On the other hand, germination of bean seeds at 15°C night temperature showed more decrease in the dry weight of bean seedlings during the first six days. Then the rate of decrease became lower during the next three days. At the 10th day (last day of experiment) there was as usual an increase in the dry weight of the whole seedlings.

When 20°C night temperature was applied, the decline in the dry weight of the seedlings was greater during the first four days than on the next three days. At the end of the experiment, there was an increase in the seedlings' dry weight.

It is interesting to note here that the increase in the seedlings' dry weight observed on the last day of experiment was the more pronounced the higher the night temperature was, therefore, 20°C night temperature seems to be the optimum for the growth of bean seedlings at this stage.

Chemical analysis of seedlings. Total nitrogen contents: It is clear from Table 5 that there was a decrease in the amount of total N per seedling with the lapse of time. The same was observed for all night temperatures. The data show also that the loss of total N content increased as the fluctuation in temperature decreased. Thus, while germinating bean seeds at a night temperature of 5°C resulted in a loss of total N content of about 5.6%, germination of seeds at higher night temperatures gave the following percentual losses: 6.9%, 8.0% and 12.3% for night temperatures of 10°, 15° and 20°C, respectively.

Table 5

Average values of total-nitrogen content of Vicia faba seedlings as influenced by the night temperature
(mg nitrogen/one seedling)

Days	Night temperature			
	5°C	10°C	15°C	20°C
1	34.92	34.71	34.48	34.17
2	34.76	34.54	34.24	33.84
3	34.52	34.33	33.97	33.39
4	34.30	34.01	33.57	32.81
5	34.13	33.79	33.24	32.24
6	34.00	33.63	32.85	31.82
7	33.91	33.32	32.68	31.22
8	33.82	33.18	32.58	30.92
9	33.74	33.13	32.48	
10	33.67	33.06	32.38	
11	33.57	32.93		
12	33.50	32.85		
13	33.43			
14	33.34			

Night temp.	5°C	10°C	15°C	20°C
Slope	− 0.1160	− 0.1762	− 0.2499	− 0.4896
95% confid. limits	± 0.0157	± 0.0080	± 0.0141	± 0.0338

Statistical analysis of the data reveals that the decrease in the total N increased with rising night temperature.

Behaviour of total soluble and protein nitrogen: Figure 1 reveals that the soluble N content increased as the seedling grew. The same tendency was observed for all night temperatures tested. The rate of increase in the soluble N contents increased with rising night temperature.

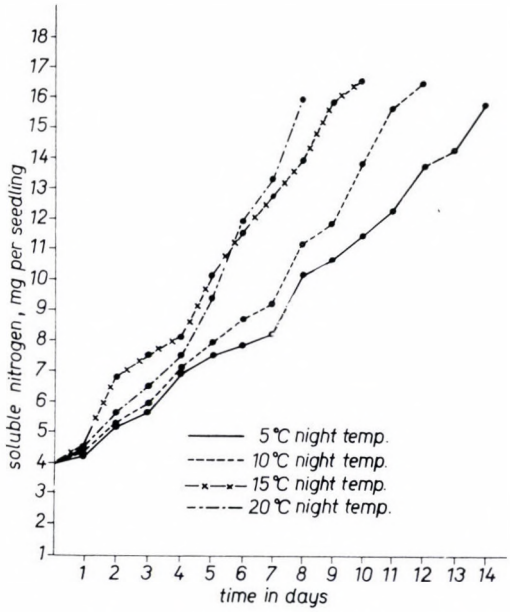

Fig. 1. The effect of different night temperature on the soluble nitrogen content of *Vicia faba* seedlings

It is interesting to note here that during the first five days of germination, the increase in the soluble N contents of bean seedlings germinated at 15°C night temperature was the highest. After six days the rate of increase was somewhat slower at 15°C than at 20°C night temperature.

Statistical analysis of the results of soluble N during the period of the experiment shows that the slope of the curve increases with rising night temperature.

The analysis of bean seedlings for their protein N contents (Table 6) revealed that there was a gradual decrease in the amounts of protein N with the lapse of time. Moreover, it is clear that higher night temperatures resulted in a more pronounced decreases protein N than lower ones.

Statistical analysis of the data shows that such decreases caused by increase in night temperature are significant.

Table 6

Average values of protein-nitrogen contents of Vicia faba seedlings as influenced by the night temperature (mg nitrogen/one seedling)

Days	Night temperature			
	5°C	10°C	15°C	20°C
1	30.69	30.29	29.85	29.66
2	29.60	29.20	27.40	28.27
3	28.88	28.47	26.43	26.90
4	27.39	27.11	25.25	25.28
5	26.60	25.92	23.17	22.83
6	26.22	24.92	21.31	19.88
7	25.58	24.11	19.99	17.93
8	23.37	22.06	18.68	15.02
9	22.27	21.20	16.65	
10	22.22	19.22	15.91	
11	21.37	17.32		
12	19.84	16.44		
13	19.27			
14	17.64			

Night temp.	5°C	10°C	15°C	20°C
Slope	— 0.9698	— 1.2796	— 1.5631	— 2.1154
95% confid. limits	± 0.0538	± 0.0956	± 0.1075	± 0.3215

Carbohydrate contents. Reducing sugars: It is clear from Table 7 that there was a continuous and regular increase in the glucose content of the seedlings during the first days of germination. The rate of increase was directly proportional to the night temperatures used. In the last three days of germination, a decrease in the glucose content of the seedlings was observed for all night temperatures under test. The rate of such decrease increased with rising night temperature.

Statistical analysis reveals that the changes in reducing sugar contents are significant.

Sucrose content: The data in Table 8 reveal that growing bean seedlings at 5°C night temperature resulted in a slower rate of increase in the sucrose content for the first eight days, then it increased suddenly during the next two days to reach its maximum amount. After the tenth day, and till the full

Table 7

Average values of glucose content of Vicia faba seedlings as influenced by the night temperature (mg glucose/one seedling)

Days	Night temperature			
	5°C	10°C	15°C	20°C
1	0.50	0.58	0.81	0.86
2	0.78	0.99	1.31	1.40
3	1.02	1.22	1.79	1.96
4	1.44	1.80	2.36	2.72
5	1.49	1.98	2.79	3.51
6	1.61	2.16	3.29	4.37
7	1.92	2.35	3.82	4.12
8	2.13	2.75	4.16	3.50
9	2.34	3.23	3.97	
10	2.65	3.44	3.76	
11	2.95	3.16		
12	3.23	2.95		
13	3.06			
14	2.96			

Night temp.	5°C	10°C	15°C	20°C
Slope	+ 0.2104	+ 0.2475	+ 0.3752	+ 0.4774
95% confid. limits	± 0.0257	± 1.0559	± 0.0985	± 0.2215

expansion of the first leaf-stage (after 14 days from planting) there was a decrease in the sucrose content of the seedlings.

The same tendency was found for the other night temperatures used, with the exception that there was a decrease in the period taken for maximum increase in the sucrose content with the increase in night temperature.

Statistical analysis shows that the increases in sucrose content as a result of rise of night temperature are significant. The sucrose content of the seedlings was in direct relation to night temperature.

Polysaccharide contents: It is clear from Fig. 2 that the polysaccharide contents decreased with the lapse of time.

At 5°C the rate of decrease was more or less low during the first three days, then slightly increased during the next nine days and reached its maximum during the last two days.

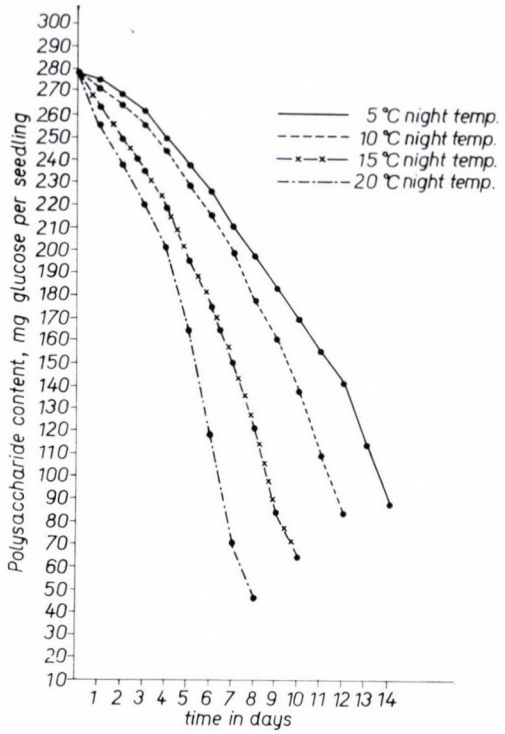

Fig. 2. The effect of different night temperature on the polysaccharide content of *Vicia faba* seedlings

At 10°C night temperature the rate of decrease was relatively low though higher than at 5°C, during the first three days; then it increased slightly for five days. The rate of decrease reached its maximum during the last four days.

At 15°C and 20°C night temperatures the decrease in the polysaccharide contents was of higher rate than at the lower night temperatures; the greatest decrease was observed at 20°/20°C day/night temperature, i.e., in the case of constant temperature.

Statistical analysis of the data shows a direct relation between the slope of the lines and the night temperature from 5°C to 20°C.

Discussion

Effects of night temperature on germination: Numerous investigators have shown that there is an almost linear relationship between temperature and germination of seeds [9, 11, 20, 21, 30].

The results of this experiment clearly show that the germination of bean seeds was remarkably affected by the night temperature applied. Thus, al-

Table 8

Average values of sucrose content of Vicia faba seedlings as influenced by the night temperature (mg glucose/one seedling)

Days	Night temperature			
	5°C	10°C	15°C	20°C
1	13.95	14.50	14.86	15.70
2	14.22	14.87	15.46	17.01
3	14.80	15.57	16.63	21.16
4	15.19	16.56	19.20	23.17
5	15.13	17.40	22.24	28.92
6	15.64	18.71	23.80	27.59
7	16.00	19.20	26.30	24.63
8	16.62	21.23	25.49	21.88
9	18.43	20.82	23.37	
10	20.14	19.54	21.13	
11	19.93	18.42		
12	19.04	17.49		
13	18.33			
14	17.42			
Night temp.	5°C	10°C	15°C	20°C
Slope	+0.4301	+0.4402	+1.0864	+1.2667
95% confid. limits	±0.1639	±0.2972	±0.6759	±1.4067

though bean seeds germinated at all temperature treatments, yet higher night temperatures showed an accelerating effect on the rate of germination. The results have shown that the lower the night temperature, the longer the period taken for the full expansion of the first leaf. KOLLER and co-workers [22] attributed the differential speed of germination to the rate of cell division and to the rate of metabolic processes.

For all night temperature treatments, radicle elongation exceeded plumule elongation in the early days of germination. Moreover, higher night temperatures induced a faster rate of growth for both radicle and plumule of bean seedlings. The length of radicle as well as that of plumule reached its maximum value at the highest night temperature used.

It is worthwhile here to note that TOOLE and co-workers [35] reported that an alteration from a temperature near the optimal constant value to some higher value, which might be above the maximum, is most effective in promoting seed germination.

As to the dry weight of seedlings, the results obtained indicate that there was a gradual decrease in the seedlings' dry weight. The decrease tended to go faster during the first days of germination but slowed down afterwards. The loss was found to be directly proportional to the night temperature. High night temperature resulted in more pronounced decrease in the dry weight.

This decrease, however, is naturally expected, since the respiration of growing cells of the seedlings utilizes the reserve food material to obtain energy required for vital processes [12, 13]. This result is in agreement with the findings of DECKER [8], who found that the rate of respiration of two species of pine seedlings was doubled when the temperature was increased.

The initial rise in dry weight of the seedlings observed in the last day of germination for all treatments may be due to a hypocotyl growth, and as increased ratio of photosynthesis, exhibited by prophylls and first leaf, to respiration.

A gain in the dry weight of pea-nut seedlings after an initial loss due to secondary growth of the hypocotyl was reported by YARBROUGH [36]. Moreover, DECKER [8], studying the effect of temperature on the photosynthesis and respiration of pine seedlings, reported a proportional decrease in the ratio of photosynthesis to respiration upon increase of temperature.

Changes in nitrogen and carbohydrate contents during germination: It was found that the total-nitrogen content of the seedlings decreased at a low rate throughout the experimental period. The decrease was found to be directly proportional with night temperature. Such decrease in nitrogen content may be attributed to leaching of the soluble nitrogenous compounds from the seedlings. This conclusion adds more support to the results reported by INGLE and co-workers [18].

With regard to the changes in nitrogenous fractions, it has been found that, whereas the protein-nitrogen contents of seedlings decreased during the period of germination; the total soluble-nitrogen fraction increased. Such changes in nitrogenous contents of the seedlings are mainly due to the breakdown of the reserve proteins in the cotyledons to form soluble nitrogenous intermediates, which are then transported to the growing organs. Similar conclusions have been obtained by numerous investigators [5, 7, 22, 26].

The hydrolysis of proteins into soluble nonprotein nitrogenous compounds is mainly due to the strength of activity of proteases present in germinating seeds. In this connection it may be mentioned that TAZAWA and HIROKAWA [34] observed a rise in protease activity in the cotyledons of soybeans in the early stages of germination.

Moreover, DILOV and co-workers [10], studying the metabolism of organic matter in barley at different temperatures, found that the change in temperature affects only the rate of conversion of the substances, and does not cause qualitative changes in the metabolism.

As regard carbohydrates, it is found that germination of bean seeds involves the hydrolysis of reserve carbohydrates (polysaccharides) into simpler soluble sugars. Thus, whereas the amounts of polysaccharides decreased during the period of germination, the amounts of reducing sugars and sucrose increased. It is evident, however, from the results of this experiment that the rate of hydrolysis was dependent upon the night temperature. Such differences are mainly due to the activity of α- and β-analyses, phosphorylase, and catalase enzymes [16, 22, 33]. In this connection, it may be mentioned that AUGSTEN [1] reported increases in catalase activity with the rise of temperature.

It is interesting to note here that high decrease of the total carbohydrate contents of the seedlings is mainly due to the utilization of the hydrolyzed sugars as substrate for respiration. The relatively smaller increases in the soluble sugars, especially sucrose, are attributed to its utilization in both respiration and cell wall formation [19].

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DIE WIRKUNG VERSCHIEDENER NACHTTEMPERATUREN AUF DIE KEIMUNG SOWIE DEN KOHLENHYDRAT- UND STICKSTOFFHAUSHALT DER BOHNENPFLÄNZLINGE *(VICIA FABA)*

Bei höheren Nachttemperaturen zeigt die Keimungsrate von *Vicia faba* einen Anstieg. Das Trockengewicht der Pflänzlinge vermindert sich. Das Ausmaß dieser Verminderung erhöht sich parallel dem nächtlichen Temperaturanstieg. Der Gesamtstickstoffgehalt der Pflänzlinge vermindert sich infolge der Auslaugung des löslichen Stickstoffgehaltes, vor allem bei höheren Nachttemperaturen. Mit der Zeit ließ sich in den Pflänzlingen ein Anstieg des Gehaltes an lösbarem Stickstoff nachweisen. Das Ausmaß dieses Anstiegs war der Nachttemperatur gerade proportional. Der Gehalt an lösbarem Zucker (reduzierendem Zucker und Sukrose) erhöhte sich anfangs langsam, dann schnell, um in den letzten drei Versuchstagen wieder abzunehmen. Der Polysacharidengehalt nahm mit der Zeit ab. Bei höheren Nachttemperaturen war das Ausmaß dieser Abnahme größer.

ДЕЙСТВИЕ РАЗЛИЧНЫХ НОЧНЫХ ТЕМПЕРАТУР НА ПРОРАСТАНИЕ СЕМЯН, НА УГЛЕВОДНЫЙ И АЗОТНЫЙ ОБМЕН БОБОВЫХ СЕЯНЦЕВ *(VICIA FABA)*

Прорастание семян *Vicia faba* повышается параллельно повышению ночной температуры. Сухой вес сеянцев уменьшается, размер уменьшения возрастает по мере повышения ночной температуры. Содержание общего азота в сеянцах уменьшается, в результате выщелачивания содержания растворимого азота, особенно при более высоких ночных температурах. С течением времени в сеянцах повышается содержание растворимого азота. Размер этого повышения прямо пропорционален высокой ночной температуре. Содержание растворимых сахаров (редуцирующий сахар и сукроза) повышается вначале медленно, затем быстро, а в три последние дни эксперимента оно вновь уменьшается. С течением времени содержание полисахаридов уменьшается. При повышении ночной температуры размер этого уменьшения возрастает.

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THE DEVELOPMENT OF THE INNERVATION AND THE MUSCULATURE OF WINGS INNERVATED BY THORACIC NERVES

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Synopsis

The brachial segments of the spinal cord of chicken embryos were replaced by thoracic segments, and the development of the wing musculature and its innervation was studied in 8- to 16-day-old embryos and in 4-week-old chickens. Wing muscles with thoracic innervation developed normally till the 10th day of incubation. From that time on the muscles gradually deteriorated, and by the 16th day the muscle tissue became replaced by adipose and connective tissue throughout the wing. Reduced silver technique and cholinesterase (ChE) methods revealed that the time of development of the primordia of neuromuscular endplates in the normal wing coincided with the time when the muscle started to deteriorate in the wings of the operated embryos. Following lesions of the 2nd brachial ventral root in chickens with thoracic spinal cord grafts, degenerated nerve fibers were found in the nerves of the wings. The findings indicate that wing muscles with thoracic innervation, despite the presence of motor fibers, degenerate in the same way as observed by others in the muscles in the nerveless limb grafts. For the normal differentiation and maintenance of muscles, specific innervation from the limb level of the spinal cord is required.

Introduction

A survey on the literature reveals that disturbances in the innervation deeply affect the development of the skeletal musculature. HAMBURGER [7] found a significant hypoplasia of limb muscles in the absence of a nerve supply in Anura. In the salamander extra limbs grafted into the thoracic region and innervated by thoracic segments of the cord can perform only small monophasic movements accompanying the trunk movements; besides a considerable reduction in the size of such limbs has been reported [3, 4, 12, 16].

The inappropriate innervation yielded a more pronounced effect on developing muscle tissue in experiments performed in chicken embryos. HUNT [10] in chorio-allantoic limb grafts, and HAMBURGER [8] in coelomic limb grafts, found an initial development of striated muscles, but after the 10th day of incubation the muscle fibers gradually degenerated into fat tissue in nerveless or weakly innervated grafts. From a series of experiments performed in chicken embryos, EASTLICK and WORTHAM [5, 6, 19] gave a detailed description of the destruction of the embryonic muscle tissue in coelomic limb grafts, which, having become attached to the mesentery or to the umbilical cord, received only vegetative innervation or none at all. Other grafts which were attached to the flanks of the hosts and became innervated by trunk or limb nerves, con-

tained well-differentiated muscle tissue even at the time of hatching. In contrast to the latter finding, SZÉKELY and SZENTÁGOTHAI [17] found no muscles after hatching in extra limbs which were grafted into the somites at the trunk level. These extra limbs were highly sensitive to mechanical stimuli. Although these authors [17] did not give a detailed account of the type of innervation, whether only sensory, or both sensory and motor nerves contributed to the innervation of the grafts, it could be assumed that in those large nerve trunks which entered the grafts, motor nerves might have been present as well. From this short and by no means complete survey on the literature, it seems that muscle tissue can develop under nerveless conditions, or with inappropriate innervation; however a specific nerve supply which arises from the limb segments of the cord is necessary to complete the differentiation and to maintain the muscle. This specificity, nevertheless, may be explained in a simple quantitative sense, assuming that thoracic segments give origin to many fewer motor fibers than brachial segments do and that because of its unfavourable position the grafted limb is penetrated only by such a small fraction of the motor fibers that the innervation is not sufficient for the maintenance of the muscles.

To study this problem, in the present work the brachial cord of chicken embryos was removed and replaced by the thoracic cord, taken from other embryos of the same age. This experimental arrangement gives the nerves from the grafted thoracic cord segments a much better chance to invade the host's own wings, which are left in the normal positions. The development of the innervation and that of the musculature of such wings was studied in different stages of incubation and after hatching.

Material and method

The present experiments were performed on chicken embryos. On the 3rd day of incubation, at stages 15—16 HAMBURGER and HAMILTON [9], the brachial segments of the spinal cord were removed (segments 13—16), and a part of the thoracic cord of equal length taken from another embryo was transplanted to fill the gap accurately. The method of transplantation was fully described in an earlier paper [14]. Out of a total of 375 operated embryos 60 successfully operated ones were killed between the 8th and 16th day of incubation, while a few of them were brought up to the time of hatching. The sacrificed embryos were fixed either in 10 per cent formalin or Susa solution for histological investigation. Serial sections were made from the vertebral column, including the transplanted part of the spinal cord. Only those cases were further investigated in which normally developed spinal cord was found. In the successfully operated embryos the development of the muscle tissue was investigated histologically. Sections prepared from of each embryo were stained with haematoxylin-eosin, those from the other wing were either impregnated with the Ábrahám—Bielschowsky [1] silver technique, or the cholinesterase (ChE) reaction was studied according to LEHRER and ORNSTEIN [11]. In hatched chickens the peripheral distribution pattern of nerves was examined in macroscopical dissections, and the ventral roots were stained for myelin according to Schultze's osmium technique.

In two of the hatched chickens at ages of 3 and 5 weeks, respectively, after hatching the 2nd brachial ventral root emerging from the transplanted thoracic cord was sectioned and 5 days later the chickens were sacrificed to show degenerating motor nerves in the wings by the Ábrahám—Bielschowsky silver technique.

Observations

Between the 8th and 16th days of incubation embryos were sacrificed daily and their wings submitted to histological investigation.

Five embryos were studied at age of 8—9 days. In haematoxylin-eosin-stained sections both homogeneous and striated muscle fibers were found. The striated fibers were starting to become organized into muscle bundles. Spaces left between the fibers were filled with embryonic mesenchyme cells. In control embryos of the same age the differentiating muscle fibers showed about the same picture. The fibers were, probably, somewhat thicker, leaving less room for interfibrillar spaces and relatively few undifferentiated mesenchyme cells were present. The innervation of wing muscles, as revealed in silver-impregnated specimens, consisted of richly sprouting plexiform nerve bundles, from which occasionally thin branches emerged and got lost among the muscle fibers. In this respect no difference was found between normal and operated embryos, except that nerves could more easily be shown in the normal wings. The histochemical localization of ChE activity showed again a similar picture in the wing muscles both in normal and operated embryos: it was found in fine brown staining particles scattered evenly throughout the muscle fibers.

Thirteen operated embryos were examined on the 10th day of incubation. The first sign which distinguished them from normal embryos was that their wings were somewhat smaller in size. Among the developing muscle fibers large giant cells appeared, 20—30 μ in diameter (Fig. 1*b*). Their cytoplasm showed the same pink colour as did the young muscle fibers stained with haematoxylin-eosin. Inside the cytoplasm one or two nuclei were found. The form and shape of these cells resembled very much the giant cells known to be present in neoplastic tissue of muscle origin. We regarded them as deteriorating muscle fibers and their presence was the first sign of muscle destruction. The interfibrillar spaces were larger, and more mesenchyme cells were present than in normal embryos (Fig. 1*a*), in which only a few mesenchyme cells and a large number of young muscle-forming cells, with occasional mitoses, were found. The cross-striation of normal muscle fibers was more distinct and the staining of nuclei lighter than in operated embryos. — The innervation of the muscles did not change too much as compared to the previous stage. In normal embryos the plexiform nerve bundles gave origin to several fine fibers, which run parallel to the muscle fibers (Fig. 1*c*). The fine branches occasionally terminated in small oval enlargements (Fig. 1*d*). Because of the absence of ChE activity on the surface of muscle fibers at this age, we could not definitely decide whether these enlargements effected neuromuscular connections or they were growth cones commonly found on the tip of growing nerve fibers in tissue culture and nerve regeneration experiments. The innervation of the muscle in the operated embryos did not differ much from that of the normal ones. The plexiform

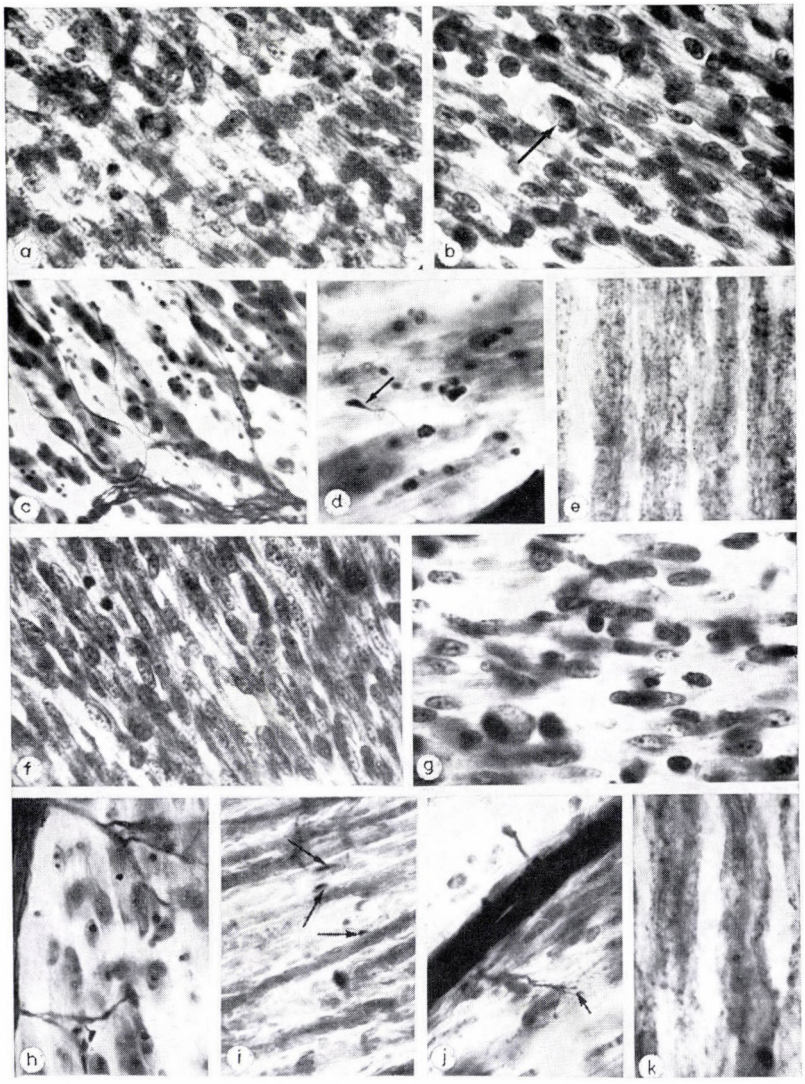

Fig. 1a. Muscle tissue from the wing of a 10-day-old normal embryo. Haematoxylin-eosin ×600

Fig. 1b. Muscle tissue from the wing of an operated embryo of the same age. The arrow points to a giant cell. Haematoxylin-eosin, ×600

Fig. 1c. Fine nerve fibers emerging from a plexiform nerve bundle in the wing muscle of a 10-day-old normal embryo. Ábrahám—Bielschowsky's silver technique. Some of the fine fibers are slightly retouched, ×600

Fig. 1d. An oval enlargement on the tip of a growing nerve fiber from the same embryo. Ábrahám—Bielschowsky's silver technique, ×900

Fig. 1e. ChE activity in the form of fine and coarse granules within the muscle fibers of a 10-day-old normal embryo. Lehrer—Ornstein technique, ×600

Fig. 1f. Muscle tissue from the wing of a 12-day-old normal embryo. Haematoxylin-eosin, ×600

Fig. 1g. The same from a 12-day-old operated embryo. Note the phagocytes and a giant cell among the muscle cells. Haematoxylin-eosin, ×600

nerve bundles were smaller in size and number, and we never could find enlargements on the tip of the fine fibers. In histochemical specimens the same fine granulation indicative of high ChE activity was shown in the muscle fibers both of the normal (Fig. 1*e*) and operated embryos, as had been found at the earlier stage. In normal muscle fibers, however, a number of larger granules appeared scattered closely under the sarcolemma, as if they were clusters of these fine granules.

Eighteen operated embryos attained the age of 11—12 days. Further reduction in the size of wings was evident relative to the controls. The muscle tissue was invaded and heavily attacked by phagocytes (Fig. 1*g*). Fibers were arranged in loosely scattered thin bundles, leaving large spaces between them. In the spaces fibroblasts and wandering cells were seen among the phagocytes. The cross-striation of fibrils stained very faintly, in most cases only a phase-contrast microscopy revealed them. At the margins of the bundles large numbers of giant cells were observed bearing two, three or more nuclei. The capillary system became prominent with signs of stasis. In the normal muscle tissue (Fig. 1*f*) fibrils were starting to thicken, hardly leaving any room for interfibrillar spaces. The structure of the cross-striation of fibrils could easily be recognized. The nuclei stained faintly and had became elongated. Between the fibers many young muscle-forming cells were still present. — In silver impregnated sections of the normal muscles the plexiform nerve bundles started to arrange into solitary nerve trunks. Several side branches consisting of one to five fine fibers emerged from the trunks. After further arborization, these fine fibers ended in oval enlargements on muscle fibers (Fig. 1*h*). In operated embryos the plexiform character of nerve bundles was more or less maintained. Several long, fine fibers were shown by silver impregnation, however these did not seem to "settle down" and terminate on muscle fibers. In rare occasions where a side branch was found leaving a nerve bundle (Fig. 1*j*), the terminal fibers were very thin and failed to form enlarged endings. ChE activity (Fig. 1*i*) appeared in the form of a diffuse faint staining inside the muscle fibers, and dark oval areas of high activity were found on the outer surface of the sarcolemma in the middle of the fibers. These dark areas matched in size and shape with the oval enlargements, being apparently the primordia of end-plates. The ChE activity in operated embryos was found in the form of fine granules

Fig. 1h. Side branches from a nerve trunk with a primitive neuro-muscular ending in the wing muscle of a 12-day-old normal embryo. Ábrahám—Bielschowsky's silver technique, ×900

Fig. 1i. ChE activity in the wing muscle of a 12-day-old normal embryo. Arrows point to the primordia of end-plates. Lehrer—Ornstein technique, ×300

Fig. 1j. An abortive neuro-muscular ending in the wing muscle of a 12-day-old operated embryo. Ábrahám—Bielschowsky's silver technique, ×900

Fig. 1k. ChE activity in the form of granules against a dark background in the muscle fibers of an operated embryo of the same age, ×600

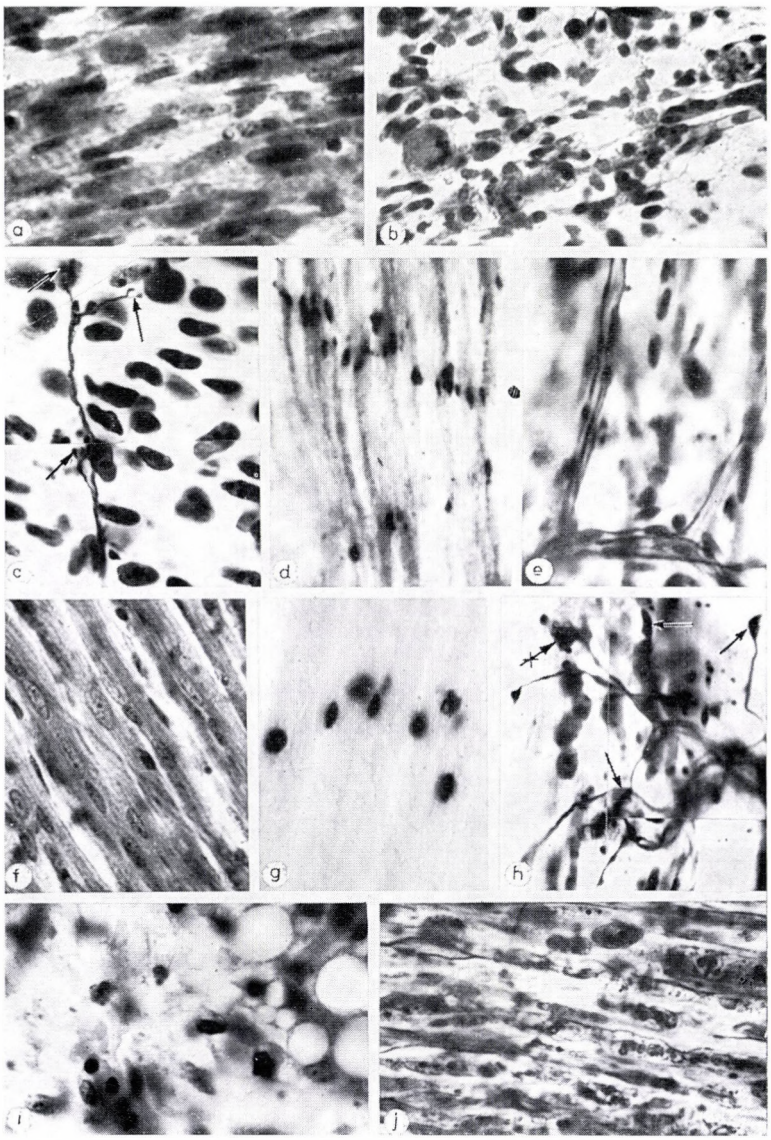

Fig. 2a. Muscle tissue from the wing of a 14-day-old normal embryo. Haematoxylin-eosin, ×600

Fig. 2b. The same from an operated embryo of the same age. A few muscle fibers, connective tissue cells and a multinucleated giant cell can be seen. Haematoxylin-eosin, ×600

Fig. 2c. Arrows point to primitive end-plates in the wing muscle of a 14-day-old normal embryo. Ábrahám—Bielschowsky's silver technique, ×900

Fig. 2d. ChE activity in the wing muscle of the same embryo. The end-plates primordia are arranged in irregular rows. Lehrer—Ornstein technique, ×300

Fig. 2e. Long nerve fibers in the wing of a 14-day-old operated embryo. Ábrahám—Bielschowsky's silver technique, ×900

Fig. 2f. Muscle tissue from the wing of a 16-day-old normal embryo. Haematoxylin-eosin, ×600

inside the muscle fibers (Fig. 1*k*), against a dark-stained background of diffuse enzyme activity.

Sixteen operated embryos were available for histological investigation at the age of 13—14 days. In haematoxylin-eosin-stained sections a further destruction of muscle was confirmed. The muscle fiber bundles were breaking up (Fig. 2*b*). In several instances solitary fibers and multinucleated giant cells, or fragments of them, occurred thickly covered with phagocytes. Because of the extensive destruction of the sarcoplasm, muscle nuclei seemed to be crowded close to each other. Regions where the muscle was removed were invaded by fibroblasts, phagocytes, wandering cells, granulocytes, and occasionally also red cells occurred. In isolated small fields preadipose tissue was being developed. The normal muscle tissue of this age showed further development. Fibers were organized into solid bundles (Fig. 2*a*). Sarcomeres become aligned laterally, showing the characteristic picture of striated muscle fibers. — In impregnated sections of normal embryos the initial organization of some primitive end-plates could be seen. The fine terminal fibers showed dichotomous branching and each branch terminated in a club-shaped ending (Fig. 2*c*). At about the middle of the muscle bundle, sites of high ChE activity were localized, lying on an irregular row perpendicular to the long axis of the muscle. The shape of the dark sites of high enzyme activity resembled very much the shape of areas embraced by the dichotomous branches of the fine terminal fibers (cf. Figs 2*c* and *d*). We could not detect any sign of end-plate formation in the wings of operated embryos. The fine terminal nerve fibers grew longer; none of the previously seen loose contacts with muscle fibers could be found, and the nerve fibers became lost among the numerous connective tissue cells (Fig. 2*e*). A diffuse strong ChE activity was seen in the muscle fibers.

Ten embryos were recovered from 15- to 16-day-old operated eggs. The reduction in the size of the wings became greater; they were about 15—20 per cent smaller than normal wings. The histological picture was dominated by scar tissue with a rich capillary system. In the scar tissue several solitary and aggregated fat cells were present, forming isolated islands, which started to conglomerate on the 16th day. A few muscle fibers, or rather fragments of them, appeared occasionally, crowded in between fibroblasts and fat cells (Fig. 2*i*). In normal wings the muscle continued differentiating and approach-

Fig. 2g. ChE activity showing the end-plates in the wing muscle of a 16-day-old normal embryo. Lehrer—Ornstein technique, ×600

Fig. 2h. Arrows point to end-plates in the wing muscle of the same embryo. Arrow with asterisk indicates a tuft-like ending as viewed from above. Ábrahám—Bielschowsky's technique, ×900

Fig. 2i. Connective tissue cells and adipose tissue replacing the wing musculature in a 16-day-old operated embryo. Haematoxylin-eosin, ×600

Fig. 2j. Degenerated nerve fibers following ventral root transsection in the radial nerve of a 4-week-old chicken bearing thoracic cord graft. Ábrahám—Bielschowsky's silver technique, ×900

ed the picture of the matured striated muscle tissue (Fig. 2*f*). — In impregnated sections of normal wings the primitive end-plates showed further signs of differentiation. Instead of the dichotomous branching seen in the earlier stage, the terminal fibers broke up in tuft-like endings covering about one half of the width of muscle fibers when viewed from above. Sections in different planes revealed different forms of the end-plates: they might appear in elongated club-shaped or in circular forms (Fig. 2*h*). Sites of high enzyme activity were increased both in number and size. They occupied oval or round fields on the surface of muscle fibers, sometimes as large as a half of the width of a fiber. The size and shape of these fields was very similar to that of the tuft-like endings found in impregnated sections (Fig. 2*g*). The diffuse sarcoplasmic activity virtually disappeared. Several nerve bundles were seen running between fat lobules and among cells of the scar tissue in wings of operated embryos. It was difficult to show the nerves since the silver was taken up mostly by the nuclei of connective tissue cells.

Five operated embryos hatched and these were kept alive for a period of 5 weeks. As was described earlier [13] the wings innervated by thoracic spinal cord were motionless and every joint became ankylotic. The macroscopic appearance of the spinal cord and the brachial plexus, as reported in detail earlier [14], gave a normal picture. The brachial plexus, as in normal chickens, was composed of four spinal nerves, but the branches were markedly thinner. Dissection of the wings revealed a normal peripheral arborization pattern of the main nerve trunks; both the median and the radial nerve followed their normal course. The musculature was replaced by fat tissue and connective tissue save for a tiny bundle at the site of the triceps muscle. This continued in a long tendon and attached to the olecranon. All shoulder muscles were present, although much smaller in size than those of normal embryos.

In 3 hatched chickens ventral roots of the transplanted, as well as of the host's own, thoracic segments and those of normal brachial cord segments were subjected to histological investigation (Table 1). The ventral root fibers

Table 1

The number of ventral root fibers in normal brachial spinal cord and in transplanted thoracic segments

Chicken		Brachial region				Total	Normal thoracic segments	
Treatment	Age, week	13	14	15	16		18	19
Operated	3	444	803	701	579	2,527	—	357
	4	—	1,212	686	582	2,480	409	397
	5	470	1,002	940	596	3,008	419	—
Normal	2	1,447	3,231	3,419	1,728	9,825	431	404
	6	1,020	3,950	6,502	2,215	13,687	549	—

of the transplanted spinal cord were considerably increased in number when compared to the number of motor fibers of normal thoracic segments. This increase was most marked in the 14th and 15th segments, somewhat less in the 13th and 16th segments. In the grafted thoracic segments there were about twice as many motor fibers as in normal thoracic segments, but only one fifth of them were coming from normal brachial spinal cord segments.

The fibers diameter spectrum showed that the larger fibers found in ventral roots of the host's own thoracic cord were 6.5 μ in diameter, and the range of fiber diameters had a peak at 3 μ. In the ventral roots of grafted segments the largest fibers were found to be 5 μ in diameter and the peak appeared at 2.5 μ.

In two chickens at ages of 3 and 5 weeks after hatching, the ventral root emerging from the transplanted thoracic cord segment that replaced the 2nd brachial segment was severed. Five days later degenerated nerve fibers were found in abundance throughout the length of the radial nerve (Fig. 2*j*), which originated mainly from the 2nd brachial segment. We also observed degenerated fibers in the scar tissue which replaced the wing musculature. A failure to show degenerated fibers in the dorsal root excluded the possibility that sensory fibers from the spinal ganglion which might have been injured during operation significantly contributed to the degeneration.

Discussion

From the results presented the question raised in the introduction can be answered in the affirmative, namely that the musculature of wings needs a specific nerve supply for the normal differentiation and maintenance of its structure. The apparent contradiction between this and EASTLICK's [5, 19] results suggesting that normal muscle tissue develops in coelomic leg grafts with thoracic innervation, can probably be explained by the fact that a coelomic graft, made after 2 to 3 days of incubation, mainly attaches to the cranial part of the body wall and receives wing nerves as well. In fact, EASTLICK [5] also describes muscle destruction in the presence of nerve fibers. These might have emerged from the thoracic segments of the cord, while those arround which a normal musculature developed might have been of brachial origin. In earlier experiments [15]we have succeeded in showing that brachial innervation is an adequate nerve supply for leg muscles, therefore a specificity of nerve supply does not exist in terms of wing or leg.

The developmental history of muscles with thoracic innervation can be summarized as follows. Embryonic myoblasts, apparently by their inherent potentiality, are capable of differentiating into striated muscle fibers without the presence of any nerves [20], or with thoracic innervation. The main difference between the further development of muscles with normal or with thorac-

ic innervation, is that in the latte rcase only a very small number of embryonic mesenchyme cells can transform into muscle-forming cells, and the majority gives rise to different connective tissue cells, which destroy and then replace the muscle. Apparently the multiplication of the muscle by the division of differentiating fibers occurs also much less frequently than in normal muscle. From 10th day on, no more new muscle-forming cells are visible, the developed fibers stop differentiating, and are gradually replaced by connective tissue within 6 days.

There is a striking difference also in the development of normal and thoracic innervation of wing muscles. Since definitive neuromuscular end-plates appear at about the second week after hatching, we could observe only the very first phase of their development. The study of this narrow section of end-plate development shows that so-called "*Plexiform Zwischenstadien*" as described by TELLO [18] and BOECKE [2] in mammalian embryos, a diffuse type of innervation, precedes the individual type of muscle innervation in the chicken as well. On the 10th day fine terminal fibers leave the plexiform nerve bundle and terminate with small oval enlargements on individual muscle fibers. One day later the high ChE activity in small oval areas on the sarcoplasm is suggestive of an effective neuromuscular contact established by the terminal fibers. According to the shape and location of these enzyme-active areas, they can be regarded as primordia of definitive end-plates, which continuously grow in size during development. In the muscles of operated embryos these end-plate primordia do not develop, and those fine branching nerve fibers which made neuromuscular connections in normal muscle, run a long course among the degenerating muscle fibers and connective tissue cells. Not being able to develop a reliable technique, we could not decide if these loose neuromuscular connections were ever functional. From a few observations made before killing the embryos, we obtained the vague impression that movements could be elicited in the shoulder joint by mechanical stimulation, and to a much less extent in the elbow and in the wrist. We again cannot tell whether these were neurogenic or myogenic movements. After the 12th day of incubation, we did not succeed in evoking any movements.

The 10th day seems to be a critical period in the development of the muscle. This is the time when an intimate neuromuscular connection begins to develop, and also when muscle destruction begins in the atypically innervated muscle. It would be, however, misleading to conclude that the muscle destruction occurs on account of the lack of end-plate formation seen first on the 11th day of incubation. The transformation of mesenchyme cells into phagocytes instead of into muscle-forming cells as early as the 9th day, may suggest a neuromuscular connection which develops in normal embryos earlier than could be shown by histological methods, and which is absent in the case of thoracic innervation.

Although the total number of the motor fibers leaving the thoracic segments was less than one fifth of the number of motor fibers from a normal brachial cord, the diminution of fibers cannot account for the extensive muscle destruction. If it were so, we could have found smaller or larger bundles around the few motor fibers available, showing normal differentiation. Instead of that, we saw degenerating muscle fibers adjacent to nerve fibers. The nerve degeneration experiments are indicative of the presence of motor fibers among degenerating muscle fibers. The present series of experiments shows that wing muscle with thoracic innervation degenerates in the same way as the muscles of nerveless limbs described by others [5, 8, 10, 19]. This suggests at the same time that limb muscles can only establish neuromuscular connections with nerves from cord segments at the limb level. Further experiments are needed to explore the nature of this specificity.

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DIE ENTWICKLUNG DER INNERVATION UND MUSKULATUR DER VON THORAKALEN NERVEN INNERVIERTEN FLÜGELN

In Hühnerembryonen wurden statt brachialen Segmenten des Rückenmarkes thorakale Segmente transplantiert. Die Entwicklung der Flügelmuskulatur und deren Innervation wurde im Alter 8 bis 16 Tagen und 5 Wochen nach der Bebrütung untersucht. Die Entwicklung der von den thorakalen Segmenten innervierten Flügelmuskulatur verlief bis zum 10. Tage der Inkubation normal. Nach dem 10. Tage begann eine graduelle Degeneration der Muskeln, so daß am 16. Tage in den Flügeln statt Muskeln nur Fett und Bindegewebe zu finden war. Es konnte mit Hilfe der Silbertechnik und Cholinesterase Methode festgestellt werden, daß die Entwicklung der ersten neuromuskulären Endplättchen in normalen Flügeln zeitlich mit dem Anfang der Degeneration der Flügelmuskulatur der operierten Embryonen zusammentrift. In Hühnern mit einem thorakalen Rückenmarktransplantat konnten nach der Läsion der zweiten brachialen Vorderwurzel degenerierte Nervenfasern in den Flügeln beobachtet werden. Die Ergebnisse sprechen dafür, daß die Flügelmuskeln mit thorakaler Innervation trotz des Vorhandenseins der motorischen Nervenfasern, ebenso zugrunde gehen, als die Muskeln des von anderen Authoren beschriebenen nervenlosen Gliedertransplantates. Zur normalen Entwicklung der Muskeln ist eine spezifische Innervation von der Gliederhöhe des Rückenmarkes nötig.

РАЗВИТИЕ ИННЕРВАЦИИ И МЫШЦ КРЫЛЬЕВ, ИННЕРВИРУЕМЫХ ТОРАКАЛЬНЫМИ НЕРВАМИ

У зародышей цыплят была произведена пересадка торакальных вместо плечевых сегментов спинного мозга. Исследование развития крыловой мышцы и ее иннервации проводилось 8—14 дней и 4 недели после начала инкубации. До десятого дня инкубации развитие крыловой мышцы, иннервируемой торакальными сегментами спинного мозга протекало нормально. Начиная с 10-го дня инкубации, однако, наблюдалась постепенная дегенерация мышцы, и на 16-ый день в крыльях находились лишь жир и соединительная ткань, мышцы не было. При исследовании с помощью серебра и холинестеразы удалось выяснить, что развитие первых нервномышечных концевых пластинков совпадает во времени с началом вырождения крыловых мышц оперированных зародышей. У цыплят с трансплантатами торакальных сегментов спинного мозга после повреждения второго плечевого корня удалось выявить вырожденные нервные волокна в крыльях. Полученные результаты указывают на то, что крыловые мышцы, иннервируемые торакальными сегментами спинного мозга, вопреки наличию двигательных нервных волокон, дегенерируют точно так же, как и мышцы неиннервированных трансплантатов конечностей, описанных другими авторами. Для нормального развития мышцы необходима специфическая иннервация. Исходящая из сегментов спинного мозга, соответствующих уровню конечностей.

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FUNCTIONAL ADAPTATION OF THORACIC SPINAL CORD SEGMENTS IN THE NEWT

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Synopsis

Primordia of brachial spinal cord segments were replaced by thoracic segments in newt larvae between stages 22 and 27. In the first group of experiments hosts and donors were of the same age, in the second the hosts were by 6 stages older, and in the third group by 2 stages younger, than the donors. In the first group limb movements gradually failed to come about in proximo-distal sequence in animals operated upon in increasingly older stages. In the second group the limbs remained motionless in the majority of the cases. Normal or slightly defective limb movements were observed in the third group. It was inferred that some specific regional influence by the surroundings might transform the undetermined grafts enabling them to replace functionally the normal brachial segments. Decline with advancing age of functional adaptation is supposed to result from a combined effect of gradually vanishing regional influence and progressing thoracic determination of the grafts. Data obtained from fibre counts in ventral roots 3 and 4 were suggestive of a preferential selection of pathways by the outgrowing nerve fibres making connections with the appropriate muscle groups.

Introduction

Various experiments performed on amphibian and chicken embryos have shown that coordinated limb movement is secured only within the normal limb segments of the spinal cord [1, 3, 10, 12]. Limb segments transplanted in the place of thoracic segments maintain the capacity to move limbs in a coordinated manner. Experimental exchanging the forelimb segments for the hindlimb segments in amphibian larvae, or substituting the leg-moving cord for the wing-moving cord in chicken embryos, results in a rhythm and character of limb movements corresponding to the origin of the grafted cord segments, irrespective of the nature of the innervated periphery [10, 12]. These experiments indicate that the capacity of the limb segments to control normal limb movements is determined very early in the embryonic life, and their functional differentiation cannot be altered by the innervated periphery.

A similar kind of functional determination was observed in the case of the eye primordium, with respect to the function of the four retina quadrants [9]. It has been shown also that this functional determination takes place separately along the two main axes of the eye primordium: first in the antero-posterior (naso-temporal) direction, and later in the dorso-ventral direction [11]. The aim of the present series of experiments is to investigate whether, as

with the eye primordium, a time sequence in the functional determination can be found in the case of the primordia of the limb-moving cord segments. In a previous attempt to investigate this problem, we found that the forelimb segments were functionally determined as soon as at stage 20, i.e., at the time of closure of the medullary tube. We also found that the primordia of the limb and trunk cord segments could replace each other in every respect, if they had been exchanged as early as in the medullary plate stage. Obviously, the determination of the forelimb segments takes place during the process when the edges of the medullary plate fold and fuse to form the medullary tube. At these stages, unfortunately, the prospective limb segments of the cord are inaccessible for operation. According to DETWILER [2] the prospective thoracic cord segments transferred to the place of brachial segments acquire the approximate size, as well as the functional capacity, of normal limb segments, if the transplantation is performed at about the mid-tailbud stage. The new position within the neural axis alters the functional differentiation of segments which are normally unable to move a limb. This possibility offers an indirect way of approach to the problem. If the early thoracic cord segments are regarded as a kind of "neutral spinal cord", and they are transferred into brachial positions in increasingly older stages, then the extent of their adaptation to move a limb may show the effect exerted by the new place upon their further differentiation. The results of such experiments may provide some indirect information about the time course of the functional determination of the normal limb segments.

Material and method

Medullary tube transplantations were performed in larvae of *Pleurodeles waltlii* at stages 20—27. To define the exact age of the larvae, stage series of *Triturus vulgaris* from SATO [7] have been adapted for *Pleurodeles*. Stages 20 to 27 include a period of development from the closure of the medullary tube to the late tailbud stage. This period lasts about 60 hours at room temperature. In the first group of experiments the three brachial segments were removed and replaced by thoracic segments of equal length taken from other embryos of the same age. In the second group the hosts were by 6 stages older, and in the third group by 2 stages younger, than the donors. Altogether 350 embryos were operated, from which 91 successful cases were selected. The movements of the forelimbs were observed, taking special notice of the excursions of the shoulder, elbow and wrist joints during ambulation.

Animals with defective limb movements started dying around metamorphosis. Therefore they were sacrificed before this time and fixed in formalin. Ventral roots emerging from the 3rd and 4th grafted segments were dissected and stained for myelin to determine the number of fibres. The spinal cords were then embedded and investigated in serial sections stained with haematoxylin-eosin.

Results

In normal animals onset of limb movement occurs at about the end of the third week. From that time on limb movements of the operated animals were regularly observed under the dissecting microscope, and were compared

with the movements of normal larvae of the same age. Limb activities were regarded as normal if the extent of movements in every joint was the same as in control animals. Movements smaller than normal were defined as defective.

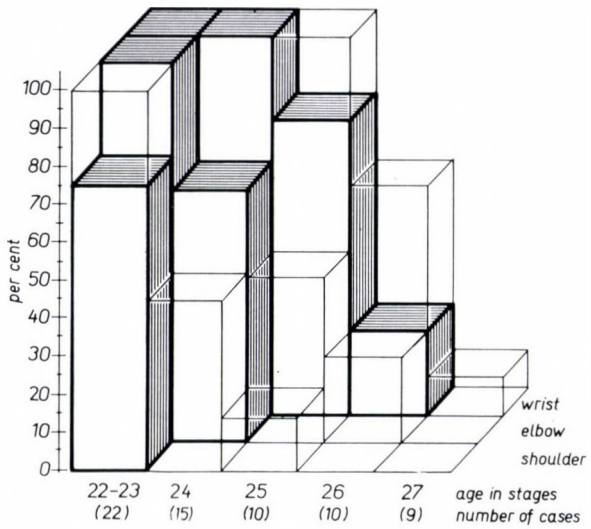

Fig. 1. Stereogram illustrating the motility of the different joints in animals operated in increasingly older stages. Columns drawn with heavy and thin lines represent complete and defective movements, respectively. The vertical axis indicates the percentage of cases in which the represented movements occurred. On the first horizontal axis the ages of operations and (in parentheses) the number of the observed cases are given. The second horizontal axis refers to the three joints of the forelimb

Group 1 (donors and hosts of the same age). 67 animals were observed in this group. The motility of the forelimbs is summarized in a stereogram (Fig. 1). The picture clearly shows that limb movements failed to come about in the proximo-distal sequence as the medullary tube transplantations were performed in increasingly older stages. Almost complete limb activity was found in animals operated not later than stage 23. The excursion of the shoulder was slightly reduced in 5 of the 22 observed cases. Defective shoulder movements appeared in 7 of the 16 animals operated at stage 24. Movements of the elbow and wrist were still normal, save for 4 cases showing defective elbow movements. With the transplantation done one stage later, complete movements exclusively of the wrist were observed in 7 out of 10 cases. The elbow and shoulder joints performed defective or no movements at all (in 4 and 1 animal, respectively). The shoulder remained motionless in animals operated at stage 26, 2 of the 10 cases yielded small movements in the elbow and the movements of the hands were normal in two, and partially normal in three cases. Finally, if the

transplantation was done at stage 27, the limbs were completely motionless except for one animal showing slight movements in the wrist.

Group 2 (donors: stage 22, hosts: stage 28). Only one out of the 18 animals yielded normal limb activities in this group. The limbs of 14 animals were completely motionless. In two cases defective shoulder movements with normal elbow and wrist motility were found, and one animal showed slight movements in the wrist.

Group 3 (donors: stage 24, hosts: stage 22). Because of large discrepancies in size in other donor-host combinations, only this combination of stages was technically feasible. Even so, not more than 6 cases recovered from a large number of operations. Three of them had normal limb activities, 2 other animals showed defective movements in every joint of the limb, while in the last one only the hand performed partial movements.

In 17 experimental and 4 control animals the ventral roots from segments 3 and 4 were dissected on one side, and treated for myelin staining to count the fibres. The experimental animals were taken from Group 1. They were operated between stages 23 and 26. The root of segment 5 contributing to the brachial plexus was neglected as, due to its small size, it could not be found in most of the cases. Care was taken to use for fibre counts animals of approximately the same length. Table 1 summarizes the data of fibre counts. The fourth and fifth columns show the average number of fibres in segments 3 and 4, respectively, calculated from the data obtained in each group. Numbers printed in italics show the averages of the total numbers of motor fibres emerging from the two segments. The last column indicates the motility of forelimbs, (+) meaning normal, (±) defective movements, and (—) lack of movement in the corresponding joints. In parallel with the failure of movement in the proximodistal direction, the number of fibres is gradually reduced to one fifth of the normal level. Normal thoracic cord segments give rise to about 60 motor fibres on the average, which is about two-thirds of the number found in the last group. It is interesting to note that the reduction in the number of fibres was approximately the same measure in the two segments investigated.

Discussion

The results presented show that thoracic cord segments grafted into the place of brachial segments before a critical stage of development are capable of replacing the brachial segments in the control of limb movements. This functional adaptation may be supposed to occur under the specific regional influence exerted by the surroundings upon the functionally undetermined graft. A similar example has already been found in the case of the functional specification of the eye primordium [11], and there is ample evidence for the

Table 1

The number of ventral root fibres in normal and in operated animals

The first row contains the data obtained from 4 normal animals; in the following rows the data of animals operated in successively older stages are shown

Stage at operation	Animal No.	Fibre counts in ventral root		Average fibre counts for groups**		Motility		
		root 3	root 4	root 3	root 4	shoulder	elbow	wrist
Normal	1	415	470	436	431	+	+	+
	2	468	496			+	+	+
	3	471	351	*866*		+	+	+
	4	385	406			+	+	+
23	1	354	281	408	387	±	+	+
	2	539	503			±	+	+
	3	419	405	*795*		−	+	+
	4	320	358			−	+	+
24	1	433	329	343	343	±	+	+
	2	292	318	*686*		−	±	+
	3	304	382			−	−	+
25	1	257	202	221	171	−	−	+
	2	231	168			−	−	+
	3	240	157	*397*		−	±	+
	4	274	164			−	−	+
	5	129	162			−	−	±
26	1	119	115	105	77	−	−	±
	2	69	81			−	−	−
	3	124	−*	*182*		−	−	+
	4	123	188			−	±	+
	5	92	−*			−	−	±

* Only root 3 was present

** The figures printed in italics indicate the average fibre counts for roots 3 + 4

influence of the surroundings upon the morphological differentiation of various grafts [4, 14]. The results of *Group 1* clearly indicate that, with increasing age, the capacity for functional adaptation gradually diminishes in proximo-distal order as projected to the limb. This may occur on account of gradual decrease of the regional influence, or increasingly advanced thoracic determination of the graft, or both. In fact the results of *Group 2* can be interpreted by assuming that the regional influence in not sufficiently effective at stage 28 to transform a graft of stage 22. On the other hand, the results of *Group 3* are indicative of still highly competent grafts taken from embryos of stage 24, and

these adapt better in 22-stage-old hosts than in hosts of similar age. We have no data, whether the grafts can be regarded as "neutral" medullary tubes at the time of transplantation, or they gradually obtain a thoracic determination. It cannot therefore be excluded that an interaction between the gradually vanishing regional influence of the surroundings and the progressing thoracic determination of the medullary tube grafts result in the gradually diminishing functional adaptation. The real background of this regional influence and of the functional determination can only be a matter of speculation, and it is not the aim of the present paper to embark on this problem.

The fact that limb movements fail to come about in proximo-distal order, may be suggestive of a progressive functional determination of the normal brachial segments occurring in the reversed direction. In other words, during the early morphogenesis of the medullary tube, the functional determination of the nervous apparatus would start with that for the shoulder movements and would become completed with that for the hand movements. However, this notion did not gain support from experiments in which we transplanted the hindlimb segments into the thoracic region at different stages of development. The function of a pair of supernumerary limbs grafted into the same region has revealed that the functional determination of the hindlimb segments took place at stage 30, but when once established, it included the movements of the entire limb without any signs of progress in any direction.

The histological signs of brachial differentiation of the prospective thoracic segments was the familiar picture of the enlarged ventral horns similar to the normal brachial segments. From the general belief that the proximo-distal order of the muscles of extremities is represented in cranio-caudal order in the spinal cord, it was expected to find a gradual reduction in size of the ventral horns in cranio-caudal direction, when the operation was made in increasingly advanced stages. Instead of this we found that, with decreasing limb activities, the size of the ventral horns decreased evenly throughout the grafted segments. For a more reliable information about the number of motoneurons, fibre counts were made in the ventral roots. This study gave essentially the same result: the number of fibres decreased evenly in both segments investigated. These findings suggest the interpretation that the central representation does not follow accurately the proximo-distal localization of the limb muscles. The problem of the arrangement of motoneurons supplying the different muscles of the limb is dealt with in detail in another paper [13].

The findings obtained from fibre counts yield interesting data concerning the outgrowth of the innervating motor nerves. From the increasingly worsening limb activities in the proximo-distal direction, the reasonable assumption could be made that the limb muscles fail to receive a proper innervation, i.e. an appropriate number of nerve fibres reaching a particular muscle group, in a proximo-distal sequence. In cases, for instance, having only hand

movements, the muscles of the shoulder and the arm received by far fewer nerve fibres than the muscles of the forearm and the hand. In fact, in a few preliminary investigation in which fibre counts were made in various sections of the limb nerves, we have found good evidence to support this idea. On the other hand, it is obvious from Table 1 that the number of fibres evenly decreased all over the grafted segments as the operations were done in more and more advanced stages. If this were so, it would be difficult to understand why the majority of the outgrowing nerve fibres should bypass the proximal muscles and terminate in the distal ones, unless by assuming that the outgrowing nerve fibres had preferentially selected the pathways which led them to their proper destination. A similar preferential selectivity in the regeneration of nerve fibres to the fin muscle [5], and of oculomotor nerve fibres [8] has been described recently. Further experiments are in progress to elucidate this question.

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DIE FUNKTIONELLE ADAPTATION DER DORSALEN RÜCKENMARKSEGMENTE BEI MOLCHEN

An die Stelle der brachialen Rückenmarksegmente von Molchlarven wurden in den 22—27 Entwicklungsstadien dorsale Segmente eingepflanzt. In der ersten Versuchsgruppe waren die Donor- und Akzeptorlarven gleich alt. In der zweiten Gruppe waren die Akzeptoren um 6

Stadien (etwa 50 Std.) älter, in der dritten Gruppe um zwei Stadien (etwa 16 Std.) jünger die als Donoren. In der ersten Gruppe, wo die Rückenmarktransplantationen in jeweils älterem embryonalen Stadium ausgeführt wurden, blieben die Bewegungen der vorderen Extremitäten in proximo-distaler Richtung (bezogen auf die Position der Gelenke) stufenweise aus. In der zweiten Gruppe waren die Extremitäten im Großteil der Fälle unbeweglich. In der dritten Gruppe wurden im allgemeinen bessere Extremitätenbewegungen erzielt, als bei den im gleichen Alter (24. Stadium) operierten Tieren der ersten Gruppe. Es wird angenommen, daß sich die in funktioneller Beziehung noch nicht determinierten überpflanzten dorsalen Rückenmarkssegmente unter einer spezifischen regionalen Wirkung der Transplantatumgebung in brachiale Segmente umwandelten, die die Bewegung der Extremitäten zu sichern vermochten. Die stufenweise abnehmende funktionelle Adaptation dürfte durch die allmählich abnehmende regionale Wirkung und die stufenweise zunehmende funktionelle Determinierung des dorsalen Typs der Transplantate bedingt sein. Die Fasern der an der Herausbildung des brachialen Bündels teilnehmenden 3. und 4. ventralen Wurzel wurden bei den Versuchstieren der ersten Gruppe sowie bei normalen Tieren gezählt. Aus den Ergebnissen läßt sich folgern, daß die wachsenden Fasern jene peripherischen Wege bevorzugen, die zu den ihnen entsprechenden Muskelgruppen führen.

ФУНКЦИОНАЛЬНАЯ АДАПТАЦИЯ ДОРСАЛЬНЫХ СПИННОМОЗГОВЫХ СЕГМЕНТОВ У ТРИТОНОВ

В 22—27 стадиях развития личинок тритонов брахиальные спинномозговые сегменты были замещены дорсальными сегментами. В первой подопытной группе личинки-доноры и личинки акцепторы были одинакового возраста. Во второй группе акцепторы были на 6 стадии (около 50 часов) старше, а в третьей группе на 2 стадии (около 40 часов) пересадка спинномозговых сегментов была произведена во все более старом эмбриональном возрасте. В этой группе движения передних конечностей в проксо-дистальном направлении (относительно положения суставов) постепенно стали прекращаться. Во второй группе конечности в значительной части случае оставались неподвижными. В третьей группе получались в общем лучшие движения, чем у животных первой группы, оперированных в одинаковом возрасте (в 24-ой стадии развития). Предполагается, что пересаженные дорсальные спинномозговые сегменты, которые еще не были функционально детерминированными, преобразовывались в брахиальные сегменты, способные двигать конечности, под специфическим регионарным действием окрестности трансплантата. Постепенное уменьшение функциональной адаптации может быть результатом постепенного уменьшения регионарного действия и постепенного повышения функциональной детерминации дорсального типа трансплантатов. Авторы проводили подсчет волокон вентральных корней 3 и 4, участвующих в образовании брахиального пучка, у подопытных животных первой группы и у нормальных животных. Из результатов подсчета они делают заключение, что растущие волокна предпочитают пути, приводящие к соответствующим их типу мышечным группам.

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